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THE PRODUCTION OF HIV SUPPRESSIVE FACTORS BY CD28, CD38 AND HLA-DR SUBPOPULATIONS OF CD8+ T CELLS

BY

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A Thesis
Submitted to the School of Graduate Studies
in Partial Fulfillment of the Requirements
for the Degree
Master in Science

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Abstract

We have examined CD8+ sub-populations to determine whether these subsets are critical to the production of CD8+ T cell nonlytic factors. The production of the β-chemokines MIP-1α, MIP-1β and RANTES, and the chemoattractant cytokine IL-16 were measured in cells derived from 24 HIV-infected and 25 uninfected subjects. Asymptomatic HIV+ subjects (CD4>200/ul) produced significantly higher levels of MIP-1α and MIP-1β from CD8+ T cells and some sub-phenotypes. Higher RANTES levels were produced by CD28-, CD38- and HLA-DR+ sub-phenotypes. However, IL-16 was only modulated in the CD38+ subset in comparison to total CD8+ T cells. Infection of CD8+ T cells and sub-populations resulted in generally increased levels of chemokine and IL-16 production, which dissipated over a 15 day time course. Moreover, CD8+ antiviral factor (CAF) activity, another major component of CD8+ T cell nonlytic suppression factors, was not associated with chemokine production. However, significantly higher levels of CAF were produced by CD38+ and HLA-DR+ sub-populations. In addition, we also showed that in CD8+ T cell populations, the production of MIP-1α, MIP-1β and IL-16 was inversely correlated with virus copy number. These findings shed light on the noncytotoxic responses of CD8+ T cells in controlling the natural course of HIV infection.
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Introduction

General Introduction:

The human immunodeficiency virus (HIV) is a member of the Retroviridae family, which is characterized by a unique enzyme, reverse transcriptase, that allows the virus to copy its RNA into double-stranded DNA in order to replicate (1, 2). Within the Retroviridae family, HIV is classified as a lentivirus. HIV infection is characterized by the depletion of the CD4+ helper/inducer subset of T-lymphocytes, leading to severe immunosuppression, constitutional symptoms, neurologic disease, and opportunistic infections and neoplasms (3). The first reports of death related to AIDS emerged in the early 1980s, and the understanding that HIV was the cause of this syndrome came soon after (4). Recently, tissue and blood samples from people who lived in Africa in the 1950s have been found to have evidence of the virus (5), so HIV appears to have been in existence longer than was previously thought.

A human being is infected with HIV every 10 seconds on average, as estimated conservatively by the World Health Organization and the Joint United Nations Programme on HIV/AIDS (UNAIDS) (5). Sexual transmission may account for 90% of new HIV cases worldwide (4). In Africa, nearly 40% of young adults are infected with HIV and other countries such as India are now experiencing continued rapid spread of the infection (6). The current estimate of the number of individuals worldwide who has become infected with HIV exceeds 30 million and an additional 10 million persons have died of HIV disease (4).

HIV is transmitted by several routes: 1) intravenous, by transfer of infected blood or blood products; 2) receptive anal intercourse; 3) receptive vaginal intercourse; 4) from infected mother to fetus or baby at or before parturition; and 5) breast-feeding by infected mothers (7).
The introduction of potent antiretroviral therapy has resulted in significant decreases in morbidity and mortality among HIV-infected persons. The primary goals of antiretroviral therapy are maximal and durable suppression of viral load, restoration and/or preservation of immunologic function, improvement of quality of life, and reduction of HIV-related morbidity and mortality (8). To date, highly active anti-retroviral therapy (HAART) is the most often used combination therapy. HAART often leads to increases in the CD4+ T cell count, which is generally related to the degree of viral load suppression (9). In addition, HAART could partially reconstitute immune function and allow for elimination of unnecessary therapies, such as those used for prevention and maintenance therapy against opportunistic infections (10). Despite the effectiveness of combination therapy, HIV-1 does develop mutations conferring resistance to the antiretroviral treatment resulting in a loss of control of HIV-1 replication in vivo. Therefore ongoing research is essential to identify new treatments for HIV-1 infection.

**Natural History of HIV Infection:**

Clinically, HIV-1 infection may be divided into three phases. During the initial phase, known as primary HIV-1 infection, virus present in the infecting inoculum replicates in the host, infecting cells that express both CD4 and the appropriate coreceptor, usually the chemokine receptor CCR5 (11). Viremia develops within the first few weeks of exposure, accompanied by infectious mononucleosis-like symptoms in some patients (12). Studies of viral dynamics during primary HIV-1 and SIV infection suggest that viral populations double every 6–10 h in the initial stages of the infection, with each infected cell giving rise to approximately 20 new infected cells (12). During symptomatic primary infection, levels of
infectious virus and of infected cells in the circulation are both very high (13). The concentration of virus particles in the blood is often $>10^6$/ml and can be as high as $10^9$/ml. The initial systemic seeding of the peripheral lymphoid organs with HIV-1 occurs as a result of the high levels of viremia that develop during primary HIV-1 infection. Interestingly, studies of the simian immunodeficiency virus (SIV) and simian/human immunodeficiency virus (SHIV) models of HIV-1 infection suggest that the large pool of CD4+ T cells in the gut-associated lymphoid tissue (GALT) is an important site for viral replication in primary infection as well as during subsequent phases of the disease (14). This likely reflects the fact that a relatively high fraction of CD4+ T cells in the GALT are in an activated state and are therefore permissive for HIV-1 replication.

Within a few weeks, the level of virus in the blood declines. This decline coincides with the development of an immune response to HIV-1. Virus-specific cytolytic T lymphocytes (CTL) appear early and may represent a critical host factor in the control of primary HIV-1 infection (15). Recent studies in the SIV model have convincingly demonstrated the importance of CD8+ CTL in the control of the viremia of primary infection. Monkeys depleted of CD8+ T cells by monoclonal antibody treatment are unable to control primary infection (15). The combined effects of CTL and other elements of the immune response cause the amount of virus in the blood to decrease to a lower plateau level.

The second phase of HIV-1 infection is the long asymptomatic period between primary infection and the development of clinical immunodeficiency (AIDS). There are two related pathophysiologic characteristics of the asymptomatic phase: ongoing viral replication in the peripheral lymphoid tissues and gradual loss of CD4+ T cells. Although the asymptomatic phase may represent a period of clinical latency, the virus replicates continuously during this
time. Free virus can be detected readily in the circulation by appropriate methods (16), although the levels are lower than those observed during symptomatic primary HIV-1 infection or in patients with AIDS, the third and final stage of the infection. Infected cells are readily detectable in the peripheral lymphoid tissues, and their number correlates directly with plasma virus levels, suggesting that the lymphoid tissues are a major source of plasma virus (17). The rate of decline in CD4+ T cells appears to be determined by the level of ongoing viral replication, as patients with higher plasma virus progress to AIDS more rapidly (18). The mechanisms underlying CD4 depletion are incompletely understood but probably include a decrease in T cell production by the thymus and an increase in the rate of destruction of T cells in the periphery (17).

The final phase of the infection is characterized by the emergence of clinical immunodeficiency. In the year or two before AIDS develops, there is often a more rapid decline in CD4+ T cells. This decline may be preceded by an increase in viral load, with viral replication occurring in many sites in addition to the lymphoid tissue (19). In some cases disease progression is associated with the evolution of more pathogenic viral species that utilize the chemokine receptor CXCR4 instead of CCR5 (reviewed in 11). As the CD4 count falls below 200 cells/µl, opportunistic infections begin to occur. The degree of CD4 decline is an excellent predictor of the risk for disease progression and provides strong evidence that the loss of CD4+ T cells is the central cause of immunodeficiency in this disease.

**HIV-1 Transcription:**

The HIV-1 particle includes two identical (+) strand RNA copies of the viral genome (20). Upon infection of a susceptible host cell, the RNA genomes are reverse transcribed by the
virally-encoded reverse transcriptase into DNA which is designated the provirus. The genetic elements of the HIV-1 provirus are shown in Fig.1. The 5' and 3' ends of the provirus include long terminal repeat (LTR) structures of 634 nucleotides. The 5'-LTR regulates the initiation of RNA transcription, whereas the 3'-LTR regulates RNA termination and polyadenylation (reviewed in 21). Structural proteins of the virus particle are encoded by the gag and env genes. The pol gene encodes for the viral enzymes which include protease, reverse transcriptase and integrase. Regulatory proteins with a potent influence on HIV-1 replication are encoded by tat and rev. Tat, a transcriptional trans-activator protein, mediates its effects by the Tat-response (TAR) element. Rev, a post-transcriptional trans-activator, enhances structural gene expression by promoting transfer of incompletely spliced viral RNAs from the nucleus to the cytoplasm, mediated by the Rev response element (RRE) in the viral RNA. Accessory proteins which are at least partially dispensable for virus replication in vitro are encoded by vif, vpr, vpu, and nef (reviewed in 21).

Basal HIV-1 transcription is determined by the interaction of cellular regulatory proteins with elements in the HIV-1 long-terminal repeat, such as NF-κB, a transcriptional activator (22). A second level of control involves the virally encoded Tat trans-activator. In the absence of Tat, initiation from the long terminal repeat is efficient, but transcription is impaired because the promoter engages poorly processive polymerases that disengage from the DNA template prematurely (23). Activation of transcriptional elongation is regulated by TAR. After binding to TAR RNA, Tat stimulates a specific protein kinase called TAK (Tat-associated kinase). This results in hyperphosphorylation of the large subunit of the RNA polymerase II carboxyl-terminal domain. Evidence, arising from experiments with inactive mutants of TAK expressed in trans which inhibit transcription and TAK inhibitors which selectively suppress
Figure 1. The HIV-1 genome. The genetic components of the HIV-1 and known functions of viral proteins are indicated.
Tat activity, has demonstrated that TAK plays a role in transcriptional regulation of the HIV-LTR (24). A final level of regulation is mediated by the viral Rev protein. Rev acts post-transcriptionally to induce the expression of HIV-1 structural proteins and thereby commits HIV-1 to the late, cytopathic phase of the viral replication cycle (25). Rev activity appears to require a critical, threshold level of Rev protein expression, thus preventing entry into this late phase in cells that are unable to support efficient HIV-1 gene expression.

**Control of HIV Replication *in vivo***:

- **Cytotoxic T cell responses in viral infection**:

  Following virus infection, a series of complex interactions occur between the virus and the host immune system. The host aims to eliminate the infection and minimize associated pathological consequences. The immune response to viral infection can be broken down into two broad categories: (a) innate or nonspecific responses and (b) adaptive or specific responses. These play critical roles at distinct points in controlling the infection. Innate responses start almost immediately but cannot eliminate the virus on their own. Specific responses are essential for clearing the infection, one of which is the cell-mediated immune response. The virus-specific CD8+ cytotoxic T lymphocyte (CTL) response particularly plays a key role in the elimination of infected cells.

  A major function of virus-specific CD8+ T cells is to kill infected cells. CTL lyse antigen-bearing target cells by two distinct pathways: (a) a secretory and membranolytic pathway involving perforin and granzymes and (b) a nonsecretory receptor–mediated pathway involving Fas (CD95) (25). Alternative mechanisms of killing, such as cytotoxicity mediated by tumour necrosis factor (TNF) and secreted adenosine triphosphate, have also been
postulated, but there is now a general consensus that perforin- and Fas-mediated pathways are the two major killing mechanisms used by CTL (25, 26).

The perforin-mediated pathway involves exocytosis of cytolytic granule contents. The CTL granules contain an array of cytotoxic molecules, including a family of serine proteases called ‘granzymes’, a pore-forming protein named ‘perforin’, calreticulin and chondroitin sulphate proteoglycans (27). Upon binding of CTL to the target cell and appropriate engagement of the T cell receptor (TCR), the cytoplasmic granules containing perforin and granzymes are released vectorially onto the target cell (28). Perforin, a 65-kDa glycoprotein mediates the formation of pores with an internal diameter of up to 16 nm and target cell death through osmotic lysis (28). The essential function of perforin for CTL-mediated lysis of virus-infected cells was clearly revealed by in vitro and in vivo studies utilizing perforin-deficient gene knockout mice developed independently by several groups (29, 30). In contrast to their heterozygous littermates, perforin knockout mice infected with lymphocytic choriomeningitis virus (LCMV) were unable to efficiently clear the infection. This inability to clear virus resulted in elevated virus titers in the serum of perforin knockout mice despite the presence of normal numbers of CD8+ T cells that became activated upon infection. Granzymes released by CTL penetrate into the target cell cytoplasm and cleave key substrates, leading to apoptosis (31). These studies have clearly established that CTL-mediated cell killing is critical in controlling the infection and that the perforin pathway is the dominant killing pathway in vivo.

The alternative pathway mediated by CTL involves membrane proteins belonging to the TNF receptor-like family (Fas (CD95) and TNFR) on the target cell which bind their respective ligands (FasL and TNF) on the CTL. Similar to the perforin pathway, the Fas-
dependent pathway is initiated by engagement of the TCR by the appropriated antigen (32). This interaction results in upregulation of FasL expression by T cells. Binding and cross-linking of FasL with Fas molecules expressed on the target cells lead to apoptosis of Fas-positive cells (33). All components of the Fas-mediated apoptotic pathway are constitutively expressed with the target cell. Once Fas is activated, the formation of a death-inducing signaling complex occurs very rapidly at the inner leaflet of the plasma membrane (34). First, Fas-associated protein death domain (FADD) is recruited and binds to the Fas cytoplasmic tail via the C-terminal death domain. The N-terminus of the FADD protein encodes a “death effector domain” which induces downstream signal transduction, recruiting FADD-like IL-1β-converting enzyme (caspase 8). The latter binds to FADD via two death effector domains located at the N terminal region, and undergoes autocatalysis to form a functional protease, which can then initiate the rest of the caspase cascade (34).

However, it is worth noting that mice deficient in Fas (lpr) or FasL (gld) do not exhibit enhanced susceptibility to viruses. Lpr mice have a loss-of-function mutation of the Fas gene, while the gld mice have a point mutation in their FasL gene, which prevents FasL binding to Fas (35). These mutant mice have essentially normal susceptibility to virus infection, with normal functioning CTL and NK cells. Moreover, Fas is only expressed on certain cell types (hepatocytes, activated T cells), and it is therefore unlikely that Fas-mediated killing can be a major effector mechanism in vivo since a variety of cells do not express this molecule and cannot be killed by this mechanism. Such a limitation does not apply to the perforin pathway (25, 32).

- **Cytotoxicity in HIV infection:**
The cellular immune response plays a vital role in HIV pathogenesis, and studies of long-term nonprogressors and exposed yet uninfected persons have suggested that certain aspects of host immunity may protect against HIV infection or slow disease progression. HIV-specific CD8+ T-cell lymphocytes, the effector arm of the cellular immune system, with aid from HIV-specific CD4+ lymphocytes (T-helper cells), kill HIV-infected cells, which present viral peptides associated with HLA class I molecules. In response to new foreign proteins, naive CTLs that recognize the antigens are selectively activated, proliferate, and become functional killer cells. Many HIV-infected persons produce HIV-specific CTLs, which are present at substantially higher levels compared with virus-specific CTLs in other chronic viral infections (36). Suppression of HIV through cytolytic mechanisms involving direct contact between infected cells and HIV-1 specific, HLA class I-restricted cytotoxic T-cell lymphocytes has been demonstrated (37).

As mentioned previously, there are two principal mechanisms of CTL killing to eliminate antigen-bearing cells. However, in HIV, Shankar et al. showed that HIV-specific CD8+ CTL did not show a major role for Fas-based cytotoxicity in CTL lysis of infected targets (38). They used CTL clones and cell lines directed against four different HIV proteins to lyse primary HIV-infected targets. Using specific antibodies, they found that the perforin pathway predominated in the lysis of HIV-infected primary CD4+ T cells by HIV-specific CTL clones. Other evidence has supported this view. A recent study of human mycobacteria-specific CTL clones showed that lysis of infected target cells by class I restricted CD8+ CTL was exclusively perforin-mediated (39). In another study, tumour killing by human melanoma reactive CD8+ and CD4+ CTL clones isolated from tumour lesions was also exclusively by the granule-dependent, Fas-FasL-independent pathway (40). Although cytotoxic T lymphocytes
provide a major host defense against viral infection, they have also been implicated in immunopathogenesis of several viral and autoimmune diseases (41, 42).

Despite being highly directional, CTL can mediate tissue damage during antigen-specific killing by lysis of neighbouring cells or by the release of soluble mediators like TNF-α (43). FasL-based mechanisms are more likely to result in bystander lysis than granule-mediated mechanisms, since FasL-expressing CTL can potentially lyse any Fas-expressing targets including uninfected cells (43). Whether CTL play a similar role in HIV infection in the process of CD4 loss has not been determined. Bystander killing is more likely caused by a Fas-mediated mechanism which is not dominant in HIV-specific CTL. It has been suggested that CD8+ CTL in HIV may not be pathogenic. Shankar et al have not observed that CD8+ CTL cause HIV immunopathology by lysing bystander cells in their experiments (38).

After more than a decade of intensive research, the precise role of HIV-specific cytotoxic T lymphocytes in determining the course of the infection remains open to argument. How do HIV-1-specific CTL respond to virus during the natural history of AIDS? After infection with HIV, 2-4 weeks elapse before clinical symptoms of acute infection ensue. In about 50-70% of infected individuals an acute retroviral syndrome develops with mild influenza-like manifestations from which most patients usually fully recover. HIV-1-specific CTL have been observed as early as a few days following the onset of acute symptoms and in general before antibody responses could be detected (reviewed in 44). The appearance of HIV-1-specific CTL usually parallels a striking diminution of the viremia in infected patients. Likewise, studies in rhesus monkeys have shown that upon deliberate infection with simian immunodeficiency virus (SIV), CTL appear as early as 4-7 days post virus inoculation, and coincide with viral clearance from blood and lymph nodes (45). Interestingly, some individuals
who were observed remained HIV-1 seronegative despite frequent exposure to the virus. In a group of women from Gambia who appeared to have escaped HIV-1 infection, despite several years of high-risk sexual behavior, three out of six patients had CTL that recognized HIV-1 and HIV-2 cross-reactive epitopes (46). This suggests that cellular immunity to HIV-2 protects against infection with HIV-1. Similarly, recent animal studies demonstrated that a live attenuated HIV-2 vaccine could protect cynomolgus macaques against development of AIDS for more than five years after infection with a pathogenic strain of SIV (47). Collectively these studies indicate that CTL are recruited very early during the encounter with HIV-1, and that in part they may be responsible for the initial control of HIV-1 replication.

After seroconversion, the plasma levels of HIV-1 RNA usually stabilize within several months around the so-called ‘virologic setpoint’. A variable asymptomatic period follows which appears to correlate with the level of this residual virus replication (48). Vigorous HIV-1-specific CTL responses have been observed in most asymptomatic individuals studied to date. The precursor frequencies of HIV-1-specific CTLs as determined in limiting dilution assays represent mainly memory CTL (49). High levels of circulating CTL effectors can often be demonstrated directly ex vivo without the need for restimulation and expansion in vitro (50). The exact relationship between the effector and memory components of the HIV-1-specific CTL responses remains unresolved. It could be hypothesized that the ratio of HIV-1-specific effector CTLs to memory CTLs reflects how successfully virus replication is contained. For example, if virus replication is well controlled, the number of circulating effector CTLs might be low while memory CTL frequencies are either high or normal. In the case of a high ratio with increased numbers of effector CTLs for a prolonged period of time, this would probably predict more-rapid disease progression.
Many studies have shown that HIV-1-specific CTL responses deteriorate during disease progression (49-52). There are two scenarios for disease progression. In rapid progressors the viral load generally seems to increase in the presence of strong HIV-1-specific CTL responses suggesting that HIV-1 has escaped antiviral CTL responses. Another pattern is observed in more-typical progressors, in whom the viral load seems to increase only after a substantial loss of HIV-1-specific CTLs. The reasons for the apparent failure of CTLs to contain HIV-1 replication may be diverse. In general, CD4+ T-cell numbers do not recover when HIV-1-specific CTL responses deteriorate during progression to AIDS (51-53), whereas it has been shown that most late-stage patients can still regain considerable numbers of CD4+ T cells during potent anti-retroviral therapy (54).

In a small proportion of HIV-1-infected individuals, an extraordinarily benign disease course beyond the median time to AIDS is observed. This group of so-called long-term non-progressors (LTNP) appears heterogeneous and seems to contain mostly very slow progressors and some true LTNP. It is expected that HIV-1-specific CTL from LTNP have distinct features that contribute to prolonged maintenance of the asymptomatic phase. Several studies of LTNP have shown robust and persistent HIV-1-specific CTL responses (50, 54) involving simultaneous recognition of multiple CTL epitopes (54). These CTL responses coincide with relatively low numbers of HIV-1-infected cells and efficient viral control. Furthermore, it has been suggested that qualitative differences in the initial immune response to HIV-1 are responsible for distinct clinical outcomes (55, 56). Pantaleo et al. have reported that simultaneous expansions of many TCR Vβ families during primary HIV-1 infection, probably resembling a broadly directed HIV-1-specific CTL response, protects against rapid progression to AIDS (55). In addition, Van Baalen et al. have shown that early CTL responses from LTNP
were more-frequently targeted at epitopes in Tat and Rev compared with those of rapid progressors (56). This suggests that CTL directed against early viral proteins are more effective in controlling viral load, because they are thought to kill HIV-1-infected cells before a major release of virions occurs.

- **Escape of HIV from immune surveillance by CTL:**

  Despite seemingly potent CTL responses, HIV-1 is almost never completely eradicated from the body but instead persists for many years at the virologic setpoint (48). It seems likely that release of virions from HIV-1-infected cells, which are relatively resistant or not readily accessible to CTL-mediated killing, or other types of immune responses, will significantly contribute to this level of residual viral replication. A variety of mechanisms have been proposed to attempt to resolve this phenomenon, including sequence variation, altered antigen presentation, latency, privileged sites of viral replication, and loss of effector cells (57, 58).

  Sequence variation has been considered as a mechanism of immune escape from CTL. The rapid rate of HIV-1 replication in infected individuals combined with low fidelity of reverse transcription result in a remarkable degree of viral heterogeneity (53, 59). Within an HIV-1-infected subject, a virus containing a mutation in any given CTL epitope will be generated on a daily basis. Phillips et al. hypothesized that sequential accumulation of mutations in CTL epitopes might serve as a dominant mechanism of immune escape (60). More compelling evidence from other studies confirmed this hypothesis and showed unrecognized variant sequences could be detected as early as 30 days after the onset of symptoms (61). Furthermore, several features of primary infection are likely to increase the probability of detecting escape from CTL response during this phase, including the high rates
of viral replication and the likelihood that the initial CTL response will be monoclonal (62). However, many unanswered questions remain. A central issue is whether sequence variation in CTL epitopes plays a leading role in the continued replication of HIV-1 despite a vigorous CTL response. Further, if there is a progressive shifting of the CTL response to subdominant epitopes, this may ultimately result in immune surveillance failure (63).

Alternative mechanisms of virus escape from CTL responses are related to the virus protein Nef. Xu et al. (64) demonstrated that CD8+ CTL in animals infected with pathogenic SIV strains underwent apoptosis. Infection of CD4+ cells in vitro with the strain SIVmacJ5 resulted in upregulation of Fas ligand, whereas only slight upregulation of Fas ligand was induced by the Nef-defective pC8 strain. The authors propose that upregulation of Fas ligand by Nef in infected cells induces lysis of effector CD8+ CTL, which are generally Fas+. Similarly, expression of Fas ligand on tumour cells may induce apoptosis of activated lymphocytes and escape from tumour-specific CTL (65). Previous reports have shown that infection of either CD4+ T cells or macrophages with HIV-1 induces upregulation of Fas ligand, although the specific proteins responsible for this effect were not identified (66). Collins et al. (67) created nef+ and nef- molecular clones of HIV-1. Following infection of primary CD4+ T cells with these HIV-1 strains, nef+ viruses induced a 20-fold down-regulation of MHC class I expression. Cells infected with nef+ viruses were also relatively resistant to lysis while cells infected with the nef- variants were efficiently lysed in an HLA-restricted manner.

Latency is another mechanism for escape from CTL responses. As it is well known that HIV-1 is able to establish two forms of latent infection at the level of individual cells: pre-integration and post-integration. In the former, HIV-1 virions bind to and fuse with resting
CD4+ T cells with the subsequent reverse transcription of the genomic viral RNA, but not productive infection (68). The latter occurs in CD4+ T cells that have undergone integration of proviral DNA and return to a resting state (69). Due to limited viral protein production, CTL may not recognize these resting and infected CD4+ T cells. Moreover, post-integration latency may provide a mechanism whereby cells may avoid both CTL surveillance and viral cytopathic effects, permitting survival of virus for the lifetime of the infected cell. The ability of virus to escape from host immune surveillance represents a further challenge to HIV-1 infection control and treatment.

- **Noncytotoxicity:**

  In addition to cytotoxicity, CD8+ T cells of HIV-1-infected individuals exert strong noncytotoxic control of virus replication (70-73). This role is through various soluble factors secreted by CD8+ T cells which interfere *in vitro* with HIV-1 replication. These factors include interferon gamma, TNF-α, the CD8+ T-cell antiviral factor (CAF), interleukin 16 (IL-16), and β-chemokines: RANTES (Regulated upon Activation Normal T Cell Expressed and Secreted), macrophage inflammatory protein-1α (MIP-1α) and MIP-1β. Among them, CAF, IL-16 and the β chemokines exert strong negative control of HIV-1 replication.

  The noncytotoxic anti-HIV response of CD8+ cells differs from the cytotoxic activity in several ways: the former is not MHC restricted, displays broad specificity and is mediated via soluble antiviral proteins, while the cytotoxic response is MHC-restricted, antigen specific and is mediated by perforin/granzymes or by Fas-induced apoptosis (74). The noncytotoxic anti-HIV response of CD8+ cells was noted in early studies that demonstrated that the removal of CD8+ T cells from PBMC cultures of infected individuals resulted in increased virus
replication in the remaining CD4-enriched cells. Reconstitution of the culture with autologous CD8+ cells suppressed virus replication in a dose-dependent manner (70). These findings demonstrated the role of CD8+ cells in controlling HIV replication and showed that the block in virus production was not due to elimination of virus-infected cells. Since then, many studies have confirmed the presence of an anti-HIV response that does not involve killing of the infected cell nor an alteration in the ability of CD4+ cells to proliferate or express activation markers (72, 75-81). It has also been shown that a correlation exists between a strong antiviral response and a healthy clinical state, and further that anti-HIV activity does not require HLA compatibility for efficient suppression of virus replication (75-79). Similar anti-HIV activities have been observed for CD8+ T cells of SIV- and HIV-infected non-human primates (82). This noncytotoxic anti-HIV response is only observed with CD8+ T cells, not CD4+ cells, B cells, NK cells or macrophages (74).

**β-chemokines:**

The β-chemokines comprise one arm of CD8+ T cell noncytotoxic anti-HIV responses. The chemokine superfamily consists of four subfamilies that display between two and four highly conserved NH2-terminal cysteine amino acid residues. The CXC (or α) family has the first two NH2-terminal cysteines separated by one nonconserved amino acid residue. In contrast, the CC (or β) family has these cysteines in juxtaposition, the C (or γ) family has one lone NH2-terminal cysteine residue, while the CX3C (or δ) family has these cysteines separated by three intervening amino acids (83). Infection of cells by HIV-1 requires the presence of the viral receptor CD4 and appropriate co-receptors on the cell surface. These coreceptors have been identified as certain chemokine receptors: CCR5 and CXCR4. To date, all primary
isolates tested use either CCR5 or CXCR4 or both receptors (84). More importantly, even viral isolates that can use multiple coreceptors (in addition to CCR5 and CXCR4) are completely inhibited by CCR5 and/or CXCR4 specific antagonists when infections are performed on primary peripheral blood lymphocytes (85), indicating that CCR5 and CXCR4 are the major coreceptors used by HIV-1. The cognate ligands to CCR5: the β-chemokines (RANTES, MIP-1α, MIP-1β) and to CXCR4: the α-chemokine stromal-derived factor-1α (SDF-1α) prevent HIV-1 from utilizing these coreceptors by either direct competition with the HIV-1 Env binding sites or down-regulation of coreceptor expression (86, 87). β-chemokines are produced by many cells but mainly by CD8+ T cells and have been an important players in CD8+ T cell noncytotoxic anti-HIV responses.

RANTES is a CC chemokine that mediates chemotaxis and activation of T cells, eosinophiles, basophils, monocytes, dendritic cells and even neurons. It is released from intracellular storage granules of platelets and eosinophils upon activation. RANTES is able to inhibit R5 (macrophage-tropic) HIV-1 entry via the CCR5 receptor (86). A key observation came from the work of Gallo and colleagues who purified soluble HIV-suppressive factors produced by CD8+ T cell clones which were identified as the CC chemokines RANTES, MIP-1α and MIP-1β (88). In addition, RANTES can also bind the CCR3 chemokine receptor, which acts as a coreceptor for dual tropic (R5X4) HIV-1 strains (89). HIV infection of microglia, which express CCR3 and CCR5 might be important in the neuropathology of AIDS (90). To date, the observation that RANTES secretion by CD8+ T cells blocks HIV infection has led some workers to champion the idea of using modified forms of RANTES as peptide therapeutics in the treatment of AIDS (91). However, there are some drawbacks due to the extremely high doses of recombinant human proteins that would be required to be administered
and the potential deleterious effects that would ensue from blocking CCR3, and CCR5 receptors.

MIP-1α and MIP-1β are other members of the CC subgroup of the chemokine family which have been shown to inhibit infection by M-tropic strains of HIV-1 (77). Many studies have shown that the kinetics of induced MIP-1α and MIP-1β are related to the severity of disease progression (92, 93). The CD8+ T cells of LTNP and exposed uninfected people produce high levels of MIP-1α, MIP-1β and RANTES, but in patients progressing to AIDS, these levels are dramatically low. Paxton et al. demonstrated that CD4+ cells from exposed uninfected individuals secreted high levels of β-chemokines and had relatively lower surface expression of CCR5 (94). This may indicate that β-chemokines do play an important role in HIV pathogenesis. Several potential approaches for the use of MIP-1α and MIP-1β in HIV disease treatment have been proposed. Yang et al. (95) inactivated the CCR-5 coreceptor by targeting a modified CC-chemokine to the endoplasmic reticulum to block the surface expression of newly synthesized CCR-5. This approach has been tested in cultured cells and rendered them resistant to HIV infection. However, none of those approaches has yet progressed to the stage of clinical testing. In addition to antiviral roles, MIP-1α and MIP-1β also regulate inflammatory cells, and normal homeostatic processes, such as: hematopoietic cell development, lymphocyte differentiation and trafficking, immune modulation, bone remodeling, and wound healing.

In addition to β-chemokines, CD8+ T cells also secrete IL-16, another component of the noncytotoxic antiviral response, which was initially described as a chemoattractant with specificity for CD4+ T cells (96). IL-16 exerts strong negative control of HIV-1 and SIV replication at the level of transcription regulation (97, 98). In transient transfection studies with
HIV-1 LTR-reporter gene constructs, IL-16 pretreatment suppressed both phorbol ester- and Tat-stimulated HIV-1 promoter activity by 60-fold (99). This effect of IL-16 required sequences within the core enhancer, but was not simply due to down-regulation of the binding activity of transcription factors such as NFκB. Data suggest that IL-16 induces the activation of a transcriptional repressor which functions through sequences within or immediately adjacent to the core enhancer. Others have demonstrated that IL-16 is capable of inhibiting both T tropic and M tropic isolates of HIV and that some antiviral effects are observed even if IL-16 is added after the establishment of infection (98).

CD8+ T-cell antiviral factor (CAF):

The inhibition of HIV-1 replication by CAF was demonstrated using a trans-well culture device in which CD8+ cells were separated from infected CD4+ cells by a permeable membrane filter (71, 80), and also by exposing infected CD4+ cells to filtered supernatants from cultured CD8+ cells (75, 80, 100). CAF is a small protein (approximately 30kD) and has been shown to be both heat and pH stable, and resistant to trypsin (74). It may be a novel antiviral factor as it has been shown to lack identity to known cytokines and antibodies against the cytokines did not inactivate the antiviral effect of CAF (100). CAF is also distinct from β-chemokines secreted by CD8+ cells. For example, the β-chemokines block replication of only primary nonsyncytium-inducing strains that use the CCR5 chemokine receptor. They do not block syncytium-inducing strains, because the latter use a different coreceptor for their entry into cells (84). In contrast, CAF can block the replication of all strains of HIV (78). In CD4+ T cells infected with a CCR5 virus, neutralizing antibodies to RANTES, MIP-1α, and MIP-1β could not substantially block the effect of CAF to suppress HIV (101, 102). The β-chemokines
and CAF suppress HIV at different points in the viral life-cycle, the former blocking the entry of HIV into the cell (84), and the latter blocking virus production at the level of transcription (103).

Studies have shown that CAF inhibits HIV replication by down-regulating viral RNA transcription. Mackewicz et al. (103) used naturally HIV-infected CD4+ cells cultured with autologous CD8+ T cells, which resulted in an 80-90% reduction in the number of CD4+ cells producing viral proteins and the number of cells expressing viral RNA. They also demonstrated that CAF-containing CD8+ cell culture fluids were able to reduce expression of HIV protein and RNA in CD4+ cells. The decreased amount of mature viral particles produced and HIV proteins expressed was likely the consequence of the HIV replication cycle being halted at the stage of RNA synthesis. They also demonstrated by northern blot analyses that the transcription of all species of HIV RNA was down-regulated upon exposure of the infected CD4+ cells to autologous CD8+ T cells (103). Furthermore, they found CAF-containing CD8+-cell culture fluids suppressed the ability of HIV to enhance LTR-driven transcription of the luciferase reporter gene in 1G5 cells (103). These findings have been confirmed by other studies. Bagasra and Pomerantz (104) found that depletion of CD8+ cells from acutely infected PBMCs greatly increased the number of cells exhibiting active transcription of HIV RNA, which indicated that the antiviral activity of CD8+ cells can modulate the expression of HIV RNA.

The HIV-1 LTR contains a negative regulatory element (NRE) which down-regulates the rate of LTR-directed transcription and HIV-1 replication. Two major protein-binding sites within the negative regulatory element have been identified. One site contains a palindromic sequence with significant identity to the chicken ovalbumin upstream promoter (COUP)
element. Mutation of this site leads to an increase in LTR-directed transcriptional activity compared with the wild type, consistent with the element being a functional part of the NRE (105, 106). Two nuclear factor of activated T cells (NFAT-1) sites have also been associated with down-regulatory activity (reviewed in 21). CD8+ T cell-mediated inhibition of the HIV-1LTR activated by phorbol ester and calcium ionophore was shown to be dependent on the NFκB element (107). In addition, CD8+ T cell-mediated inhibition of the HIV-1 Tat-activated LTR was shown to be dependent on the NFAT-1 site (108). Culturing Jurkat T cells with CD8+ T cell supernatant demonstrated an inhibition in binding at the NFκB sites and increased binding at an AP-1 site which overlaps the COUP site (109). This may be the mechanism used by CAF to suppress HIV-LTR transcription.

CAF activity is not antigen-specific, and is not restricted to human immunodeficiency virus. Le Borgne et al. (110) showed that Epstein-Barr virus (EBV)-specific CTL line was able to suppress T cell-tropic strain of HIV-1 after reverse transcription. They found that an EBV-specific CTL line from an HIV-seronegative donor was as efficient as HIV-specific effectors in suppressing replication of macrophage- and T cell-tropic HIV-1 strains in vitro. The HIV-suppressive activity mediated by a soluble factor(s) present in the culture supernatant was detectable for up to 14 days following stimulation of EBV-specific CD8+ cells with the cognate epitope peptide. Following acute infection of the CEM T cell line with a T cell-tropic strain of HIV-1, EBV-specific CTL line supernatant containing HIV-suppressive activity did not block virus entry but was shown to interfere with virus replication after the first template switching of reverse transcription. Similar CAF activity was observed in a simian immunodeficiency virus model (111), which showed a suppressive effect by CD8+ cells and by CD8+ cell culture fluids on LTR-driven chloramphenicol acetyltransferase (CAT)
transcription. Other studies have observed CAF activity with SIVagm (82), HIV-1 (LAV) and HIV-2 (111), as well as in chimpanzee and baboon models of HIV infection (112).

The production of CAF by CD8+ T cells is dependent upon activation which can be mediated by lectin, mitogen as well as antibodies to CD28 and CD3 (113). Variation in CAF production between individuals may be due to differences in levels of particular CD8+ T cell subsets and the response of subsets to activation (70). Studies have shown CAF activity corresponds to CD29+/CD45RA- memory cells and CD28+/HLA-DR+ cells (76). The ability to suppress HIV replication from CD8+ T cells was increased by the presence of autologous macrophages, particularly macrophages expressing CD86, which costimulated CD8+ cells to produce IL-2 and increase CD8+ cell antiviral activity (114). In the feline immunodeficiency virus (FIV) infection model, CD8 antiviral activity was shown to be mediated by the CD8 alpha+ beta- (CD62L-) phenotype which expanded throughout the asymptomatic infection, and was the dominant CD8 phenotype in long-term infected cats. Infection with FIV also increased expression of CD44, CD49d, and CD18, activation phenotypes. CD8 alpha+ beta- subpopulations demonstrated strong antiviral activity in the FIV acute-infection assay (115). However, similar data has not been determined in HIV-infected patients.

High levels of CAF correlate directly with high numbers of circulating CD4+ cells and a healthy clinical state, as demonstrated by increased CAF production by CD8+ T cells of LTNP (77, 81). In acute infection assays, CD8+ T cells of many asymptomatic individuals can control HIV replication at a ratio of 0.25, whereas CD8+ T cells of AIDS patients require a CD8+: CD4+ cell ratio of 2-4 or higher (76, 77). Moreover, in infected individuals followed over time, the noncytotoxic anti-HIV response of CD8+ cells decreases concomitant with the development of disease (77). These findings were confirmed by observations with lymph
nodes. Subjects with long-term asymptomatic disease courses have strong noncytotoxic suppressing activity associated with CD8+ cells in lymphoid tissues (116). In contrast, symptomatic patients show a reduced antiviral response by CD8+ cells concomitant with high levels of productively infected cells detected in the lymph node (116). However, opposite results have been documented, which showed no correlation between the extent of CAF mediated suppression and either clinical stage of infection or CD4+ T cell count (117). Numerous reasons may contribute to the diverse outcomes, including the type of bioassays used to measure CAF activity.

Here, we summarize CD8+ effector immune response to HIV in Fig. 2., which includes cytotoxicity and non-cytotoxicity.

**Rationale**

HIV infection is one of common infectious diseases in the world. The control of viral spread and effective treatment of infected individuals are major challenges for research in this field. Despite the rapid pace of novel discoveries in HIV research, the host immune functions which impede HIV replication and disease progression remain unresolved. Within the host immune response, CD8+ T cells play important roles in the control of HIV-1 infection, mediating the removal of HIV-1-infected cells and the release of soluble factors capable of suppressing HIV-1 replication. HIV infection results in an increased level of cellular activation with accompanying increases in cell surface receptor expression by CD8+ T cells. These include CD28 which is associated with good prognosis (118, 119) and CD38 which is associated with disease progression (120-123). In addition, CD8+ T cells which express high levels of CD38 and HLA-DR are associated with faster disease progression whereas
CD8+HLA-DR+CD38- cells are associated with good health (120, 121, 124). However, the complete relationship between these subpopulations and CD8+ T cell antiviral activities remains unknown. Further research regarding subpopulations which produce antiviral activity and the effect of infection of these on cellular function may provide insight into the pathogenesis of HIV infection.
Figure. 2. CD8+ effector immune response to HIV. Classical CD8+ cytotoxic T cells recognize viral determinants on infected cells in the context of MHC class I presentation and lead to apoptosis by perforin/granzymes, Fas/FasL and TNF. CD8+ T cells carry out noncytotoxicity by producing β-chemokine which inhibit viral entry, and CAF and IL-16 which suppress viral transcription.
CD8+ Effector

Cytotoxicity
- Perforin/Granzymes
- Fas/FasL
- TNF
  - Apoptosis

Non-cytotoxicity
- β-Chemokine
- CAF
- IL-16
  - Viral Entry Inhibition
  - Suppression Viral Transcription
Hypothesis

In HIV infection, evidence has shown that the cell-mediated immune response is the most important line of defense in the HIV-infected host and that CD8+ T cell noncytolytic antiviral control is a major part of this response. We hypothesize that the level of noncytolytic antiviral factors might be different between infected individuals and healthy controls and in addition, different levels of antiviral factors may be produced by subpopulations of CD8+ T cells associated with disease pathogenesis. Further, we hypothesize that infection of these cells may compromise their noncytolytic antiviral activity.

Objectives

1. Examination of antiviral factors, such as β-chemokines, CAF and IL-16, produced by CD8+ T cells and subpopulations of uninfected and HIV-1-infected individuals, including those which modulate transcription and viral entry.

2. Determination of the effect of infection with the T-tropic strain HIV11B on the noncytolytic antiviral abilities of CD8+ T cells and subpopulations.

3. Determination of RNA viral copies of infected individual CD8+ T cells and subpopulations, and correlation with the production of noncytolytic factors.
Material and Methods

HIV-1+ subjects and controls.

Heparinized peripheral blood samples were obtained from HIV-1-infected subjects at different stages of disease progression. A total of 24 HIV-1 infected subjects were included in our studies. The majority of the infected cases were being treated with antiviral drugs at the time of the study. A total of 25 HIV-1 seronegative blood donors were randomly selected as comparisons. All samples were collected from the Ottawa Hospital after informed consent (approved by Ottawa Hospital Research Ethics Board). In the study, AIDS was defined either by CD4+ T-cell count <200/ml.

Cell selection.

PBMCs were isolated by Ficoll-Paque gradient separation. The CD8+ T cells were positively selected using CD8 MultiSort magnetic beads (MultiSort Kit, Miltenyi, Auburn CA). Selected cells were incubated with MultiSort Release Reagent to release the magnetic beads, allowing for a second selection of a CD8+ sub-population. The CD8+ T cells were then incubated with MultiSort Stop Reagent, followed by incubation with specific antibodies against sub-phenotypes (CD28, CD38, HLA-DR) conjugated with FITC. Anti-FITC-conjugated microbeads were then used to select sub-populations. Purity of the CD8+ T cells was routinely >98% and sub-phenotypes were >90%, as determined by flow cytometry. CD3 staining of CD8+ cells confirmed that the CD8+ cell population was >94% T cells.
**Measurement of β-chemokines and IL-16 by purified CD8+ T cell and subpopulations.**

The selected cells were cultured for 3 days in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), penicillin (100 units/ml), streptomycin (100 µg/ml), phytohemagglutinin (PHA, 5 µg/ml, Sigma), and interleukin 2 (IL-2, 20 U/ml, Sigma) at a concentration of 1 x 10^6 cells/ml. The cells were then washed twice, resuspended in the same medium without PHA, and re-cultured for 3 days. Supernatants were collected at both time points and stored at -70°C. The β-chemokines (R&D Systems, Minneapolis, MN) and IL-16 (Biosource, Auburn CA) levels were analyzed in CD8+ T cell supernatants at the first time point supernatants by ELISA (according to the manufacturer's instructions). The second time point supernatants were stored for the measurement of CAF.

**HIV infection assays.**

PBMCs of HIV-1 seronegative donors were isolated by Ficoll gradients and CD8+ T cells and subpopulations were positively selected with magnetic beads as described above. Infection was performed using cells which had been previously stimulated with PHA (5 µg/ml) and IL-2 (20 U/ml). Cells were pretreated with polybrene (2 µg/ml) for one hour, and then infected with 300 TCID₅₀/10^6 cells of the T cell-tropic laboratory strain HIV₃₉₉ (AIDS Research and Reference Program Division of AIDS, NIAID, NIH) for 3 days. The cells were then washed and recultured in RPMI containing PHA and IL-2. Samples were collected at days 5, 10 and 15 post-infection. Virus replication was assessed by p24 production as detected by ELISA (Organon Teknika, Durham, NC). The β-chemokines and IL-16 in cell supernatants were determined as previously described.
Transfections and vectors.

The vector pLTRCAT incorporates the HIV-LTR of the BRU strain at position +77, from which it drives CAT expression (125). The vector pSVtat (126) was used to enhance HIV-1 transcription. The CD4+ Jurkat T cell line (3 × 10^7 cells) was transfected with 10 μg of pLTRCAT and 5 μg of pSVtat, using a DEAE-dextran procedure (127). Transfected cells were cultured in supernatant derived from CD8+ T cells or subpopulations at ratio 1:1 with medium. After 24 hr, transfected cells were stimulated with phorbal myristate acetate (PMA, 25 ng/ml; Sigma) and ionomycin (2 μM; Sigma) for 18 hr. The cells were then lysed by three rounds of 5 minute freeze-thaw cycles. Lysates were standardized for protein concentration and assayed for the presence of chloramphenicol acetyltransferase (CAT) by ELISA (Roche, Indianapolis, IN). Results were calculated as percent inhibition of CAT compared to control samples cultured in RPMI.

Semiquantitative PCR assays.

Total DNA and RNA were purified from infected subject’s CD8+ T cells and subphenotypes by phenol-chloroform extraction and Trizol purification (Gibco-BRL, Burlington, ON) respectively. Complementary DNA (cDNA) was synthesized using the First-Strand cDNA Synthesis Kit (Amersham Pharmacia, Piscataway, NJ). Prior to the semiquantitative evaluation of viral DNA and cDNA content, the samples were serially diluted and subjected to PCR analysis, using primers and probe for HIV-1 p24. Amplification reactions were carried out in a mixture containing 1X PCR buffer, 2mM deoxynucleoside triphosphate, and 2 U of Taq polymerase (5U/μl, Gibco) using 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min in a DNA thermal Cycler (Progene, TECHNE, Princeton, NJ). The amplified PCR products were
denatured at 94°C for 5 min and hybridized with a p24 specific 32P-labeled oligonucleotide probe in liquid phase at 55°C for 10 min as described previously (128). The results were analyzed by densitometric analysis of the autoradiograms after electrophoresis on a SDS-4% polyacrylamide gel. The primer and probe sequences used for HIV p24 were as follows:

primer1: 5'-ATAGAGGAAGAGCAAAAAACAAA-3';
primer2: 5'-GTTCTGAAGGGGTACTAGTGT-3';
primer3: 5' -AAAAATTACCTATAGTGCA-3';
primer4: 5' -ATGTCACTTCCCCCTGGTCT-3'; and
probe: 5'-ACCCCGCTGTAGTTGTCGTTAGA-3'.

End labeling of oligonucleotide probes.

The oligonucleotide probes used in the semiquantitative PCR analysis were end-labeled with [γ-32P]ATP (4,500Ci/mmol; NEN Life Science Products Inc. Boston, MA) by using the T4 polynucleotide kinase. Reactions were incubated at 37°C for 1 h, and the reactions then stopped by the addition of 60ul of 25mM EDTA. The labeled probes were purified on a G50 column (Amersham Pharmacia). The radioactivity in 1 ul of the labeled probe was counted in 5ml of scintillation liquid with a scintillation counter (Beckman LS6500, Fullerton, CA). A specific activity of >10^5 cpm/ul was routinely obtained.

Densitometric analyses.

Densitometric analysis of the autoradiograms was carried out with a Personal Densitometer (Storm 862, Baie d’Urfé, QU) with ImageQuant NT.
**Statistical analysis.**

Data were entered into a Microsoft Excel 97 spreadsheet. Descriptive statistics were generated using the Statistical Package for the Social Sciences (SPSS). Analysis was conducted using parametric and nonparametric methods where appropriate after normalization. For independent variables. Student’s t-test was used to assess differences between groups, as used for chemokine measurement in other published papers. Using Pearson’s correlation coefficient and testing for a significant correlation by the t-test assessed relationships between continuous variable. For all analyses, a $P$ value of 0.05, two-tailed, was set as the level of statistical significance for excluding type I error.
Results

β-chemokine and IL-16 production by CD8+ T cells and sub-populations:

Activation-induced β-chemokine production by CD8+ T cells and CD8+ sub-populations derived from peripheral blood mononuclear cells of uninfected and HIV-infected individuals was analysed. In comparisons of HIV-infected individuals with CD4 counts greater than 200/µl with uninfected controls, significantly increased production (indicated by asterisk) of MIP-1α and MIP-1β by total CD8+ T cells was associated with infection (Fig. 3A). Overall, the CD8+CD38- subset produced significantly increased levels of MIP-1α, MIP-1β and RANTES as compared to uninfected controls (Fig. 3E). Low levels of IL-16 were produced by total CD8+ T cells and a significant difference was noted only by the CD38+ sub-population. While RANTES production by total CD8+ T cells was not modulated by HIV infection (CD4>200/µl), significantly increased levels of RANTES were produced by CD28- (Fig. 3C), CD38- (Fig. 3E), HLA-DR- (Fig. 3F) and HLA-DR+ (Fig. 3F) subsets.

In comparisons of the HIV+ CD4 counts>200/µl and CD4 <200/µl groups, the former secreted higher levels of β-chemokines and IL-16 (indicated as # in Fig.3). The HLA-DR+ subset produced significantly increased levels of MIP-1α, MIP-1 and RANTES (Fig. 3F). RANTES production was increased in the CD4>200/µl by total CD8+ T cells and all subsets examined (Fig. 3A-G). Significantly increased chemokine production was also observed for MIP-1α by CD28+ (Fig. 3B), CD28- (Fig. 3C), CD38- (Fig. 3E) and HLA-DR- (Fig 3G) subsets; MIP-1β by CD28+ (Fig. 3B) and HLA-DR+ (Fig. 3F). Increases in IL-16 production were observed for CD38+ (Fig. 3D) and HLA-DR- (Fig. 3G) subsets, however, the levels of IL-16 produced by total CD8+ T cells and all subsets were very low. The observations indicate
Figure 3. Analysis of β-chemokine and IL-16 production by unseparated CD8⁺ T cells and sub-populations from HIV-1 infected individuals and controls after stimulation with PHA and IL-2. (A) Unselected CD8⁺ T cells; (B) (C) CD8⁺CD28⁺ and CD8⁺CD28⁻; (D) (E) CD8⁺CD38⁺ and CD8⁺CD38⁻; (F) (G) CD8⁺HLA-DR⁺ and CD8⁺HLA-DR⁻, respectively. (*) Values from cells taken from infected individuals were compared to cells from healthy controls. (P = 0.00004 - 0.034). (#) Values from cells taken from infected individuals with CD4 < 200/ml were compared to CD4 > 200/ml, (P = 0.002 - 0.04). The results are presented as mean± SD of duplicate wells. P values were calculated using Microsoft Excel.
A: Unselected CD8+ T cells

- **MIP-1α**
- **MIP-1β**
- **RANTES**
- **IL-16**

Mean secreted chemokine level (ng/ml)
that overall total CD8+ T cells and sub-populations of HIV+ individuals with CD4+ T cells counts greater than 200/μl produced higher levels of soluble antiviral suppressive factors.

**Effect of in vitro infection of CD8+ T cells and sub-populations on antiviral factor production:**

HIV infected CD8+ T cells have been detected *in vivo* [36-38], however, studies have not been performed to indicate how infection might affect cellular function. To determine the effect of infection of CD8+ T cells and sub-populations on β-chemokine and IL-16 production, positively selected CD8+ T cells and sub-populations were infected with HIVMB and p24 was measured at days 5, 10, and 15 post-infection in cell-free supernatant (Fig.4). As it was seen in figure, the virus replication was increased over time course. However, Levels of MIP-1α, MIP-1β, RANTES and IL-16 measured at the same time points (Fig.5), showed decreased trend. Cells were stimulated by PHA and IL-2 throughout the infection. Infection of total CD8+ T cells resulted in 3-8 fold increases in the production of β-chemokines and IL-16 at day 5 post-infection (Fig 5B), compared to uninfected CD8+ T cells (Fig. 5A). While chemokine and IL-16 production rose over the course of the 15 day culture of uninfected CD8+ T cells, infection resulted in a dissipation of the higher 5 day levels over time, with the exception of IL-16 levels which increased again after day 10 of infection (Fig.5B). MIP-1α was produced more strongly by CD28+ (Fig. 5C), CD38- (Fig. 5E) and HLA-DR- cells (Fig. 5G) and production was increased over the time course in CD28+ and HLA-DR- cells (Fig. 5C, 5G).
Figure 4. P24 production by *in vitro* infected unselected CD8+ T cells and subpopulations. P24 was measured by ELISA at post-infection D5, D10 and D15. Over the time course, P24 level was increased. Values represent mean ± SD of duplicate wells.
P24 Value (pg/ml) of infected CD8 T cells and subpopulations
Figure 5. Impact of HIV\textsubscript{M} in vitro infection on the production of β-chemokines and IL-16. Data is representative of one of five experiments. (A) – (B) Uninfected CD8+ T cells and infected CD8+ T cells; (C) – (D) Infected CD8+CD28+ and CD8+CD28-; (E) – (F) Infected CD8+CD38+ and infected CD8+CD38-; (G) – (H) Infected CD8+HLA-DR+ and CD8+HLA-DR-, respectively.
Similar to infected total CD8+ T cells (Fig. 5B), CD28+ (Fig. 5C) and CD38- (Fig. 5F) subsets produced sustained levels of IL-16. Both CD28- (Fig. 5D) and CD38+ (Fig. 5E) subsets produced enhanced levels of RANTES. Increased production of MIP-1β was observed for CD28- (Fig. 5D), CD38- (Fig. 5F) and HLA-DR+ (Fig. 5G) subsets.

**CAF activity in CD8+ T cells and sub-populations:**

CAF is another CD8+ non-cytolytic antiviral factor which inhibits HIV-1 gene expression. We examined differences in CAF production of total CD8+ T cells and different CD8+ sub-populations from healthy controls and patients, on LTR-mediated transcription in Jurkat T cells. Figure 6A shows the result of culture of transfected Jurkat T cells with supernatants of total CD8+ T cells, CD8+CD28+ and CD8+CD28- T cells of HIV-infected and uninfected individuals. There were no differences found in the ability of these cell types to produce CAF. However, both CD38+ (Fig.6B) and HLA-DR+ (Fig.6C) sub-populations of infected individuals demonstrated significantly stronger suppression of LTR-CAT gene expression, compared to HIV infected individuals (P<0.0168 and 0.0175, respectively). Increased CAF activity by these sub-populations did not correlate with differences in chemokine production as determined by regression analysis, suggesting again that CAF production is independent of chemokine production by CD8+ T cells.
Figure 6. CAF production by CD8+ T cell sub-populations. Jurkat T cells transfected with pLTR-CAT plus pSVtat were cultured with RPMI or with CD8+ T cell supernatant:RPMI (1:1) for 16 hours. Cells were additionally treated with PMA and ionomycin for 18 hours. CAT activity in cell lysates was measured by ELISA. Results show the mean and standard deviation of 4 independent experiments. Asterisks indicate statistically significant CD8-mediated decreases from infected subjects in LTR-mediated gene expression as compared to healthy controls.
**Virus copy number, chemokine/cytokine production and clinical status:**

The issue of whether virus copy number in CD8+ T cells of infected individuals is associated with chemokine/cytokine production has thus far been controversial. This issue was investigated using semiquantitative PCR. As a standard, 8E5/LAV cells were used which bear one virus copy per cell (107). Identical amounts of DNAs and cDNAs from samples were serially diluted 3-fold prior to a pre-quantitative PCR (Fig.7). Virus copy number per ng of PCR product was compared to chemokine/cytokine production using Pearson’s correlation coefficient. As shown in Table 1, a significant reverse linear correlation was evident between CD8+ T cell virus copy number and production of MIP-1α (γ = -0.879, P = 0.001), MIP-1β (γ = -0.663, P = 0.01) and IL-16 (γ = -0.578, P < 0.05). There was no significant correlation between CD8+ T cell virus copy number and RANTES production. We also examined the association between virus copy number from sub-populations and β-chemokine and IL-16 production, however, no correlation was demonstrated when the data were grouped according to CD4 counts (data not shown).
Figure 7. Semi-quantitative PCR. The total cellular DNAs and cDNAs were isolated and amplified by PCR, followed by hybridization with labeled probe. Autoradiograms were analyzed by densitometry. 8E5/Lav cells which bear one copy of virus/cell, were used as a standard. Standards and samples were diluted as indicated.
Semi-quantitative PCR

8E5/Lav cells as standard

sample

$10^4$  $10^3$  $10^2$  $10$  $1$

$3^{-1}$  $3^{-2}$  $3^{-3}$
Table 1. Relationship between β-chemokine and IL-16 production with virus RNA copy number from CD8+ T cell and sub-phenotypes.

<table>
<thead>
<tr>
<th></th>
<th>CD8+</th>
<th>CD8+</th>
<th>CD28-</th>
<th>CD28+</th>
<th>CD8-</th>
<th>HLA-DR+</th>
<th>HLA-DR-</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIP-α</td>
<td>-0.8799*</td>
<td>-0.376</td>
<td>-0.5069</td>
<td>-0.6121</td>
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<tr>
<td>MIP-1β</td>
<td>-0.6636*</td>
<td>-0.269</td>
<td>-0.6034</td>
<td>-0.0369</td>
<td>-0.608</td>
<td>-0.608</td>
<td>-0.258</td>
</tr>
<tr>
<td>RANTES</td>
<td>-0.4711</td>
<td>-0.294</td>
<td>-0.594</td>
<td>-0.0294</td>
<td>-0.069</td>
<td>-0.069</td>
<td>-0.136</td>
</tr>
<tr>
<td>IL-16</td>
<td>-0.5787*</td>
<td>-0.4702</td>
<td>-0.369</td>
<td>-0.395</td>
<td>-0.089</td>
<td>-0.089</td>
<td>-0.069</td>
</tr>
</tbody>
</table>

Data showing linear correlation (γ). * indicates significant difference (MIP-1α, P = 0.001; MIP-1β, P = 0.01; IL-16, P = 0.036).
Discussion

CD8+ T cells mediate the control of HIV-1 replication by three distinct mechanisms. Infected cells are recognized and lysed in an MHC-restricted manner by CD8+ cytotoxic T lymphocytes (66). Two noncytolytic responses by CD8+ T cells have also been reported. One involves blocking the entry of HIV-1 into the cell, which is mediated by the binding of chemokines to receptors, which also serve as co-receptors for the virus (11). However, CD8+ T cells also elicit an activity which inhibits HIV long terminal repeat (LTR)-mediated transcription (103, 107, 108). This activity is mediated by a soluble factor which is distinct from previously identified cytokines and chemokines and has been termed the CD8+ T cell antiviral factor (CAF). CD8+ T cells also produce IL-16 which has been shown to strongly inhibit LTR-mediated transcription.

In recent years it has been shown that chemokines play a major role in the pathogenesis of HIV infection. Since the discovery of chemokine receptors as necessary coreceptors for viral entry (11) an increased understanding of the pathogenesis of HIV and potential therapeutics have evolved. The β-chemokines, MIP-1α, MIP-1β and RANTES, effectively block entry of macrophage-tropic HIV-1 into CD4+ T cells and monocytes (11, 84) by binding the chemokine receptor CCR5, which serves as a coreceptor required for entry of macrophage-tropic HIV. Similarly, the α-chemokine, SDF-1, has been shown to bind the CXCR4 coreceptor utilized by T cell-tropic viruses, thus blocking viral entry (11, 84).

A wide range of clinical outcomes has been observed in HIV-infected individuals. Some patients might progress rapidly to AIDS while others might be asymptomatic and maintain high CD4+ cell counts for many years (3). CD8+ T lymphocytes are believed to play a pivotal role in limiting HIV immunopathogenesis both by lysing HIV-infected cells and by
secreting factors that control viral replication. In this study, we have focused our attention on the importance of understanding the nonlytic, antiviral activity of CD8+ T cells and CD8+ sub-populations, including the ability to produce β-chemokines, IL-16 and the CD8+ T cell antiviral factor.

It has been shown that the production of CD8+ T cell nonlytic suppressive factors is associated with phenotypes expressed on the cell surface (97). Some of them, such as CD28, CD38 and HLA-DR also closely relate to an infected individual’s clinical state. CD28, a co-stimulatory molecule necessary for optimal T cell function, is expressed by approximately 75% of CD8+ T cells (129). The interaction of CD28 with its natural ligand B7 (expressed on antigen-presenting cells) stimulates T-cell proliferation activated by the CD3/T cell receptors or CD2 membrane receptors (130). It increases T-cell mediated cytotoxicity in resting lymphocytes and enhances the production of IL-2 and other cytokines produced by T1 helper lymphocytes (130). An altered CD28 expression on the CD8+ cells of HIV-infected patients has been hypothesized to explain defects in the generation of cytotoxic T cells. Studies have demonstrated that in HIV infection, CD8+CD28+ T cells, including naïve cells and memory CTL precursors, were decreased in AIDS patients compared with uninfected subjects (118, 119). Moreover, a reduction in the CD4+CD28+ cell subset was also observed in HIV-infected subjects (131). The decrease in CD28 expression on the CD4+ and CD8+ T-cell populations of HIV-infected patients may well explain some peculiar defects since the asymptomatic stage of infection, e.g. impaired T-cell proliferation in response to alloantigen and mitogen stimulation, and defective MHC-restricted and unrestricted cytotoxic capability.

Because of the apparent importance of CD28 in T cell activation, it is interesting to understand the function of the CD8+CD28- subpopulation, which lacks expression of a
membrane receptor for the B7/BB1 B cell differentiation Ag involved in T cell activation. CD8+CD28- T cells are not observed in the thymus and are present at only low frequency in cord blood, suggesting that these cells may represent a type of "memory" population (129). Consistent with this interpretation, CD8+CD28- T cells were morphologically large, granular lymphocytes, without expression of Ag associated with acute activation (e.g., HLA-DR, CD25, CD69). Freshly isolated CD8+CD28- T lymphocytes mediated potent anti-CD3 redirected cytotoxicity against FcR-bearing targets, demonstrating that the CD3/TCR complex is functional and that these cells possess cytolytic activity (129). CD8+CD28- cells are observed dramatically increased with HIV disease progression (131). Data reported in the literature support the hypothesis that decreased CD28 expression on CD8+ cells does not involve a down-modulation phenomena but might be a result of the appearance of a specific CD8+CD28- lymphocyte subpopulation with peculiar cytotoxic features. Several attempts to down-modulate CD28 expression on T cells by HIV infection or by treating cells with HIVgp120, cytokines failed (132). Gruters et al. (133) reported a longitudinal increase in the absolute numbers of CD8+CD28- cells in five out of six HIV positive patients, monitored over a period of 2–4 years.

Functional studies of the nonlytic differences between CD8+CD28+ T cells and CD8+CD28- cells have not been performed. In the present study, differences in the production of chemokines by CD28+ and CD28- subsets of HIV-infected versus uninfected cells were demonstrated. In HIV+ individuals with high CD4 count, CD8+CD28+ T cells produced much more MIP-1α and MIP-1β as compared to uninfected controls and HIV infected individuals with low CD4 counts. This indicates that CD8+CD28+ cells may have higher overall antiviral
activity. There findings are consistent with the observation that the CD8+CD28+ sub-population is usually associated with better prognosis (118, 119, 131).

Human CD38 is a 45kDa single-chain type II transmembrane glycoprotein with multiple functions. Its expression varies significantly with age. In adults, the CD38 protein is present on the majority of natural killer cells, T cells, B cells, monocyte/macrophages, and to some extent on platelets and erythrocytes (134). CD38 has functions as an ectoenzyme, an adhesion receptor, and a signaling molecule (135). Ligation of CD38 with agonistic antibodies induces variable effects on hematopoietic cells including growth stimulation, protection from apoptosis and the induction of cytokines (135). CD38 may play a role in T cell signaling as it has been demonstrated that ligation of CD38 on peripheral blood mononuclear cells and T cell lines induced activation and proliferation signals (136). Subsequent experiments revealed that ligation of human CD38 with specific mAbs induced the transcription of cytokines interleukin-1 (IL-1), tumor necrosis factor-α, and granulocyte-macrophage colony-stimulating factor at levels similar to those obtained after triggering T cell receptor CD3. However, the cytokines triggered in response to CD38 are quantitatively distinct from those induced via CD3 (137). For example, IL-6 mRNA and protein expression induced in response to CD38 ligation is greater than that induced via the T cell receptor CD3. In contrast, interferon-γ, IL-2 and IL-10 transcription is higher after activation by CD3 than after activation by CD38. Another apparent difference in these two pathways is that CD38-mediated cytokine induction did not require either T cell proliferation or addition of antigen-presenting cells (138). Further, signaling via CD38 in a T cell acute lymphoblastic leukemia (Jurkat) cell line led to Ca^{2+} mobilization with kinetics that were distinct from those induced via T cell receptor CD3 (138).
What is the role of CD38 in HIV pathogenesis? As in most viral infections, HIV-1 infection causes activation of B and T cells (both CD4 and CD8 cells), which consequently upregulates the surface expression of CD38 and other activation markers. One marker of HIV infection and progression to disease is an increase in the CD8+ population with expression of CD38. Several studies suggest that high CD38+/CD8+ ratios not only closely correlate with HIV stage, but are also strongly correlated with higher plasma viremia and lower CD4 count. Furthermore, increased CD38 expression by CD8+ T cells characterizes patients with poor prognosis and progressive disease (120-123). One Multicenter AIDS Cohort Study (MACS) strongly suggested that elevated CD38 on CD8+ T cells was the most predictive marker of those studied for the development of a clinical AIDS diagnosis and death (138). CD38 may also be a useful tool for monitoring highly active antiretroviral therapy (HAART). In HIV-infected subjects, individuals with high proportions of CD8+CD38+ cells will respond to HAART more rapidly than individuals with low levels. This evidence suggests that these cells may contribute to viral clearance and become effective when HAART decreases viral load (139). In addition, studies have shown that decreased CD38 expression on CD8+ T cells is a marker of effective response to HAART in adults, and persistence of high expression of CD38 is a marker of therapeutic failure in HIV-1 infected children (140).

In the present study, CD8+CD38+ T cells from HIV infected individuals did not produce higher levels of the three β-chemokines, in comparison to controls. Low β-chemokine production by the CD8+CD38+ T cell sub-population may contribute to its role as a predictor of poor prognosis. However, the CD8+CD38- subset from HIV infected individuals with high CD4 counts produced greater levels of all β-chemokines than individuals with low CD4 T cell counts, but not IL-16. Interestingly, CD8+CD38- cells from HIV+ individuals with low CD4
counts also secreted high levels of MIP-1α and this is the only sub-population through all subsets that we studied which displayed a difference in β-chemokine production in groups with low CD4 count. This may imply that even in late stage HIV infection, when host immune responses are reduced, there is still partial suppression against virus replication.

HLA-DR is a MHC class II antigen that is expressed on all hematopoietic progenitor cells as well as on mature resting B cells (141). HLA-DR expression increases on CD8+ T cells at HIV seroconversion, and remains high and relatively stable throughout disease progression (141). Individuals whose CD8+ T cells express HLA-DR but not CD38 after seroconversion experience a stabilization of their CD4+ T-cell counts and a less fulminant disease course, whereas individuals whose CD8+ T cells express both HLA-DR and CD38 experience a more aggressive course with rapid CD4+ T-cell depletion and a poorer (120,121,124). Significantly increased levels of MIP-1α and RANTES by CD8+HLA-DR+ cells from HIV+ individuals with high CD4 count was observed in our study. This may contribute to host protection against the virus. Our data also showed that in HIV infected subjects, the high CD4 count group generally produced increased levels of β-chemokines and IL-16 than the low CD4 count group. Therefore β-chemokine production may be closely related to an infected subject’s clinical state.

Based on the results of in vitro infection with the T-tropic lab strain HIVIIIb, CD8+ T cells and sub-populations from seronegative donors generally secreted increased β-chemokine and IL-16 levels, however these levels dissipated over a 15-day time course. This may suggest that virus infection impairs the ability of cells to produce chemokine/cytokine over time, thus reducing the nonlytic control of virus replication. This would be consistent with the inverse correlations found between virus copy number and chemokine/cytokine production.
Interestingly, a recent study from Gallo’s group has indicated that MIP-1α and MIP-1β production by CD8+ T cells correlated with asymptomatic HIV infection (93). However these correlations did not apply to RANTES. This findings is consistent with our results.

Reports of correlations between the β-chemokine production and viral copy number have thus far not been consistent. In a previous study, plasma levels of MIP-1α correlated with both CD4+ and CD8+ T-cell counts, but this did not apply to MIP-1β and RANTES (93). We have also shown that the production of MIP-1α and MIP-1β, as well as IL-16 are negatively correlated with virus RNA copy number in HIV-infected CD8+ T cells. However, Aleman and colleagues (135) found a reverse relationship between infected individual’s PBMC viral RNA copies and MIP-1β levels, but no correlation between MIP-1α or RANTES. Here we demonstrated that the production of MIP-1α, MIP-1β and IL-16, but not RANTES, from purified CD8+ T cells, were negatively correlated with viral RNA copy number in HIV-infected CD8+ T cells. No correlation was seen between β-chemokines or IL-16 and viral RNA copies in CD8+ T cell subpopulations. Different results from study to study may reflect different methods or systems used. This issue deserves further study.

The importance of β-chemokines in HIV-1 pathogenesis has opened new perspectives for the development of chemokine-based therapeutic approaches for HIV infection. This is currently an area of intense interest. Much attention has been paid to N-terminal modifications of the chemokine RANTES such as aminooxypentane (AOP)-RANTES and N-nonanoyl (NNY)-RANTES have drawn a lot of attention. Modified RANTES compounds induce rapid CCR5 internalization, downregulation and much slower receptor reexpression than native RANTES, further, NNY-RANTES is more efficient than AOP-RANTES (142) and further, both RANTES and AOP-RANTES have been shown to promote CCR5 desensitization.
involving G protein-coupled receptor kinases-2 and β-arrestin equally well (143). An important difference between the two molecules is that (AOP)-RANTES is more efficient than RANTES in promoting Ser/Thr phosphorylation of the receptor and association of G protein-coupled receptor kinases-2, beta-arrestin, and clathrin to CCR5 (143). After stimulation, either ligand induces rapid, transient association of dynamin to CCR5, implicating this protein in receptor sensitization. This association is faster and longer-lasting following (AOP)-RANTES stimulation (142).

A non-allelic isoform of MIP-1α, termed MIP-1αP, is another active naturally occurring inhibitor of HIV entry. Townson et al. (144) has shown that, like RANTES, the addition of AOP to MIP-1alphaP enhances its interactions with CCR1 and CCR5, allows more effective internalization of CCR5, and increases the ligand's potency as an inhibitor of HIV entry through CCR5. Moreover, they have demonstrated that AOP-MIP-1alphaP is about 10-fold more active than AOP-RANTES at inhibiting HIV entry, making it the most effective chemokine-based inhibitor of HIV entry through CCR5 described to date.

The CD8 antiviral factor (CAF) suppresses HIV-1 replication in a nonlytic, MHC class I-unrestricted manner, at the level of transcription. Levy et al. demonstrated that CAF, which is distinct from known cytokines or chemokines, is a protein of approximately 30 kD (74). Chun et al (145) measured the incidence of CAF at different disease stages in a comparison of patients who were drug-naïve or treated with HAART. What they found is that in chronically infected patients who were not receiving antiretroviral therapy, CAF played a major role in suppression of HIV replication and that this activity could not be abrogated by anti-CC-chemokine antibodies. CAF also completely suppressed HIV replication in acutely infected patients, in whom HAART was initiated early. However, less CAF activity was found in long
term non-progressors (LTNPs) as well as in chronically infected patients without HAART. In contrast, in study of HIV-infected individuals who were either drug-naïve or had commenced combination therapy within 6 months of infection, combination therapy resulted in decreased in CAF activity (146). Conflicting reports such as these may be the result of the timing of combination therapy following diagnosis, the level of virus replication at the time of treatment, the particular drug combinations used as well as compliance with the drug regimen.

Certain activated phenotypes might modulate the production and strength of CAF activity. Barker et al. have shown increased production of CAF by CD8+ T cells in the presence of autologous macrophages (114) which was mediated by CD86B7 expressed on macrophages (114). However, the specific phenotype of CD8+ T cells providing factors that suppress HIV-1 LTR-driven gene expression has not yet been determined. We demonstrated that CD38+ and HLA-DR+ subsets derived from uninfected subjects, but not infected subjects, had stronger suppression of LTR-mediated gene expression. We did not observe correlations between CAF activity and β-chemokine or IL-16 production. This was particularly evident with CD8+CD38+ T cells which produced high CAF activity but low β-chemokine production. Other sub-populations which had high β-chemokine production did not demonstrate strong CAF activity. Taken together, CAF is an important antiviral factor produced by CD8+ T cells and increased CAF activity can be attributed to particular subpopulations. This factor may be a potential therapeutic and here we have identified sub-populations which could be used to facilitate its identification.

In summary, this study has shown compelling evidence that the production of CD8+ T cell nonlytic, antiviral factors, including β-chemokines, CAF and IL-16 may be associated with specific sub-populations. In addition, MIP-1α, MIP-1β and IL-16 were found to be negatively
correlated with virus copy number in CD8+ cells. Further, no correlation was found between CAF activity and chemokine or IL-16 production. The ability of CD8+CD38+ T cells to produce greater levels of CAF and low levels of MIP-1α, MIP-1β, RANTES and IL-16 identifies a subpopulation which would be advantageous to the isolation of CAF.
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