Innate, non-cytolytic CD8+ T cell-mediated suppression of HIV replication by MHC-independent inhibition of virus transcription

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Introduction

The Human Immunodeficiency Virus (HIV) is the causative agent of the acquired immunodeficiency syndrome (AIDS), characterized by a severe deterioration of the immune cell-mediated response due to the loss of T cells, and infects around 36.7 million people worldwide. UNAIDS estimates 1.8 million new HIV infections annually and 1 million AIDS-related deaths (1). The origin of the HIV has been traced to the simian immunodeficiency virus (SIV), found in African apes and monkeys (2). Epidemiologic and phylogenetic analyses currently available imply that HIV was introduced into the human population through several cross-species transmissions that are estimated to have occurred during the first part of twentieth century in the West Central Africa, but it was only 40 years ago the recognition and identification of the virus began. In 1983, the researchers Dr. Luc Montagnier and Dr. Francoise Barre-Sinoussi and Robert Gallo separately isolated HIV (3,4) and in 2008 they received the Nobel Prize for their findings. HIV is grouped to the genus *Lentivirus* within the family of *Retroviridae,* subfamily *Orthoretrovirinae*. On the basis of genetic characteristic and differences in the viral antigens, HIV is classified into the type 1 and 2 (HIV-1, HIV-2). HIV-1 virus has spread to all continents, but, as of today, Sub-Saharan Africa is the most affected area of the world, where 22.9 million people live with HIV (5) (Figure 1), whereas HIV-2 is still found predominantly in West Africa. The global epidemiology of HIV infection has changed markedly as a result of the expanding access to antiretroviral therapy (ART), with a decrease of global incidence and an increase of global prevalence due to the longer life expectancy of HIV-infected subjects (6). While ART has dramatically reduced the mortality and morbidity of HIV infection and has transformed HIV from a fatal disease to a chronic condition, it fails to provide a cure. The main obstacle to the development of a HIV cure is the presence of HIV-infected cells containing integrated DNA without the expression of the virus, defined as latently infected cells (7). This population is termed "HIV viral reservoir" and occurs primarily within resting memory CD4+ T cells, persisting for the lifetime of the patient.
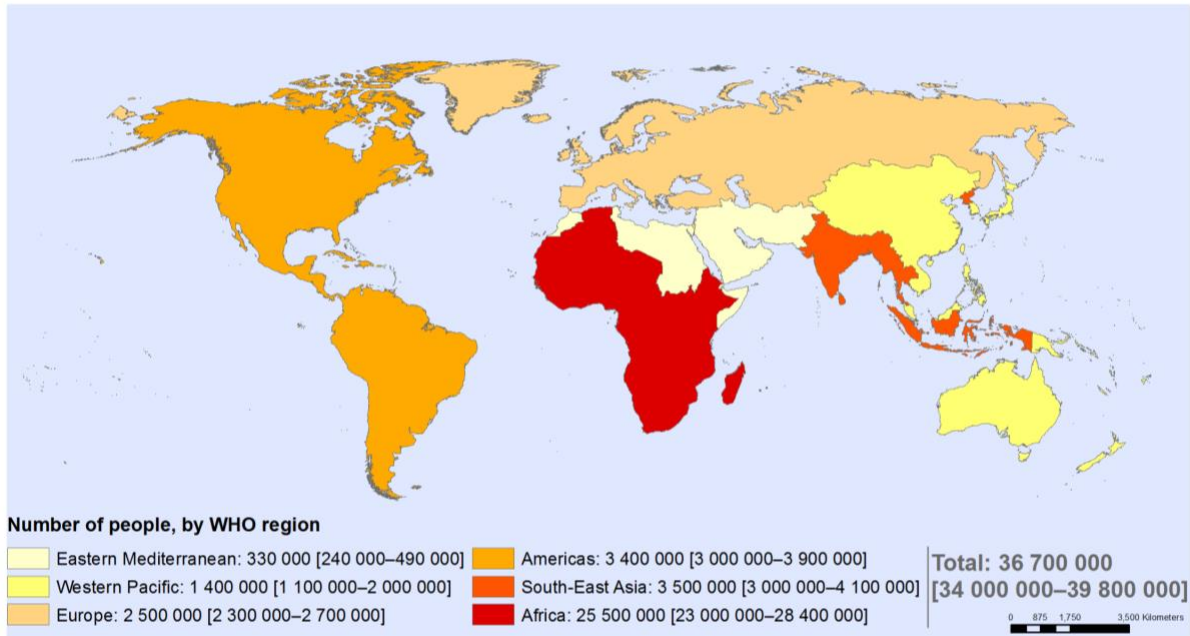
Figure 1 The global HIV distribution

Data Source: World Health Organization, 2016

HIV-1 Virology

Virion structure

The HIV-1 virion is spherical in shape, with a diameter of about 100 nm (Figure 2). It is delimited by an outer membrane, the envelope, that is composed of a lipid bilayer, derived from the plasma membrane of the producer cell when the virion buds from the cell surface (8). Embedded within the envelope is the viral envelope protein, called Env, composed of two proteins, a surface glycoprotein called gp120 (SU) and a transmembrane protein called gp41 (TM). These two proteins together form a single structural unit. Three of these units comprise the Env trimer. Each Env trimer forms a "spike," which protrudes away from the surface of the viral envelope. Constituted by the association of the viral protein p17, the matrix of HIV-1 forms an inner coat directly underneath the lipid envelope of the virion and surrounds the capsid, ensuring the integrity of the virion particle. It is known that the myristoylation of the p17, an addition co-translational of myristic acid to the amino terminal glycine residue, plays an important role for the virus assembly

during the late stages of the HIV-1 replication cycle, while, in the early stages, some p17 molecules dissociate from the viral membrane to direct the pre-integration complex to the host-cell nucleus (9). The conical capsid is assembled from the inner capsid protein p24 (CA) and, depending on the section plane, the capsid appears as a cone, a ring or an ellipse in electron micrographs. Its role is to protect the two identical molecules of viral genomic RNA, located inside the so-called nucleocapsid and bound to its associated enzymes reverse transcriptase (RT), protease (PR) and integrase (IN) and to the nucleocapsid proteins p6 and p7. HIV-1 virions adopt two distinct morphological states: the virion buds from a producer cell in its immature form, with a spherical capsid composed of the immature or precursor structural protein called Gag (p55). To become infectious, the immature virion undergoes a morphological change into the mature form at the end of the budding process. This process, termed "Maturation" is triggered by site-specific proteolytic processing or cleavage of the precursor Gag protein by the viral protease (10), that generates new structural proteins including CA, which reassembles into a new, cone-shaped capsid.
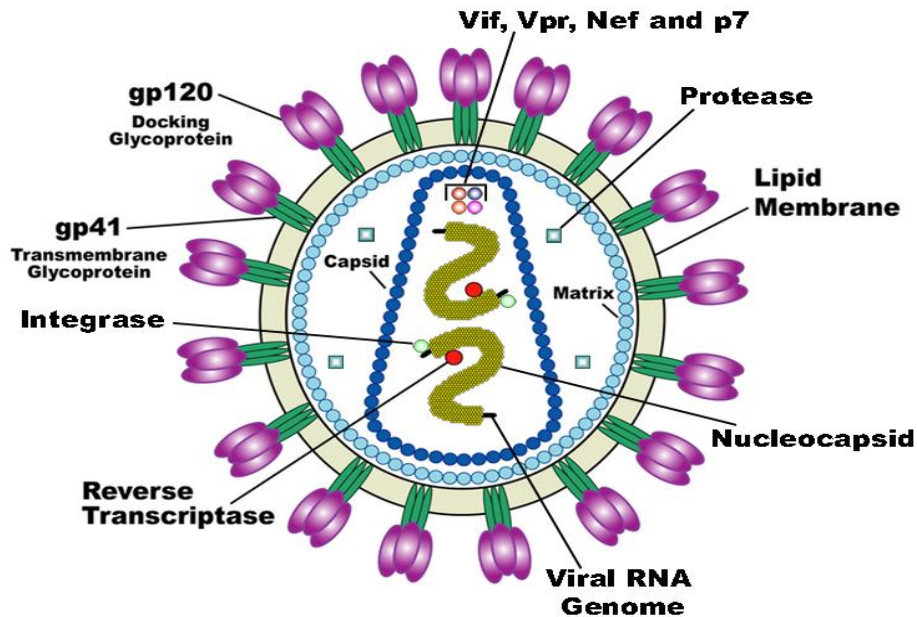
Figure 2 The HIV-1 virion structure

Data Source: https://www.projectinform.org/glossary/hiv-structure-function/

Genome structure

The HIV virus is grouped to the genus Lentivirus within the family of *Retroviridae*, subfamily *Orthoretrovirinae*. The HIV-1 genome consists of two identical, unspliced, positive-sense single-stranded RNA molecules of ~9.2 kilobase that are enclosed within the core of the virus particle, non-covalently linked via a palindrome sequence within the dimer initiation site (DIS) stem-loop, 5′-capped and 3′-polyadenylated. Long Terminal Repeat (LTR) sequences, divided in structural (U3, R, U5) and functional (modulatory, enhancer, core) regions (11), flank the genome at both ends; they drive the HIV-1 gene expression, containing crucial regulatory elements (as TATAA sequence), the trans-activation response element (TAR), that interfaces with the viral transcription activator protein Tat, transcription terminator and polyadenylation signals, the primer binding site (PBS), required for initiation of reverse transcription.

HIV-1 genome has several major genes coding for structural proteins that are found in all retroviruses as well as several non-structural genes unique to this virus (Figure 3). In particular, the identified nine open reading frames, encoding fifteen viral proteins, can be divided into three classes:

1.   The major structural genes, gag, pol, and env
2.   The regulatory genes, tat and rev
3.   The accessory genes, vpu, vpr, vif, and nef
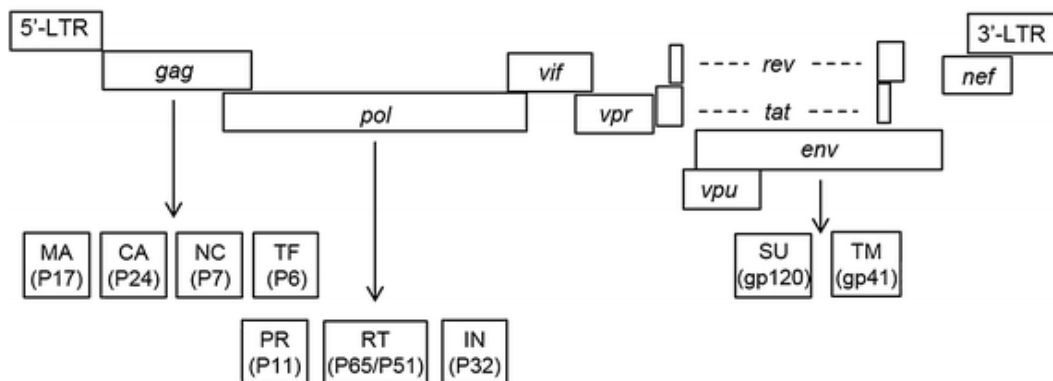
Figure 3 Genomic organization of HIV-1

Modified by https://cellandbioscience.biomedcentral.com/articles/10.1186/s13578-015-0037-7/figures/1

Structural genes

In the direction 5' to 3' the reading frame of the *gag (*group-specific antigen*)* gene follows and gives rise to the 55-kilodalton (kda) Gag precursor protein, also called p55, which is expressed from the unspliced viral mRNA and is required for both virion assembly and maturation. After budding, p55 is cleaved by the viral encoded protease (a product of the *pol* gene) during the process of viral maturation into its final four smaller proteins:

- the outer core membrane protein p17 (MA) participates in the RNA targeting to the plasma membrane prior to viral assembly as well as in the incorporation of the Env glycoprotein into virions.
- the capsid protein p24 (CA) forms the distinctive conical core of viral particles that encapsulates the viral RNA-protein complex, providing structural stability of the virion; the N-terminal domain of the protein is important for infectivity, apparently by participating in viral uncoating through its association with a putative cellular chaperone, cyclophilin A (12).
- the nucleocapsid protein p7 (NC), a nucleic acid chaperon, coats the genomic RNA inside the virion core and is responsible for specifically recognizing the so-called packaging signal of HIV-1. It also has a critical role in mediating highly specific and efficient reverse transcription (13).
- Finally, p6 is a small proline-rich protein important for detachment of maturing virus particles and for packaging of other viral proteins within the virion.

The *gag* reading frame is followed by the *pol (*polymerase*)* reading frame, coding for the enzymes protease (PR, p12), reverse transcriptase (RT/RNAseH, p51/p66) and integrase (IN, p32).

- PR (p12) is an aspartyl protease that acts as a dimer. Protease activity is required for cleavage of the Gag and Gag-Pol polyprotein precursors during virion maturation. Knowledge of this structure has led to a class of drugs directed toward inhibiting the HIV protease function, greatly improving the treatment for HIV-infected individuals.
- Before the viral genome can be integrated into the host chromosome, it must first be reverse transcribed into duplex DNA. RT (p51/p66), the viral reverse transcriptase,

catalyzes both RNA-dependent and DNA-dependent DNA polymerization reactions and contains an RNAse H domain that cleaves the RNA portion of RNA-DNA hybrids generated during the reverse transcription reaction. RT has also been a major target for drug design.

- The IN (p32) protein mediates the insertion of the HIV proviral DNA into the genomic DNA of an infected cell by an exonuclease activity, a double-stranded endonuclease activity and finally, a ligase activity (14).

Adjacent to the pol gene, the *env* (envelope) reading frame encodes a glycosylated precursor protein (gp161), which is cleaved to two envelope proteins:

- located on the viral membrane surface, gp120 (SU) is involved in recognition and binding to specific cell surface receptors, causing structural changes in Env that facilitate coreceptor binding and subsequent viral entry.
- The primary function of gp41 (TM), a smaller hydrophobic transmembrane protein, is to mediate fusion between the viral and cellular membranes, following receptors binding.

Regulatory genes

In addition to the structural proteins, the HIV genome codes for several regulatory proteins necessary for the initiation of HIV replication in all host cells:

- tat (Transactivator of Transcription) is essential for HIV-1 replication, enhancing the efficiency of viral transcription. It is an RNA binding protein of two esons that acts by binding to an RNA short-stem loop structure, known as the transactivation response element (TAR), that is located at the 5' terminus of HIV RNAs (15).

- rev (Regulator of Virion Expression) is a 13-kD sequence-specific RNA binding protein, that induces the transition from the early to the late phase of HIV-1 gene expression. The binding of Rev to the RRE (Rev Responsive Element) site facilitates the

export of unspliced and incompletely spliced viral RNAs from the nucleus to the cytoplasm region (16).

Accessory genes

In addition to the gag, pol, and env genes contained in all retroviruses, and the tat and rev regulatory genes, HIV-1 contains four additional genes: *nef, vif, vpr* and *vpu*, encoding the so-called accessory proteins. Nef is expressed from a multiply spliced mRNA and is therefore rev independent. In contrast, vpr, vpu, and vif are the product of incompletely spliced mRNA, and thus are expressed only during the late, rev-dependent phase of infection from singly spliced mRNAs.

- Nef (negative expression regulatory factor) is a 27-kD myristylated protein that downregulates the cell surface expression of CD4, the primary receptor for HIV-1, may enhancing the Env incorporation into virions and virion budding. Nef also down-regulates the cell surface expression of Class I MHC, decreasing the efficiency of the cytotoxic T cells killing of HIV infected cells and stimulates the infectivity of HIV virions (17).

- The Vpr protein is incorporated into viral particles and induces the G2 cell cycle arrest. This protein also directs the nuclear localization of the pre-integration complex (PIC) in the nondividing cells, through an NLS (Nuclear localization sequence).

- The 16-kD Vpu polypeptide is an integral membrane phosphoprotein that in HIV-infected cells promotes the degradation of the viral receptor CD4 when it forms complexes with the viral envelope protein in the endoplasmic reticulum, causing the trapping of both proteins to within this compartment, that interferes with virion assembly. It can also increase the release of HIV from the surface of an infected cell.

- Vif (viral infectivity factor) is a 23-kD polypeptide that is essential for the production of infectious virus in a cell type-specific manner. It inhibits an anti-retroviral cellular factor expressed in non-permissive cells named APOBEC3G (18).

Replication

The HIV-1 replication cycle can be divided into an early and a late phase. The early phase encompasses virus binding to cell surface receptors, cell entry, reverse transcription of the viral RNA to DNA, uncoating of the viral capsid, nuclear import of viral DNA and DNA integration. The late phase refers to the transcription of viral RNA, translation and modification of viral proteins and release of progeny virions by budding through the cell plasma membrane (Figure 4).
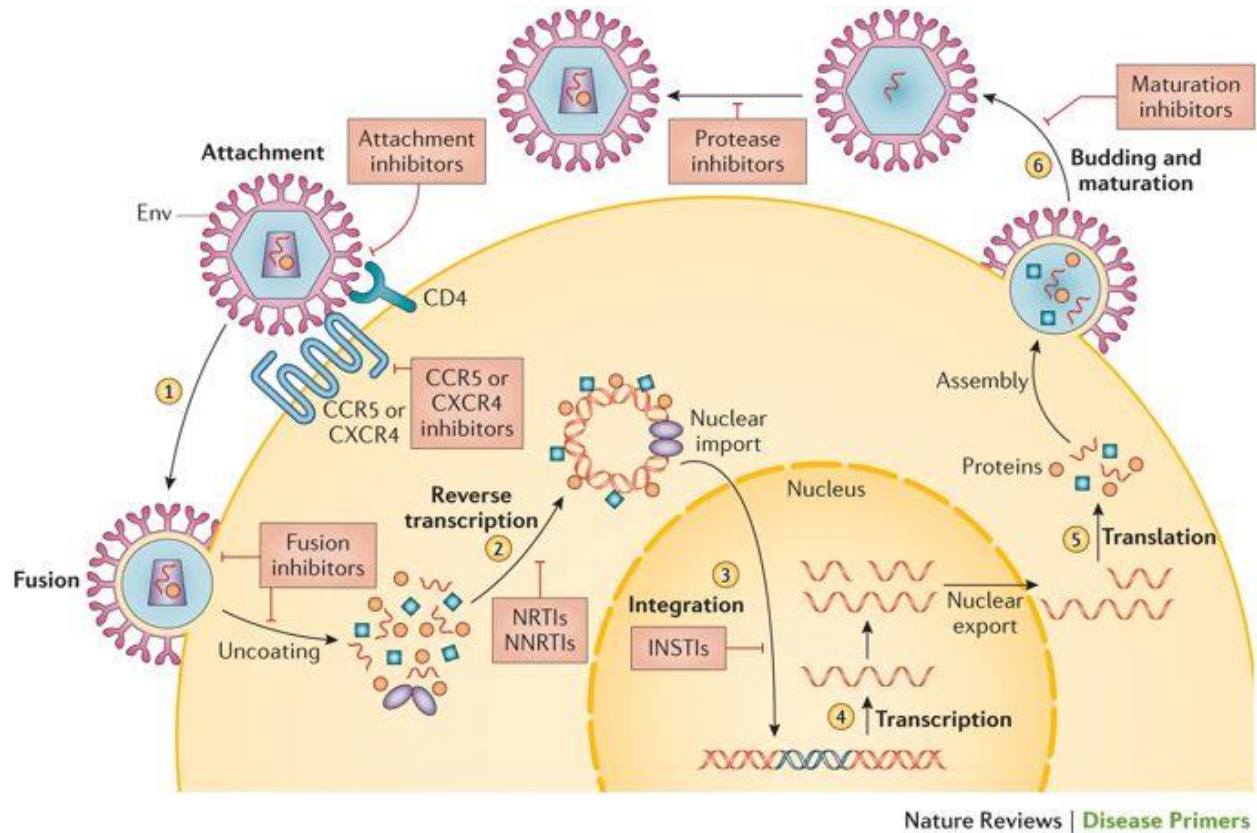

Figure 4 HIV-1 life cycle

Data source: https://www.nature.com/articles/nrdp201535?draft=collection

Binding, entry and uncoating

The initial step of entry involves a high-affinity binding of the surface envelope glycoprotein gp120 with the cellular receptor, CD4, which is present on the surface of a subset of helper T lymphocytes, on monocyte-macrophages, dendritic cells and astrocytes (19). This results in a conformational change in gp120 that allows interaction with the coreceptors, chemokine receptor 5 (CCR5) or chemokine receptor 4 (CXCR4 or fusin) on the host cell (20). HIV tropism (the type of cell that the virus is able to infect) is determined by the type of coreceptor recognized by gp120. Macrophage (M-tropic) strains of HIV-1 use the β-chemokine receptor CCR5 for binding and are able to infect macrophages, dendritic cells, and CD4+ T cells. T-lymphotropic HIV-1 attaches first to the CD4 receptor and then to the α-chemokine receptor CXCR4. Binding of gp120 to CD4 and to the co-receptor triggers an additional conformational change in gp120 and subsequently in gp41 that, presenting its N-terminus on the viral membrane, forms a highly hydrophobic channel inserted into the plasma membrane of the target cell. Fusion of cell membrane and viral envelope is therefore completed, leading to translocation of the viral capsid into the cytoplasm.

Once entered the cytoplasm of the cell, the conical capsid composed of p24 (CA) protein dissociates from the rest of the cytoplasmic viral complex by a process called uncoating (21).

Reverse transcription

Once the viral core enters the cytoplasm of the cell, HIV-1 RT, through the RNA-dependent DNA polymerase activity, converts the single-strand HIV RNA genome into DNA (complementary DNA or cDNA) with a generation of a DNA-RNA duplex, beginning with the annealing of a host transfer RNA (tRNAlys) to a specific primer binding site (PBS) at the 5' end of the viral genome (22). In parallel to DNA synthesis, the RNaseH activity, also found in reverse transcriptase, removes the 5' end of the viral RNA, leaving a purine-rich sequence, called PPT, that serves as a primer for positive-strand DNA synthesis. The nascent minus-strand viral DNA "jumps" and reanneals at the 3' end of the RNA. After this transfer, DNA synthesis proceeds until the minus-strand completion. Then, there is a second priming event and a second template switch with degradation of the RNA component of RNA-DNA hybrids during the course of the positive-strand

viral DNA synthesis. The complete double-stranded viral DNA copy contains duplicated terminal U3RU5 regions (long terminal repeats), which are necessary for subsequent integration. Finally, the proviral DNA is actively transported into the cell nucleus in the form of a complex (the pre-integration complex or PIC) consisting of the integrase, the Gag matrix protein (p17) and Vpr.

Integration

The first step of the integration process occurs in the cytoplasm of the host cell following the completion of reverse transcription of the HIV RNA into c-DNA and involves binding and recognition of specific sequences within the LTRs of viral cDNA by the integrase protein, that cleaves the viral DNA at each 3' end. Subsequently, the pre-integration complex is transported into the nucleus where it binds to the host DNA through the host tethering protein LEDGF/p75 (23). The following step is characterized by the colinear insertion of the HIV DNA into a selected region of the host genome, with the consequent generation of unpaired regions both in the HIV and host DNA junctions, referred to as DNA "gaps". The gap repair process completes the integration of the HIV DNA into the host DNA, with the fully integrated HIV DNA, now being referred to as "proviral DNA". Although viral integration can take place in various locations in the host cell genome as heterochromatin regions, HIV-1 preferentially targets regions that have high gene density and transcriptional activity (24). The viral genome is immediately transcribed in productively infected cells, while it can undergo transcriptional silencing in resting $CD4_+$ T cells, leading to the generation of a latent viral reservoir.

Expression of viral proteins

The genes in the integrated HIV provirus are only expressed in cells in which cellular transcription factors have been induced. In fact, after activation of infected cells, the 5' LTR promotor of the proviral genome serves as attachment site for cellular DNA-dependent RNA polymerases and for both viral and cellular transcription factors (as NF-AT, NF-kB, Sp1, AP-1), initiating the synthesis of viral mRNA products. A crucial role in the activation and maintenance of the high levels of

transcription of viral RNAs is played by the Tat protein, that interacts with Tar; it has been also suggested to be involved in remodeling nucleosomes to relieve transcriptional blockage imposed by chromatin (25). The proviral gene expression is a sequential event: in early infection most of the viral mRNAs found in the cytoplasm have undergone multiple splicing events and encode mainly for Tat, Nef, and Rev. Later, the predominant mRNA species are unspliced and singly spliced RNA (Gag, Pol, Env, Vpr, Vif e Vpu). Through interaction with the Rev-response element (RRE) located in the env region of the full-length transcript, the regulatory protein Rev is involved in the export into cytoplasm of unspliced and incompletely spliced mRNAs (26, 27). All mRNAs are translated in the cytoplasm near the endoplasmatic reticulum (ER) by the normal cellular transcription machinery. Finally, viral and cellular proteases cleavage the precursor peptides to generate the mature HIV-1 proteins.

Assembly, Budding and Maturation

HIV-1 virion assembly takes place at the lipid rafts of the plasma membrane, specialized membrane microdomains reached in cholesterol and sphingolipids, that probably serve as platforms for the targeting of the Gag polyprotein precursor. This protein contains all the necessary elements for the assembly and release of virus-like particles from many eukaryotic cell types: the matrix (MA) domain directs Gag to the plasma membrane and promotes the incorporation of the viral envelope glycoproteins into the forming virions; the capsid (CA) domain drives Gag– Gag interactions during assembly; the nucleocapsid (NC) domain packages the viral genomic RNA; and the p6 domain recruits the endosomal sorting complex required for transport (ESCRT) apparatus, which catalyzes the membrane fusion step to complete the budding process of spherical immature particles (28). A final step is required for conversion of the immature virion into its mature infectious form: the processing of the polyproteins by the viral protease with rearrangement of the structural proteins into a cylinder-shaped organization.

HIV genetic variation

Sources of genetic variation

HIV is one of the fastest evolving organisms known. Due to the fast-evolutionary rate, the virus evades the host immune system and has the capacity to develop resistance to antiretroviral drugs during suboptimal treatment. There are several mechanisms that contribute to the high genetic variation of HIV:

1. The error-prone RT enzyme generates on average 0.1-0.3 mutations per genome and replication cycle (29,30) and it is considered to account for most of the point mutations seen in HIV-1. These mutations remain uncorrected since RT lacks proofreading activity.

2. The high rate of virus turnover, approximately 10^9 virions per day, sustained by HIV-1 infection *in vivo*, strongly contributes to the continuous generation of new viral variants (31).

3. Retroviral recombination is another important strategy by which HIV generates genetic diversity (32). Each retroviral particle contains two copies of single-stranded RNA, and template switches occur frequently during reverse transcription, thus generating mutations and recombination by intramolecular and intermolecular jumps.

Genetic variants of HIV

Two types of HIV are known to cause AIDS: HIV-1 and HIV-2. Geographically, whereas HIV-1 is responsible to the worldwide AIDS epidemic, HIV-2 is mainly restricted to West Africa, with small epidemics reported in Portugal, France, Spain and Brazil. Although HIV-2 is morphologically indistinguishable from HIV-1, it is characterized by lower transmissibility and reduced likelihood of progression to AIDS (33). Sequence comparisons suggest that both HIV-1 and HIV-2 are the result of cross-species transmissions of simian immunodeficiency virus from chimpanzees (SIVcpz) and sooty mangabeys (SIVsmm), respectively. The reduced pathogenicity

of HIV-2 in humans is thought to be the result of lower levels of virus replication, perhaps reflecting incomplete adaptation of SIV to the human host.

Due to an extensive genetic heterogeneity, HIV-1 variants are classified into four major phylogenetic groups: group M (main), group O (outlier), group N (non-M/non-O) and group P. Group M is the predominant circulating HIV-1 group, with a global distribution. It splits into 10 phylogenetic subtypes (A to K), or clades, phylogenetically linked strains with a genetic distance from one another of 25 to 35%. Within a subtype, it is possible recognize sub-subtypes, denoted with numbers, with a genetic variation of 15% to 20% (34). HIV-1 group O, relatively rarely found, seems to be endemic to Cameroon and neighboring countries in West Central Africa; likewise, group N is a very distinctive form of the virus and has been identified only in a limited number of individuals from Cameroon. The M and N HIV-1 groups have a common ancestor, closely related to the SIV strain found in chimpanzees, while, recent evidence suggests that the HIV-1 O and P groups may have originated in wild gorillas (35).

All retroviruses have a propensity to recombine with other relatively closely related retroviruses, and HIVs are no exception. Inter-subtype recombinant genomes are common, but many of them, called unique recombinant form (URF) are found only in the one dually-infected (or multiply-infected) individual patient in which they arose. If an inter-subtype recombinant virus is transmitted to many people, it becomes one of the circulating strains in the HIV epidemic, and it can be classified as a "circulating recombinant form (CRF)", an epidemiologically relevant contribution to the HIV-1 M group epidemic.

HIV infection

HIV-1 Transmission

The virus is able to enter the body through three main routes: sexual contact, parenteral inoculation and vertical transmission. The sexual contact is the predominant mode of HIV transmission worldwide, mediated by exposure to HIV-1-infected cells and/or infectious virus in mucosal secretions or semen. The stratified mucosal epithelium and the endocervical epithelium form effective barriers against HIV-1 or HIV-1-infected cells; however, breaches in the integrity of

these barriers are frequent, increasing susceptibility to infection. Once the epithelial barriers have been breached, HIV-1 can target cells in the underlying epithelial layers, including T cells, dendritic cells and macrophages. The probability of HIV-1 sexual transmission has been shown to vary based on different factors; amongst them, the clinical stage of infection is one of the most important because the early and late stages of HIV-1 infection have long been known to be associated with high rates of transmission, related to the high viral loads observed for these periods (36). Also, the sexually transmitted diseases (STD) and the male circumcision are co-factors for the HIV acquisition, increasing the shedding of the virus and the transmission efficiency. Receiving HIV contaminated blood through transfusions and organ/tissue transplants or needles or syringes sharing for drugs injection results in the so-called parenteral inoculation, the most effective mode of HIV-1 transmission. Luckily, the introduction of HIV antibody testing of blood donors significantly reduced the risk of transfusion-associated HIV transmission. HIV-1 can be also spread from mother to child during pregnancy, occurring in utero, intrapartum or postnatally through breastfeeding. Factors associated with higher probabilities of vertical transmission include higher maternal HIV RNA levels, lower $CD4^+$ counts, infant prematurity, chorioamnionitis, and duration of rupture of membranes. Use of combined antiretroviral therapy, with or without cesarean section to further diminish risk of transmission intra-partum, has resulted in transmission risk of less than 2% among infants born to HIV-1 infected mothers in resource rich areas, while the vertical transmission is nowadays a leading cause of infant mortality in resource-limited countries in sub-Saharan Africa (37).

HIV-1 infection and pathogenesis

There are three stages of HIV infection: the acute infection, the chronic infection and the acquired immunodeficiency syndrome, known as AIDS. As of now there is no cure for HIV-1 infection and, without treatment, chronic HIV infection usually advances to AIDS in 10 years or longer, resulting in the death of the infected subject, with the exception of the elite controllers (EC), HIV-1 infected people who maintain high $CD4_+$ and $CD8_+$ T cell counts and remain therapy naïve.

Typical Course of HIV Infection
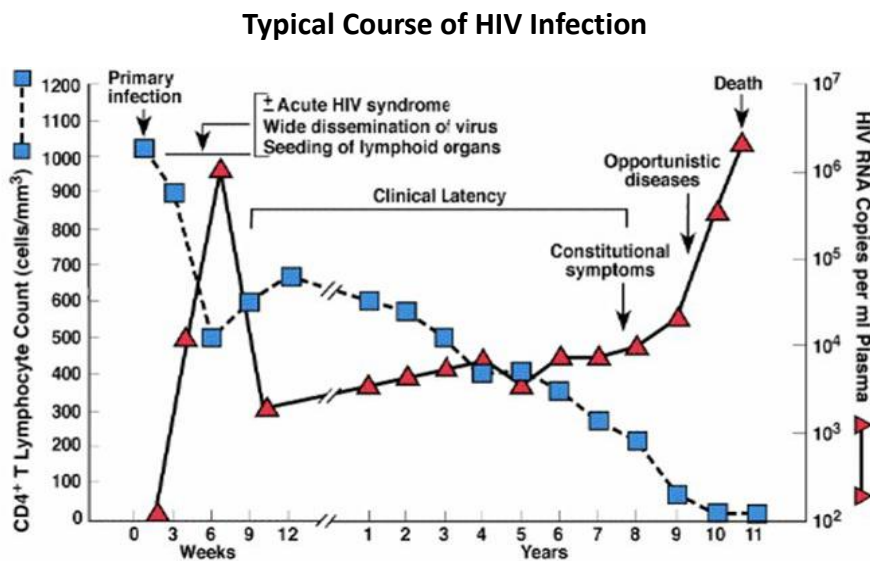
Figure 5 Typical course of HIV infection

Modified from: Fauci, A.S., et al, Ann. Intern. Med., 124:654, 1996

Acute or primary HIV infection is the earliest stage and it generally develops within 2 to 4 weeks following the exposure and transmission. During this time, the virus replicates rapidly in the mucosa and in draining lymphoreticular tissues (especially gut-associated lymphoid tissue), enriching high levels in plasma (10_6-10_7copies per ml). Before the antibodies begin to be produced by the immune system, the so-called "seroconversion", there is an "eclipse-phase", a period generally lasts 7 to 21 days, in which the virus can't be detected in plasma (38). The early HIV-1 infection is also characterized by a transient drastic decline in the CD4+ lymphocytes count, the preferred target for infection, since a normalization of the viral values occurring once the antibody response is activated, which is known as the "viral set point". Importantly, the set point level is correlated with clinical outcome: individuals with high viral load set point typically progress more rapidly to AIDS and death than those with lower viral set point levels (39). Acute infection often occurs asymptomatically or people may experience flu-like illness marked by fever, generalized lymphadenopathy, a nonspecific rash, myalgias and/or malaise. The severity of symptoms is strongly correlated with peak viral load during this phase of the infection.

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The second step during the HIV infection is the chronic stage (also called asymptomatic HIV infection or clinical latency). During this stage of the disease, HIV continues to multiply in the body, but at very low levels, with also progressive decrease in the number of circulating $CD4_+$ lymphocytes and the appearance of functional defects affecting both $CD4_+$ and $CD8_+$ lymphocytes and monocytes and macrophages, associated with a progressive deterioration of the immune system. This phase, clinically latent, can last several years and does not correspond to a biological latency as viral replication continues in the lymphoid organs, but it is rather characterized by lack of symptoms.

AIDS is the final, most severe stage of HIV infection. Because HIV has severely damaged the immune system, the body can't fight off multiple opportunistic infections and neoplasms, that contribute to a rapid clinical deterioration, such as visceral toxoplasmosis, *Pneumocystis carinii* pneumonia and Cytomegalovirus. Moreover, the progressive immunodeficiency and chronic immuno-activation are reflected in a decrease in $CD4_+$ count of less than 200 cells/mm$_3$ and in progressively increase of the viremia (40). People diagnosed with AIDS present severe symptoms as persistent lymphadenopathy (Lymphadenopathy Syndrome, LAS) followed by a phase of weight loss, asthenia, anemia, fever, diarrhea, hypergammaglobulinemia (AIDS-related complex, ARC) and, without treatment, typically survive about 3 years.

Antiretroviral treatment

The development of combination ART (cART) is often considered as one of the greatest achievements of modern medicine, helping people with HIV live longer and healthier and reducing the risk of HIV transmission.

Currently the drugs approved by the FDA (Food and Drugs Administration) for the treatment of HIV infection are divided, according to their action mechanism capable of blocking various stages of viral replication, into four main categories (41):

- Nucleoside and non-nucleoside reverse transcriptase inhibitors

Nucleoside reverse transcriptase inhibitors (NRTIs) act by blocking reverse transcriptase. These drugs are analogues of natural nucleosides and nucleotides, and are preferentially incorporated into HIV DNA, leading to termination of DNA synthesis (Tenofovir, Abacavir, AZT). Non-nucleoside

reverse transcriptase inhibitors (NNRTIs) bind to a pocket near the active site of the reverse transcriptase, which causes a conformational change of the enzyme and inhibition of reverse transcription (Nevirapine, Rilpivirine, Etravirine).

- Protease inhibitors

As the virion matures and buds from the cell, long polypeptide chains are enzymatically cleaved into mature functional proteins by the HIV enzyme protease. Protease inhibitors block this late step in the life cycle of the virus (Atazanavir, Darunavir, Ritonavir)

- Entry inhibitors

Entry inhibitors prevent HIV from entering the cell, Maraviroc binding to CCR5, Enfuvirtide binding to HIV directly.

- Integrase strand transfer inhibitors

Integrase strand transfer inhibitors prevent the HIV genome from being integrated into the host genome (Dolutegravir, Raltegravir, Elvitegravir).

The history of HIV infection treatment began in 1987 with the introduction of the first NRTI, zidovudine (42); subsequently, other drugs of the same class were used, first used as monotherapy and then as dual therapy (1994). The real "therapeutic revolution" began in 1996-1997 with the introduction of protease inhibitors and, after, with non-nucleoside reverse transcriptase inhibitors; with the advent of the new drugs the era of cART has started and consists of the use of three or more drugs belonging to two or more different classes of inhibitors (43). Thanks to the combination of antiretroviral therapy, mortality has significantly decreased, turning a deadly disease into a long-term chronic disease. While monotherapy usually produces drug-resistant HIV mutant forms fairly rapidly, the therapeutic combination generally slows the selection of mutants because it acts at various levels of HIV replication. Although the cART can suppress viral replication and reduces the plasma viral load to below limits of detection of the most sensitive clinical assays (<20 RNA copies/mL) for decades, there isn't currently a cure for HIV infection, due to the persistency of the virus in latent reservoirs.

HIV-1 Latency

Although antiretroviral therapy suppresses HIV to undetectable levels, interrupting ART causes the virus to rapidly rebound to pretreatment levels, due to the natural phenomenon of HIV latency. Viral latency is a state of reversibly non-productive infection of individual cells, providing an important mechanism for viral persistence and escape from immune recognition, established early during acute infection, likely within days of initial infection. Two forms of viral latency are known: pre- and post-integration latency (44). Pre-integration latency is characterized by an unintegrated virus, due to the inhibition of integration and nuclear import mediated by the low metabolic state of the host cells. The unintegrated forms are located in the cytoplasm for days and constitute the pre-integration complex which eventually decays over time. During post-integration latency, the provirus is able to successfully integrate into the host genome, but exists in a transcriptionally inert state.

For HIV-1, the term latency was initially used in the clinical sense to describe the long asymptomatic period between initial infection and the development of AIDS. However, it became clear that HIV-1 replicates actively throughout the course of the infection, even during the asymptomatic period and that it can establish a state of latent infection at the level of individual T cells.

Naive $CD4^+$ T cells exist in a resting state until they encounter an antigen, after which they undergo cellular proliferation and differentiation, giving rise to effector cells. Most effector cells die quickly, but a subset survives and reverts to a resting G_0 state, persisting as memory cells. Activated $CD4_+$ T cells are highly susceptible to HIV-1 infection and typically die quickly as a result of the cytopathic effects of the virus or host immune responses. However, some activated infected $CD4_+$ T cells survive long enough to revert back to a resting memory state. Due to the long-lived survival capabilities, they represent an ideal cellular reservoir for the maintenance of a stably integrated, but transcriptionally silent form of the latent virus, even though the frequency of these latently infected cells is very small (in the order of approximately $\sim 1 \times 10_6$ cells per infected individual). Moreover, the host cell can be activated again by an encounter with antigen or cytokine, leading to the induction of transcription factors like NF-kB and NFAT that, in turn, promote reactivation of the latent HIV proviruses (45).

Cellular and anatomical reservoirs

A reservoir is defined as 'a cell type or anatomical site in which a replication-competent form of virus accumulates and persists', in spite of long periods of cART-suppressed viremia (46).
The best-characterized cellular reservoir of HIV comprises resting memory CD4+ T cells. Chomont et al. identified two viral reservoirs within the memory CD4+ T cells pool, known as Central memory (TCM) and Transitional memory (TTM) cells, able to survive for decades, whereas Naive (TN) and effector T cells only marginally contribute to the viral persistence (47). Other immune cells have been implicated as reservoirs as well, especially myeloid cells. Macrophages are known to be susceptible to infection and more resistant to the cytopathic effects of the virus compared to CD4+ T cells, but their role remains debated due to their highly specialization per organ: macrophages in the gut-associated lymphoid tissue (GALT) appear to be quite resistant to infection, except in the rectum, whereas alveolar macrophages seem relatively permissive (48). In addition, dendritic cells (DCs), natural killer (NK) cells, and other specialized cell types (e.g., renal, mucosal, and cervical epithelial cells; mastocytes, astrocytes, and microglia in the central nervous system; skin fibroblasts; and bone marrow stem cells) can represent persistent populations with integrated HIV proviruses, but their specific role is not yet clearly understood.
HIV-1 also hides in several immune privileged anatomic sites that are poorly penetrated by antiretroviral drugs: after entering the body, it quickly disseminates to the lymph nodes and later on to the bloodstream, spreading throughout the entire body as individual virions, by the transport of infected immune cells and by cell-to-cell transmission. Indeed, anatomical reservoirs that may harbor such cells include the brain and the central nervous system, the gastrointestinal tract and the gut-associated lymphoid tissue and other lymphoid organs, and the genital tract (49-52).

HIV-1 molecular mechanisms of latency

A critical and unanswered question is what distinguishes between productive and latent infection at the molecular level. It is known that the maintaining of the latent state is likely a multifactorial process; it can involve changes in chromatin condensation state in the host cells, subsequently affecting the HIV gene expression: transcribed genes are usually found within "relaxed" chromosomal DNA, termed euchromatin, while analysis of latent integration sites showed that

latent HIV-1 proviruses preferentially integrate within the heterochromatin, a more condensed transcriptionally repressed chromosomal form in which the blocking of the transcriptional machinery is mediated by nucleosomes (53). As fundamental structural unit of chromatin, Nuc-o and Nuc-1 regulate the basal transcriptional activity of the 5′ LTR of HIV genome because they overlap with the binding sites for many key transcription factors that drive HIV-1 gene expression, making the promotor region inaccessible to them; other aspects clearly related to latent conditions are post-translational modifications of the histone tails of the nucleosomes, including acetylation, methylation, ubiquitylation, phosphorylation, sumoylation, and poly ADP-ribosylation. Nuc-1 is in fact rapidly and specifically disrupted following treatment with HDAC (histone deacetylase) inhibitors, thus creating a less-repressive chromatin state and allowing for increased HIV-1 transcription (54).

DNA methylation is another possible mechanism that reinforces HIV-1 latency. Regulation of transcriptional silencing by DNA methylation is catalyzed by DNA methyltransferases and occurs largely at CpG dinucleotides. CpG methylation at the $5^{'}$LTR of HIV-1 genome acts by preventing the binding of essential transcription factors such as NF-kB and Sp1. In a study by Kauder et al., it was demonstrated that, during latency, the HIV-1 promoter is hypermethylated at two CpG islands surrounding the transcriptional start site in both J-Lat cell lines and in primary CD4$^+$ T cells (55).

Considering that latent provirus is able to integrate also into actively transcribed regions, it isn't really surprising that some forms of latency mechanistically involve transcriptional interference. One mechanism depends on the orientation of the HIV-1 provirus relative to the host gene: if both share the same polarity, "promoter occlusion" can occur, in which read-through by the host RNA Pol II displaces key transcription factors on HIV-1 LTR, thereby reinforcing viral latency. Conversely, the "convergent transcription" occurs when the provirus integrates in the opposite orientation or polarity relative to the host gene, leading to collision of the RNA Pol II complexes from the host and viral promoters and early arrest of transcription from both promoters or the weaker of the two (56). This form of transcriptional interference could also generate double-stranded viral RNAs, resulting in silencing of viral transcription or translation through RNA interference, RNA- directed DNA methylation, or the generation of antisense RNA (57).

Initiation of HIV-1 gene expression is critically dependent on a range of host transcription factors, including NF-κB, NFAT, and AP-1, whose activities are induced by extracellular stimuli,

including T-cell receptor ligation or the action of various cytokines. Sp1 also plays a key role by binding at three sites in the core promoter, through its associations with TATA-box-binding protein (TBP), TBP-associated factor (TAF) 250, and TAF55, increasing both basal and Tat-dependent activation of the HIV-1 LTR. Members of the NF-κB/Rel family of transcription factors exert both activating and inhibitory effects on the HIV-1 LTR: in the cytoplasm of resting cells, NF-kB exists in an inactive form, the p50/RelA heterodimer, while in the nucleus of HIV-infected resting cells, NF-kB p50/p50 homodimers are bound to the 5'LTR; the binding of homodimers of p50 recruits HDAC-1 and leads to transcriptional inhibition because of histone deacetylation and chromatin condensation, promoting latency. On the contrary, after their activation and translocation into the nucleus, the p50/RelA heterodimers displace the p50 homodimers and promote a strong transcriptional activation, recruiting histone acetyl- transferases (HATs) like p300/CBP (58).

The HIV transactivator Tat play a major role during the HIV transcription, exclusively stimulating transcription elongation by binding to the transactivation-responsive element TAR, a rather simple RNA stem-loop structure located at the 5′ end of all viral transcripts. Following TAR binding, Tat recruits the P-TEFb complex, that mediates the phosphorylation of the CTD of RNA Pol II, primarily at serine-2. In addition, P-TEFb mediates phosphorylation of two transcription inhibitors, DSIF (DRB sensitivity-inducing factor) and NELF (negative elongation factor). These events result in highly effective RNA Pol II elongation. In the latent proviruses transcription elongation is very inefficient due to the relative absence of Tat, that is able to produce only short abortive transcripts, additionally subjected to elongation restrictions by NELF (59). Transcriptional activation also depends on the acetylation state of Tat, that leads to the dissociation of Tat from TAR, thus promoting the switch between the early and late phases in HIV transcriptional elongation.

Another rather unexpected mechanism has emerged. Specifically, resting CD4+ T cells from HIV-1-infected patients on antiretroviral therapy retain both Tat and Rev transcripts in their nuclei (60) through the ectopic expression of polypyrimidine tract binding protein (PTB), an RNA export protein. Interestingly, a short form of PTB is present in resting CD4+ T cells, whereas full-length PTB is virtually undetectable.
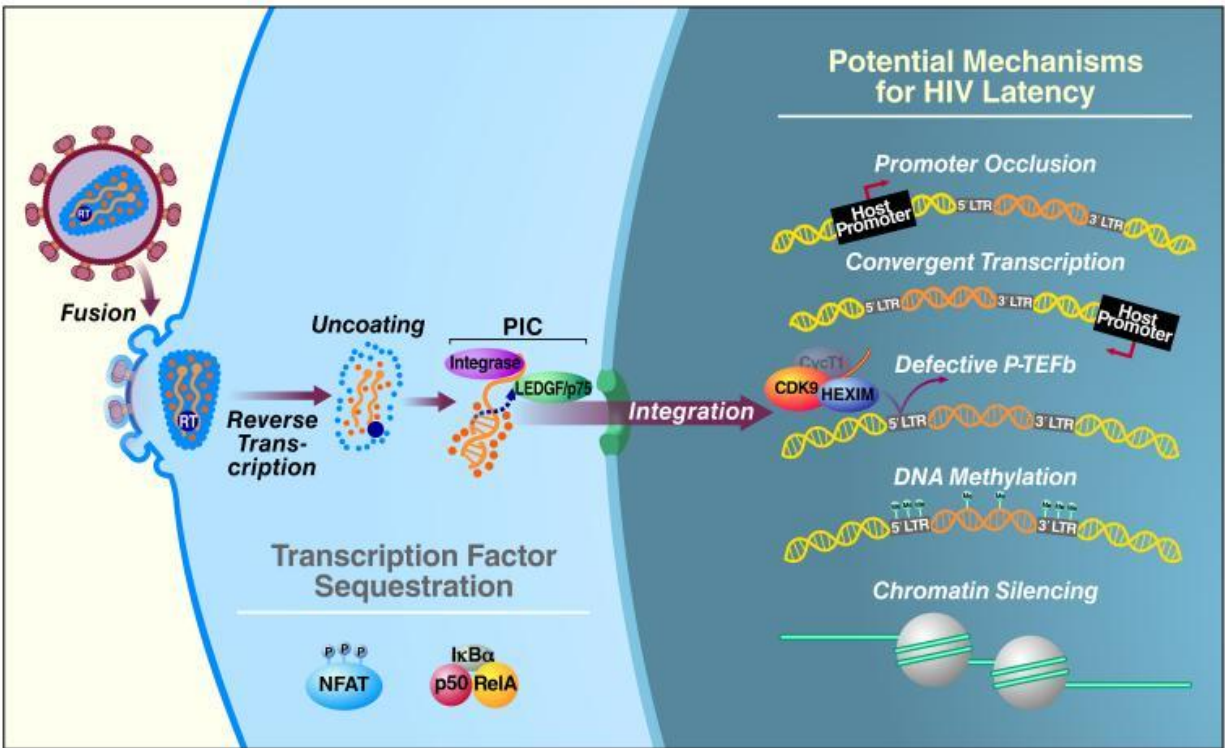
Figure 6 Potential mechanisms for HIV Latency

Data source: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4361081

CD8+ T cells

Role and Importance of CD8+ T Cells During the Antiviral Response

CD8+ T cells are a specialized lymphocyte subset in the adaptive immune system that provide defense against intracellular pathogens, mainly against viruses and tumor cells. To produce an effective and robust immune response, Ag-specific CD8 T cells are characterized by the expression of antigen receptors on their surface, known as the T cell receptor (TCR). The TCR receptor is a transmembrane heterodimeric protein, normally consisting of two disulfide-linked polypeptide chains: in humans, the vast majority of T lymphocytes express alpha and beta chains on their surface, but a minority has a TCR made up of a gamma and a delta chains (61); it features a variable region involved in antigen recognition, a constant region, a hinge region, a transmembrane region and a short cytoplasmic tail. The TCR is always expressed with the associated CD3 complex,

comprised of multiple independently expressed units, required for signal transduction once presented antigen is encountered (62). TCRs recognize antigenic peptides that are bound and presented by endogenous major histocompatibility complex (MHC) molecules, which in humans are called HLA. HLA genes are located on chromosome 6 and are known to be the most polymorphic genes in humans (63). Their diversity allows accommodation of a range of different peptides for presentation to T cells. There are two types of HLA molecules, HLA class I and HLA class II. Human HLA class I molecules are glycoproteins generally categorized into two groups: classical (HLA-A, B and C) and non-classical (HLA-E, F and G). HLA genes produce proteins that would form the major histocompatibility complex (MHC) (64). HLA class I molecules present processed peptides to CD8$_+$ T cells, whereas HLA class II molecules present peptides to CD4$_+$ T cells. MHC I molecules are heterodimeric and composed of two polypeptide chains, α- and β2-microglobulin. The two alpha helices (heavy chains) are highly polymorphic, whereas the light chain β2-microglobulin is less polymorphic. The third α3 domain is quite conserved and interacts with the CTL co-receptor (65). Initial recognition of antigen by naïve Ag-specific CD8 T cells or "priming" usually occurs in a lymph node to which professional antigen-presenting cells (APCs) transport antigen from the site of infection. Through the formation of the so-called "immunological synapse", α and β domains of the CD8 molecule bind to the α3 domain of the MHC class I molecule (66). This binding is stabilized by the presence of costimulatory molecules, including CD80 and CD86 on the APC (67), and generates a signaling cascade that initiates activation of the CD8$_+$ T cells. The effective response against virally infected cells requires a "clonal expansion", an intense proliferation within the lymph nodes that gives rise to many CD8$_+$ T cells of identical TCR specificity. This expansion is necessary for activated cells to differentiate into effector cells and acquire antimicrobial activity to fight the ensuing infection: the differentiating CD8$_+$ T cells exit the local lymph node to enter circulation and begins to express chemokine receptors and adhesion molecules appropriate for trafficking to the effector site and gene expression profiles appropriate to carry out effector functions (68). Virus-specific CD8$_+$ T-cell responses can become evident as early as 4–5 days after infection, with the peak numbers of effector CD8$_+$ T cells usually observed between 7–14 days after infection (69). At the end of the primary response, in order to preserve the flexibility to respond new foreign Ags and limit immunopathology during a chronic infection, the majority of responding CD8$_+$ T cells dies by apoptosis (contraction phase); however, a small fraction remains as long-lived memory T cells. These memory CD8$_+$ T cells

respond with strong proliferation and rapid conversion into effector cells upon re-exposure to the cognate antigen (70, 71), a process defined "the secondary response".

CD8+ T cells differentiation pathways

T cells develop from lymphoid progenitor cells originating in the bone marrow, but they mature in the thymus. There, they rearrange their TCR genes and undergo the processes of positive and negative selection (72): T cells which weakly recognize self-peptides presented by MHC class I and II molecules on epithelial cells receive survival signals, while T cells that strongly recognize self-peptide-MHC complexes are eliminated, in order to avoid the subsequent effector function mediated against self-antigen. During positive selection, thymocytes bearing "useful" TCRs undergo differentiation to the CD4+ helper or CD8+ cytotoxic lineage, and mature CD4+ or CD8+ single-positive (SP) cells exit the thymus and circulate in the periphery (73), in search of an antigen that matches the specificity of their TCR. To increase their chances of meeting antigen, they use lymph nodes as meeting points with APCs. From its exit from the thymus as a naïve CD8+ T cell, to its targeted execution of effector function and formation of a memory compartment, there are many complex steps in the development of virus-specific CD8+ T cell responses. Once activated, they acquire an effector phenotype, characterized by high cytotoxic ability and production of different types of cytokines; therefore, they can be divided in:

(i)   CD8+ T cells with a Th1-like cytokine pattern (Tc1), which produce IFN-γ and TNF-α and exhibit a high cytotoxic function
(ii)   CD8+ T cells with a Th2-like cytokine pattern (Tc2), which produce IL-4, IL-5, IL-6, and IL-10 and have lower cytotoxic ability than Tc1 cells (74).
(iii)   CD8+ T cells that produces IL-17, but no granzyme B, with a pattern recently described as Tc17. These cells exhibit some functional plasticity, since they can produce IFN-γ while losing the expression of IL-17 (75).

Once the pathogen is eradicated, some cells acquire a memory phenotype that persists even in the absence of the antigen (76). They are distinguished from naïve and effector CD8+ T cells that express the marker CD45RA, by the loss of this marker and the gain of CD45RO after differentiation. These memory CD8+ T cells have been classified into the following subsets,

considering differences in the degrees of effector functions, proliferative capacity, and tissue homing properties (77):

(i)   central memory cells, restricted mainly to lymphoid tissues because their expression of the lymphoid homing molecules CD62L and CCR7, which are also considered as the source for self-renewal of the pool of memory cells, generating a second wave of effector T cells

(ii) effector memory cells that provide a first line of defense against infections through immediate effector functions and are present in circulation and non-lymphoid tissues due to the low expression of the lymphoid homing molecules CD62L and CCR7 (78)

(iii) a recently described subset of tissue-resident memory cells, that are located at sites of pathogen entry.

The CD8+ T cells can also be classified according to the level of activation; based on the expression of the activation markers HLA-DR and CD38, four phenotypes of cells have been identified: (i) HLA-DR+CD38+; (ii) HLA-DR+CD38−; (iii) HLA-DR−CD38+; and (iv) HLA-DR−CD38−. In addition to these described populations, some CD8+ T cells exhibit the ability to suppress T helper activity and induce anergy, called regulatory CD8+ T cells (79,80). These cells seem to be related to a memory phenotype since they are CD45RA negative; in addition, they can express the CD25 and the FoxP3 markers, and their regulatory function seems to be mainly achieved by IL-10 production.

CD8+ T cells function

CD8+ T cells display considerable functional heterogeneity. Induction of apoptosis in target cells is one of their most important effector functions. Direct lysis of infected host cells is caused by the release of pro-apoptotic enzymes and antiviral chemokines. The secretion of perforin from lytic granules in a calcium-dependent process results in the formation of pores (81, 82). With the integrity of the cell membrane destroyed, the cells die rapidly through osmotic lysis. In addition, proteases co-secreted with perforin by lytic granule exocytosis enter the target cell and induce apoptosis. Amongst them, Granzyme B, a serine protease with a caspase cleavage activity, is the major mediator of target cell DNA fragmentation in this perforin-dependent pathway (83). Studies

have shown that the exocytosis of these two secretory proteins is MHC-I restricted and antigen specific (84).

There is a second perforin-independent mechanism of cytotoxicity, Calcium-independent. This process involves specific death-inducing ligands, such as Fas-Ligand (FasL), which trigger apoptosis following the engagement of receptors on the target cell surface. Fas (CD95) is a member of the tumor necrosis factor receptor superfamily; crosslinking of Fas by FasL, which is expressed on activated CTLs, leads to apoptotic target cell death (85,86). Of interest is a study that hinted at the contribution of Fas-mediated cell death to the granule exocytosis-mediated pathway attributed to CD8+ T cells. Kojima et al. reveled by immunoelectron microscopy that FasL was localized to the outer membrane of the cytoplasmic granules during degranulation (87). These findings indicate that these two lytic pathways may be more intertwined than previously thought.

One non-lytic pathway used by CD8+ T cells to modulate viral infection is through the secretion of soluble factors that aid in the neutralization of foreign pathogens. This mechanism does not directly eliminate virally infected cells like the perforin/granzyme and FasL-mediated routes, it however does play a large role in the control of viral replication. Almost all CD8+ T cells can secrete potent amounts of the antiviral cytokine IFN-γ, which can block viral replication and cause up-regulation of MHC molecules on the surface of an infected cell. IFN-γ can also activate macrophages, NK cells and promote isotype switching in B cells (88). Moreover, CD8 T cells are an important source of one of the members of the TNF superfamily, TNF-α. It has been shown to be an important antiviral factor due to its role as a mediator of apoptosis as well as inflammation and immunity (89). TNF-α also promotes an antiviral state by enhancing expression of MHC class I and by inducing expression of IL-12 and IL-18 which are both important for upregulating production of IFN-γ by CD8$^+$ T cells (90). However, similar to IFN-γ, activation of cells induced by TNF-α can also result in increased production of virus.

Although typically considered a CD4+ T cell cytokine, CD8+ T cells are also quite capable of producing IL-2. It has no direct antiviral effector function, but it does promote expansion of CD4+ and CD8+ T cells, thereby amplifying the effector response to pathogens (91). IL-2 may also be important for programming CD8+ T cells for better memory generation (92).

Both CD4$^+$ and CD8$^+$ T cells secrete a variety of chemotactic cytokines (chemokines) upon activation. Chief among them are the β-chemokines macrophage inflammatory protein-1α (MIP-1α) and -1β (MIP-1β) and regulated upon activation normal T cell expressed and secreted

(RANTES). MIP-1α and MIP-1β can be found in cytotoxic granules while RANTES is stored in a separate secretory compartment called the RANTES secretory vesicle (RSV) (93). Both types of granules are rapidly released following CD8+ T cells activation. New MIP-1α and MIP-1β synthesis occurs within a few hours of activation, while RANTES can take several days to be upregulated following its initial release. All three contribute to an inflammatory response primarily by recruiting leukocytes to the site of injury or infection.

The role of CD8+ T cells in HIV infection

Numerous lines of evidence suggest that CD8+ T cells exert potent antiviral effects also during HIV infection. First, the emergence of HIV-specific CD8+ T cell responses during the primary infection has been associated with a post-peak decline of HIV viremia, resolution of clinical symptoms and the development of a semi-stable viral setpoint (94). This is consistent with experimental animal model of simian immunodeficiency virus (SIV) of rhesus macaques (RM) showing that the depletion of CD8+ T cells during acute phase of infection results in the abrogation of the post-peak decline of viremia (95).

Additionally, the immunologic pressure exerted by virus-specific CD8+ T cells is manifested by the rapid evolution of virus escape variants following acute infection (96, 97). These mutations can result in HIV-infected cells either failing to present the viral epitope or, if presented, evading recognition by CD8+ T cells. This process, termed "escape" is first observed within weeks of transmission and continues through infection.

Unlike most viral infections, HIV leads to a marked and durable activation not only in HIV-specific (representing only the 10% of total circulating CD8+ T cells), but also in non-specific CD8+ T cells, referred to as "bystander" activation (98). This mechanism seems to occur through a T-cell-receptor-independent response, conversely initiated mainly by cytokine and chemokine stimulation: in fact, activated CD8+ T cells include subsets specific for persistent pathogens such as cytomegalovirus (CMV), Epstein-Barr virus (EBV), as well as subsets for non-persistent pathogens, such as influenza virus and adenovirus (99).

While HIV-specific CD8+ T cells cytotoxic activity appear to be essential for the post-peak decline in viremia during the early acute infection, HIV-infected individuals experience a progressive

dysfunction and "exhaustion" of virus-specific CD8+ T cells, due to persistent exposure to HIV antigen and chronic inflammation with the ongoing viral replication (100). Altered differentiation, impaired function and decreased proliferation characterize the exhausted phenotype of CD8+ population, as well as the expression of a variety of coinhibitory molecules, including programmed death 1 (PD-1), T cell Ig and mucin domain 3 (TIM3), CD160, the NK cell receptor 2B4, lymphocyte activation gene 3 (LAG-3), and cytotoxic T lymphocyte antigen 4 (CTLA-4) (101-103).

CD8+ T cells continue to exert some level of control over HIV and SIV replication after the acute phase of infection, as shown by studies in which depletion of these cells during chronic SIV infection results in increased viral replication (104). Despite their efforts to maintain the antiviral immune response also during the chronic phase of infection, the combination of the continuous appearance of virus escape mutants (105), the progressive T cell dysfunction and immune exhaustion increasingly limit the HIV specific CD8+ T cells efficacy, contributing to the disease progression. Additionally, the functional impairment of CD8+ T cells is accompanied by a higher level of expression of activation markers CD38 and HLA-DR during the chronic phase of infection and most closely associated with shorter patient survival than viral load or CD4+ T cell count (106). The understanding that CD8+ T cells play a central role in natural control of HIV derives first from several studies in non-human primates, the best-characterized and most widely studied animal model for the study of AIDS pathogenesis. In particular, SIV infection of sooty mangabeys (SMs), a natural host species, does not cause AIDS despite high-level virus replication. In contrast, SIV infection of non-natural hosts such as RMs induces an AIDS-like disease. In the first study, Friedrich et al. showed that the depletion of CD8+ lymphocytes from controller Rhesus Macaques (with symptoms resembling human elite controllers) resulted in a transient increase in viremia, suggesting a key role for CD8+ T cells in controlling levels of SIV replication (107). By the contrast, only minor changes in the levels of plasma viremia were observed in most SIV-infected SMs during the period of CD8+ cell deficiency (108).

Strong evidences suggest the prominent role of HIV-specific CD8+ T cells in keeping the immune control of the virus also in particular groups of human HIV-infected subjects, named long-term non-progressors (LTNP) and elite controllers (EC), who achieve a spontaneous and sustained control of the infection, maintaining low or undetectable viral loads without cART. Amongst them, very high levels of escape mutations found in the EC population highlight the selective pressure

on the virus exerted by the CD8+ T cells (109). In support, EC cohorts show enrichment with certain HLA class 1 type alleles, such as *HLA-B27* and *HLA-B57*, that drive potent and effective CD8+ T-cell responses, as the preservation of T cell proliferation coupled to their perforin expression, which presumably enables them to kill HIV-1-infected cells (110, 111).

Cytolytic activity during HIV pathogenesis

As described before, viruses are typically eliminated by virus-specific CD8+ T cells, which recognize processed viral proteins that are presented as a complex with an HLA class I molecule at the surface of an infected cell. They are also able to suppress HIV replication in vitro by direct cytotoxicity (112).

However, the specific contribution of CTL responses to the control of HIV infection remains incompletely understood. During acute infection the emergence of HIV-specific CD8+ T cells correlates with resolution of peak viremia and of clinical symptoms. The critical role of CD8 T cells in controlling viral replication in primary infection is also manifested by CTL escape mutations and the association of certain MHC class I alleles with superior control of viral replication. During the chronic phase of infection, the HIV specific CD8 T cells are functionally impaired, showing a reduced proliferative and differentiative capacity, loss of perforin expression, inability to secrete cytokines in response to antigen re-stimulation, and a reduced capacity to lyse target cells in vitro. This loss of HIV specific CD8 cytolytic function is thought to be a contributing factor to progressive HIV infection (113). Together, these data suggest that CTL activity by CD8+ T cells is present and likely very important during the acute phase of HIV/SIV infection and in determining the relatively rare EC phenotype, while its role during chronic progressive infection is not clear and possibly much less important.

Non-cytolytic activity during HIV pathogenesis

In addition to cytotoxic activity, CD8+ T cells can suppress HIV-1 replication by releasing soluble factors. Non-lytic control of HIV-1 infection by CD8+ involves the β chemokines, MIP-1α, MIP-1β, and RANTES, as major mediators of the suppressive activity against R5 HIV-1 isolates that depend upon CCR5 for entry (114).

HIV/SIV-specific CD8+ T cells also secrete IFN-γ which may play a role in the non-cytolytic immune response, however there is no demonstrable correlation between IFN-γ expression and viral load, viral set point, viral clearance, or chronicity, with considerable variation between patients (115).

An unidentified soluble factor, called CD8+ cell antiviral factor (CAF), seems to play a critical role in the non-cytolytic immune response by the CD8+ T cells. Its activity inhibits HIV transcription, particularly at the step of long terminal repeat (LTR)-driven gene expression through activation of STAT1 (signal transducers and activators of transcription) (116), while having little effect on other stages of the virus life cycle, such as entry into the cell and integration into the host cell genome. The identity of CAF and its precise mechanism for suppressing HIV replication have remained unclear. Interestingly, the relative importance of cytolytic properties of CD8+ T cells has been questioned by *in vivo* CD8+ depletion experiments in SIV-infected nonhuman primates, in which the in vivo lifespan of productively infected cells was measured in CD8+ lymphocyte-depleted versus non-depleted SIV-infected RM (117, 118). The lack of a prolonged lifetime of infected CD4+ T cells in the absence of CD8+ lymphocytes suggested that non cytolytic responses might represent another strong element of the protective CD8+ T cell-mediated response to HIV or SIV infection. In conclusion, a number of independent experimental investigations suggest that conventional CTL activity does not fully explain the antiviral role of CD8+ T cells in HIV/SIV infection.

CD8+ T cells and ART

Although effective ART achieves the viral control and CD4 T-cell recovery in the majority of patients, it fails in restoring the quantitative and functional defects in the CD8 T-cells population also after treatment: elevation and expansion of CD8+ T cells occur from the very early days of HIV infection, contributing to a partial control of viremia, but, unlike other viral infections, the CD8 counts remain consistently elevated and relatively stable over time, resulting in a persistently low CD4:CD8 ratio even during virologic control (119, 120).

Interestingly, a decrease in CD8 count was reported in HIV-infected patients treated with ART during the early primary infection (121). Moreover, the progressive loss of the proliferative capacity and of the immune function, as the capacity to produce antiviral cytokines and release lytic molecules following antigenic stimulation, that characterize the HIV-specific CD8+ T cell during the natural course of HIV infection, do not return to normal level in ART-threated HIV-infected subjects (122-124). Factors associated with this phenomenon seem to include the persistent immune activation, exhaustion and immune senescence, as well as increased risks of non-AIDS related events, to which CD8+ T cells are subjects to.

Nevertheless, numerous lines of evidence in animal models suggest that CD8+ lymphocytes exert a potent antiviral effect also under ART, as recently demonstrated in a group of ART-threated SIV-infected RM, characterized by a rebound of plasma viremia after the CD8+ T cells depletion (125) In support, in this study the re-emergence of viral control was consistently coupled to the reconstitution of CD8+ T cells. Of note, the longitudinal viral sequencing analysis, performed during peak viremia, immediately prior the ART initiation and at the time of the virus rebound after CD8+ depletion, suggested that the increased plasma viremia after CD8+ depletion is linked to the reactivation of virus transcription from a pool of long-lived, latently infected cells, infected prior to ART initiation. In fact, the virus that emerges after CD8+ T cell depletion shows sequences that overlap with the peak viremia quasi-species and not the pre-ART quasi species. In addition, having observed an increased CD4+ T cell activation and proliferation after the CD8+ T cells depletion, a CD4+ T cell depletion was also performed in a small group of SIV-infected RM under ART, not resulting in a viral rebound (126). These results support the hypothesis that CD8+ T cells

play a previously unappreciated, but important direct role in the control of virus production in ART treated SIV infected RM.

Surprisingly, these findings also highlight the possible contribute of CD8+ T cells in maintaining of the HIV viral reservoirs under ART. In this regard, while clearance of infected cells via CTL activity during the early infection results in a decrease of the reservoir size, non-cytolytic mechanism can act on infected CD4+ T cells to block virus expression and promote latent infection. This hypothesis is supported by the fact that HIV-1 latent in resting CD4+ T cells under cART constitutes a barrier to CD8+ T cell mediated eradication (127). The ability of these long-lived cells to persist against the viral cytopathic effect of the immune response depends on several factors, including presence of viral escape variants. A recent study reported that, while escape mutations of common CD8+ T cells epitopes are relatively rare in individuals treated during acute infection, more than 98% of proviruses in patients treated during chronic infection harbored escape mutations in dominant epitopes that rendered the proviruses unrecognizable to CD8+ T cells (128).

Moreover, functional defects limit the HIV-specific CD8+ T cells efficacy as the persistent CD8+ T cell exhaustion, due to their chronic immune stimulation. The anatomical immune compartmentalization may also contribute to HIV persistence, considering that HIV-specific CD8+ T cells are largely excluded from lymph node follicles, one of the major sanctuaries of the virus.

Aim of the study

Aim of the study was to examine the mechanisms underlying the CD8+ T cell-mediated control of HIV replication. Therefore, we developed an experimental system consisting of a dual-color CD4/CD8 co-culture assay using *in vitro* HIV infection of healthy, uninfected donors to evaluate whether:

- the autologous TCR-activated CD8+ T cells modulate the ex-vivo infection of CD4+ T cells through a non-specific, cell-type-independent mechanism.
- the CD8+ T cell-mediated suppressive activity is affecting pre- or post-entry viral replication
- the effect of CD8+ T cells on HIV replication is related to a modulation of infected CD4+ T cells activation and proliferation levels.
- the CD8+ T cell-mediated virus suppression is due to a non-cytolytic mechanism
- the CD8+ T cell mediated effect affects the fraction of CD4+ T cells infected with HIV

- the CD8+ T cell-mediated HIV suppression activity is mediated by soluble factors
- the molecular mechanisms underlying virus suppression by CD8+ T cells are related to changes in the transcriptome profile of infected CD4+ T cells.

Methods

Study subjects

HIV-negative individuals (n = 42) were recruited from the LifeSouth Community Blood Centers (Atlanta, GA). Exclusion criteria included history or current infectious diseases. Written informed consent was obtained from each patient and the study was approved by the Emory University Institutional Review Board (IRB) IRB00045821. All enrolled participants underwent peripheral blood collection.

Enrichment and Stimulation of Primary Cells

Starting from ~60 mL of buffy's coats collected from the Life South Community Blood Centers, peripheral blood mononuclear cells (PBMCs) were obtained by Ficoll-Hypaque density gradient centrifugation (Lymphocytes Separation Media; Lonza, USA).

Resting $CD4_+$ T cells and $CD8_+$ T cells were isolated by negative selection (Miltenyi Biotec, USA) and stimulated with human CD3/CD28/CD2 antibody-coated beads (Miltenyi Biotec, USA) at 1:1 bead-to-cell ratio, in the presence of 50 IU/mL of rhIL-2 (Miltenyi Biotec, USA) for three days.

$CD19_+$ B cells were purified by positive selection (Miltenyi Biotec, USA) and stimulated with 8 IU/ml of CD40 L (Miltenyi Biotec, USA) in the presence of 50 IU/ml of IL-4 (Miltenyi Biotec, USA) for three days.

$CD14_+$ monocytes were purified by positive selection (Miltenyi Biotec, USA) and stimulated with 100 ng/ml of LPS (InvivoGen, USA) and 500 ng/ml of R848 (InvivoGen, USA) for three days.

All cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 (R10) (Corning, USA) supplemented with 10% Fetal Bovine Serum (FBS) (Gem Cell, USA), (200mM) L-glutamine (Gemini, USA) and (10,000 U/ml) penicillin/ (10 mg/ml) streptomycin (Gemini, USA) (37°C, 5% CO_2).

Virus production

F. Kirchoff (129) (Ulm University Medical Center, Ulm, Germany) provided us a replication-competent and an Envelope (Env)-defective NL4-3 reporter virus expressing eGFP under control of the HIV-1 LTR. Envelope (Env)-defective NL4-3_eGFP was then complemented in trans with

a dual-tropic Env (pSVIII-92HT593.1) obtained through the NIH-NIH AIDS Reagent Program from Dr. Beatrice Hahn (Cat#3077).

In addition, we decided to modify at the Emory Custom Cloning Core Division the (Env)-defective NL4-3 IRES eGFP reporter virus by adding a destabilized, short-lived version of the eGFP protein. The so-called D2eGFP protein was created by fusing amino acids 422-461 of the degradation domain of mouse ornithine decarboxylase (MODC) to the C-terminal end of an enhanced variant of GFP (eGFP), as previously described (130).

(Env)-defective NL4-3 IRES D2eGFP viral stocks were generated by single or co-transfection of Lenti-X 293T cells (Takara Bio, USA) with the plasmid encoding (Env)-defective NL4-3 IRES D2eGFP and the plasmid encoding a dual-tropic Env (pSVIII-92HT593.1). First, the Lenti-X 293T Cell Line, a subclone of the transformed human embryonic kidney cell line HEK 293, were seeded in Dulbecco's Modified Eagle Medium (DMEM) (ThermoFisher, USA) supplemented with 10% FBS, L-glutamine, penicillin/streptomycin (37° C, 5% CO_2) to approximately reach 75% confluence on transfection day. Fugene6 (Promega, USA) in OptiMem (ThermoFisher, USA) was used to execute the transfection of the HIV-1 constructs into the cultured Lenti-X 293T cells. Medium was changed 6-12 h post-transfection and supernatants were collected after 48 h, filtered through a 0.45 µM pore size filter (Corning, USA) to clear cell debris, and then concentrated by ultracentrifugation (28,000 rpm, 90 minutes, 4°C). Concentrated virions were resuspended in R10. Finally, aliquots of virus were stored at -80° C.

Dual color co-culture assays

We developed a dual color co-culture assay, in which TCR-activated target $CD4_+$ T cells were labelled with 5 µM of CellTrace Violet dye (Vio) per $10x10^6$ cells (Thermo Fisher, USA) and effector TCR-activated $CD8_+$ T cells, activated $CD19_+$ B, or activated $CD14_+$ monocytes were separately labelled with 5 µM of Cell Trace Red eye (Red) per $10x10^6$ cells (Thermo Fisher, USA), prior to infection. $CD4_+$ T cells were subsequently infected with the replication-competent NL4-3 reporter virus expressing eGFP under control of HIV-1 LTR by spinoculation (2h, 37° C, 2,500 rpm) using 100 µl of virus stock/$0.6x10^6$ cells in flat-bottom 96 well-plates. We also prepared uninfected negative controls (mock infection) by adding 100 µl of cell culture media/$0.6x10^6$ cells.

Subsequently, autologous TCR-activated CD8+ T Cells, activated B cells or activated monocytes were added to the Vio+CD4+ T cells at 1:1 and 5:1 effector-to-target cell (E:T) ratios in the presence of 50 UI/ml of rhIL-2 after infection in 48-well plates.

We also performed single cycle infections by blocking the production of mature particles from replication-competent NL4-3_eGFP virus with 100 nM of protease inhibitor (Darunavir), or by using the replication-incompetent, Envelope (Env)-defective NL4-3_eGFP or (Env)-defective NL4-3 IRES D2eGFP complemented in trans with a dual tropic Env.

Moreover, we set up heterologous co-culture assays, under the same conditions described above, mismatching CD4+ T cells and CD8+ T cells from different donors. Additionally, we physically separated target from effector T cells in a transwell assay, placing Vio+CD4+ T cells in the top chamber of 6.5 mm plates with 0.4 μm pore membrane insert (Corning, USA) and Red+CD8+ T cells in the bottom chamber.

We examined the MHC-based T cell response by adding after infection for the duration of co-culture 20 μg/ml of anti-pan HLA-ABC blocking mAb W6/32 (Biolegend, USA) or 20 μg/mL of anti-pan HLA-E blocking mAb 3D12 (Biolegend, USA). Corresponding isotype control antibodies (Purified Mouse IgG2a, k clone MOPC-173; Purified Mouse IgG2a, k clone MOPC-21; Biolegend, USA) were used as negative controls for comparison. All the antibodies described above, before using, were 30KDa-filtered in phosphate-buffered solution, pH 7.2 (Amicon, Millipore Sigma, USA)

In all experiments, the total number of cells were kept constant at a cell density of 0.6×10^6 cells/ml/well in mono-culture and co-culture to control for variations in media consumption between conditions.

Flow Cytometry analysis

Multiparametric flow cytometry was performed on mono- and co-cultures three days post infection to evaluate cell activation, proliferation, survival and eGFP expression. Cells were first stained with cell viability dye (Live/Dead Fixable Aqua; Thermo Fisher, USA) for 10 min and then stained with monoclonal antibodies to CD4-BV650 (clone OKT4), CD8-PE-Cy5 (clone RPA-T8) and HLA-E-PE (clone 3D12) (Biolegend, USA); CD3-PE-CF594 (clone SP34-2) and HLA-DR-PE-Cy7 (clone G46-6) (BD Biosciences, USA); and HLA-ABC (clone W6/32) (Thermo Fisher, USA)

for 30 min. All specimens were acquired on an LSR II flow cytometer (BD Biosciences) and the acquired data (100,000 live CD3+ T cells) were analyzed with FlowJo software (version 10.1r5).

Cell Sorting

FACS-sorting of productively infected (eGFP+) and non-productively infected as well as uninfected CD4+ T cells (eGFP-) was performed by the Emory Vaccine Center's Flow Cytometry Core, on a BSL-3 FACS Aria II cell sorter (BD Biosciences) equipped with FACS Diva software, three days post-infection using the replication competent NL4-3_eGFP under single cycle condition with 100nM Darunavir to assess the cell host and viral RNA, as well as integrated and inducible provirus. Prior to sorting, cells from mono- and co-cultures were stained with the previously described Live/Dead-Aqua, and with CD3, CD4 and CD8 antibodies. Populations for sorting were defined as follows: productively infected (eGFP+), LiveCD3+Vio+Red-CD8-eGFP+; non-productively infected as well as uninfected CD4+ T cells (eGFP-), LiveCD3+Vio+Red-CD8-eGFP-. The uninfected (mock) wells were used as negative controls to draw a gate for the HIV-infected (eGFP) wells.

Quantification of inducible provirus

On day 3 post HIV-infection, 1×10^6 FACS-sorted eGFP-CD4+ T cells from the mono - and co-cultures were placed back in culture and re-stimulated 24 hours with human CD3/CD28/CD2 antibody-coated beads (bead-to-cell ratio 1:1) or with plate-bound anti-CD3 (clone OKT3; eBioscience, USA) and soluble anti-CD28 (clone 28.2; eBioscience, USA) at 2 μg/ml in the presence of 50 IU/ml of rhIL-2. De novo infection and pre-integration events were controlled by adding respectively 100 nM Darunavir and 1 μM Dolutegravir. The inducible provirus was quantified measuring the frequencies of eGFP+ cells obtained 24 hours post-reactivation by Flow Cytometry. Analysis of the acquired data was performed using FlowJo software (version 10.1r5).

Purification of total viral nucleic acids

For the total viral nucleic acid extraction, we first lysed 0.5×10^6 FACS-sorted eGFP-CD4+ T cells and at least 0.5×10^5 FACS-sorted eGFP+CD4+ T cells from the mono- and co-cultures using RLT buffer (Qiagen, USA) with 1% of 2-Mercaptoethanol (BME) (Sigma, USA) and stored them at -80_o C immediately.

Subsequently, we performed the purification of total viral nucleic acids by using the QIAamp Viral RNA Mini Kit (Qiagen, USA) and following the manufacturer's instructions.

Quantification of integrated HIV-DNA

The integrated HIV-DNA was determined using a real time nested polymerase chain reaction (PCR) (adapted from (131)) and performed at the Laboratory of Biochemical Pharmacology, in the Department of Pediatrics of the Emory University. Serial dilution of lysed ACH-2 cells, which carry a single copy of the integrated HIV genome, were used to generate a standard curve for the integrated HIV DNA assay. Additionally, in all PCRs, primers specific for the human CD3 gene (primers HCD3OUT5′ and HCD3OUT3′) were used to quantify the exact number of cells present in the reaction tube.

Briefly, 2.5 µl of the purified viral acid nucleic was used in a 25 µl first-round PCR reaction consisting of a denaturation step of 8 min at 95°C and 12 cycles of amplification (95°C for 1 min, 55°C for 1 min, 72°C for 10 min, followed by an elongation step of 15 min at 72°C.)

The second-round of PCR reaction was performed in a final volume of 20 µl containing 6.4 µl of a 1/10 dilution of the first PCR products. The same amplification steps were used for all reactions: a denaturation step (95°C for 4 min), followed by 40 cycles of amplification (95°C for 3 s, 60°C for 10 s).

Integrated HIV-DNA was amplified using primers and probe specific for the LTR-Gag region and for the human Alu sequences (131). Results were expressed as numbers of HIV copies per million cells.

Quantification of cell-associated Tat/rev multiply spliced HIV RNA

The frequency of cells harboring Tat/rev multiply spliced HIV RNA was determined using a real time quantitative reverse transcription polymerase chain reaction (qRT-PCR) (adapted from (132)) and performed at the Laboratory of Biochemical Pharmacology, in the Department of Pediatrics of the Emory University. Briefly, 1 µl of the purified viral acid nucleic was used in a 10 µl first-round RT-PCR consisting of a reverse transcription step at 50° C for 15 min, a denaturation step at 95°C for 2 min, and 24 cycles of amplification (95°C for 15 s, 60°C for 4 min). The second-round of PCR reaction was performed in a final volume of 10 µl containing 1 µl of a 1/10 dilution of the first PCR products (a preincubation step at 95° C for 10 min, 45 cycles of 95°C for 10 s,

60°C for 30 s, 72°C for 1 s and a cooling step at 40°C for 30 s). Multiply spliced HIV RNA was amplified using primers and probe specific for the tat/rev region (132).

RNA copy numbers were calculated based on the RNA controls from an *in vitro* transcription of target DNA (T7-Tat/Rev).

RNA-seq library preparation

50,000 FACS-sorted eGFP- and eGFP+CD4+ T cells from mono- and co-cultures were stored in 350 μl of RLT buffer (Qiagen, USA) with 1% of 2-Mercaptoethanol (BME) (Sigma, USA) at -80°C. Subsequently, RNA was purified using RNeasy Micro columns kit (Qiagen, USA) and RNA quality was assessed using an Agilent Bioanalyzer. RNA-seq analysis was conducted at the Yerkes Nonhuman Primate Genomics Core Laboratory (http://www.yerkes.emory.edu.proxy.library.emory.edu/nhp_genomics_core/). The SMART-Seq v4 Ultra Low Input RNA kit (Clontech, USA) was used to perform the cDNA synthesis, starting with ten nanograms of total RNA. Amplified cDNA was simultaneously fragmented and tagged with dual-indexed bar codes, by using the NexteraXT DNA Library Preparation kit (Illumina, USA)

Libraries were validated by capillary electrophoresis on an Agilent 4200 TapeStation, pooled, and sequenced on an Illumina HiSeq3000 at 100 single-reads (SR) at an average read depth of 15 million reads/sample. Reads were aligned to the human Refseq (GRCh38) using STAR software (v2.5.2b) (https://github.com/alexdobin/STAR/releases) (133). Transcript abundance estimation was performed internally in STAR with reference to the GRCh38 annotation using the algorithm of htseq-count. Library size normalization, differential expression and log2 transformation were performed using DESeq2 (134). Gene set and pathway analysis was performed with Gene Set Enrichment Analysis (GSEA) (135). Ranked datasets contrasting co-cultures versus mono-cultures were tested for enrichment of the BIOCARTA collection gene sets from the Molecular Signatures Database (136) using gene set permutation to test for statistical significance. Heat maps and other visualizations were generated using Partek Genomics software, v.6.6.

Cytokine Array

We used a semi-quantitative human cytokine antibody array (C Series 4000, Ray Biotech, USA) detecting 274 proteins to compare the secretome profile of CD4+ T cells monoculture and CD4/CD8 co-cultures. To investigate, in our experimental system, the role of potential soluble factors that mediate the observed CD8+ T cells suppressor activity, we selected only the supernatant of CD4/CD8 co-cultures at 5:1 (E:T) and compared those to the supernatant of CD4 mono-cultures. After three days of infection with the replication competent NL4-3_eGFP under single cycle condition with 100nM Darunavir, we collected the supernatants described above, centrifuged at 18,500 rpm for 10 min, diluted 2-fold with an inactivation buffer containing 1% 4-Nonylphenyl-polyethylene glycol (Sigma-Aldrich, USA) and 1% protease inhibitor cocktail (Thermo Fisher, USA), and stored at -80o C immediately.

An incubation at 4 °C overnight, with gentle shaking, was performed by adding 1.5 ml of the diluted mono- and co-culture cell supernatants to each cytokine array. The chemiluminescent signals were captured using the ChemiDoc XRS+ Imaging system (BioRad), and numerical signal densities were processed with ImageJ software. The average signal of the pair of duplicate spots representing each cytokine was determined and average background signal from blank was subtracted from each spot. Values that were below background after blank subtraction were set to 0. Values were then normalized using the average internal positive control signals according to manufacturer's data analysis instructions. This normalization process allows for a consistent comparison of results across multiple arrays. Comparisons between 5:1 (E:T CD4/CD8) co-cultures and CD4 mono-cultures were carried out using parametric donor-matched pairwise t-test (test of variance) and batch t-test (test of the mean and standard deviation of the set). Normality was assessed using Kolmogorov-Smirnov test, and significance was attributed at $p<0.05$.

Statistical analysis

The statistical analysis was done using GraphPad Prism 7.0., except for RNA-seq and Cytokine Array. Comparisons between mono-and co-cultures were determined by paired Wilcoxon matched-pairs signed-rank test, and significance was attributed at p values of <0.05. RNA-seq differential expression analysis and statistical analysis were performed as previously described using the DESeq2 package (134), and Cytokine Array statistical analysis were determined using the Scipy v.1.1.0 library implemented in Python v.3.3.1 as previously described using parametric

donor-matched pairwise t-test and batch t-test. Normality was assessed using Kolmogorov-Smirnov test, and significance was attributed at p<0.05.

Results

TCR-stimulated CD8+ T cells effectively mediate suppression of HIV replication in CD4+ T cells

We evaluated antiviral effects of autologous TCR-stimulated CD8+ T lymphocytes from healthy donors in controlling HIV replication in infected CD4+ T cells. Prior to infection with a replication-competent NL4-3_eGFP reporter virus expressing eGFP under control of the HIV-1 LTR, we labelled separately CD4+ T cells with CellTrace Violet dye (Vio) and CD8+ T cells with CellTrace Red (Red). After infection, cells were co-cultured for 72 hours at an effector (CD8) to target (CD4) (E:T) ratio of 1:1 and of 5:1. This *ex-vivo* CD4/CD8 dual color co-culture assay provides a practical method to distinguish between the productively infected CD4+ T cells, defined as live CD3+Vio+Red-CD8-eGFP+ cells and non-productively infected as well as uninfected cells, defined as live CD3+Vio+Red-CD8-eGFP- (Figure 7a, 7b).

By comparing the frequencies of productively infected cells between the CD4/CD8 co-cultures and the CD4 mono-cultures, we observed in the presence of CD8+ T cells a mean reduction of 56 % at 1:1 ratio in the HIV_eGFP expression and a 73% in the 5:1 ratio under multiple cycle of infection (p=0.0020; Figure 7b).

The specific role of CD8+ T cells in modulating HIV expression was confirmed in a subsequent experiment in which, using the same previous co-culture assay, we substituted autologous CD8+ T cells with autologous activated Red+CD19+ B cells or autologous activated Red+CD14+ monocytes cells. Indeed, co-cultures of infected Vio+CD4+ cells with B cells or monocytes resulted in a significant increase of the frequency of productively infected CD4+T cells (p=0.0078; Figure 7c).

Starting from our dual color CD4/CD8 assay, we next set up two distinct systems, both based on a single cycle of infection, to differentiate which stage of HIV life cycle, between the pre-or post-entry replication, is susceptible to inhibition by CD8+ T cells. In the first model, we added the protease inhibitor Darunavir to the replication-competent NL4-3_eGFP reporter virus, while, in the second, we used a replication-incompetent Envelope (Env)-defective NL4-3_eGFP complemented in trans with a dual-tropic Env. Opposite to the effects observed in the multiple round infection assay, CD8+ T cells didn't exhibit significant levels of suppression activity in the

co-cultures with the infected CD4+ cells, compared to the CD4+ mono-cultures, under single cycle infection conditions (Figure 7d). Therefore, we hypothesized that the unexpected lower level of viral reduction mediated by CD8+ T cells in the experiments described above was probably linked to the strong stability and the consequent long life of 26 hours of the eGFP protein.

So, we decided to generate an Env-defective NL4-3_eGFP construct expressing a destabilized-eGFP protein (130) under the control of the viral HIV-1 LTR, with a short half-life of 2 hours, called NL4-3_D2eGFP.

Interestingly, as shown in Figure 7e, significant decreased levels of both the frequency and the mean fluorescence intensity (MFI) of D2eGFP+ cells, obtained in our dual color CD4/CD8 assay using the modified D2eGFP virus complemented in trans with a dual tropic envelope, suggest that the CD8-mediated suppression activity acts not only on the HIV spread, but also on the HIV LTR-driven expression on a per-infected cell basis.
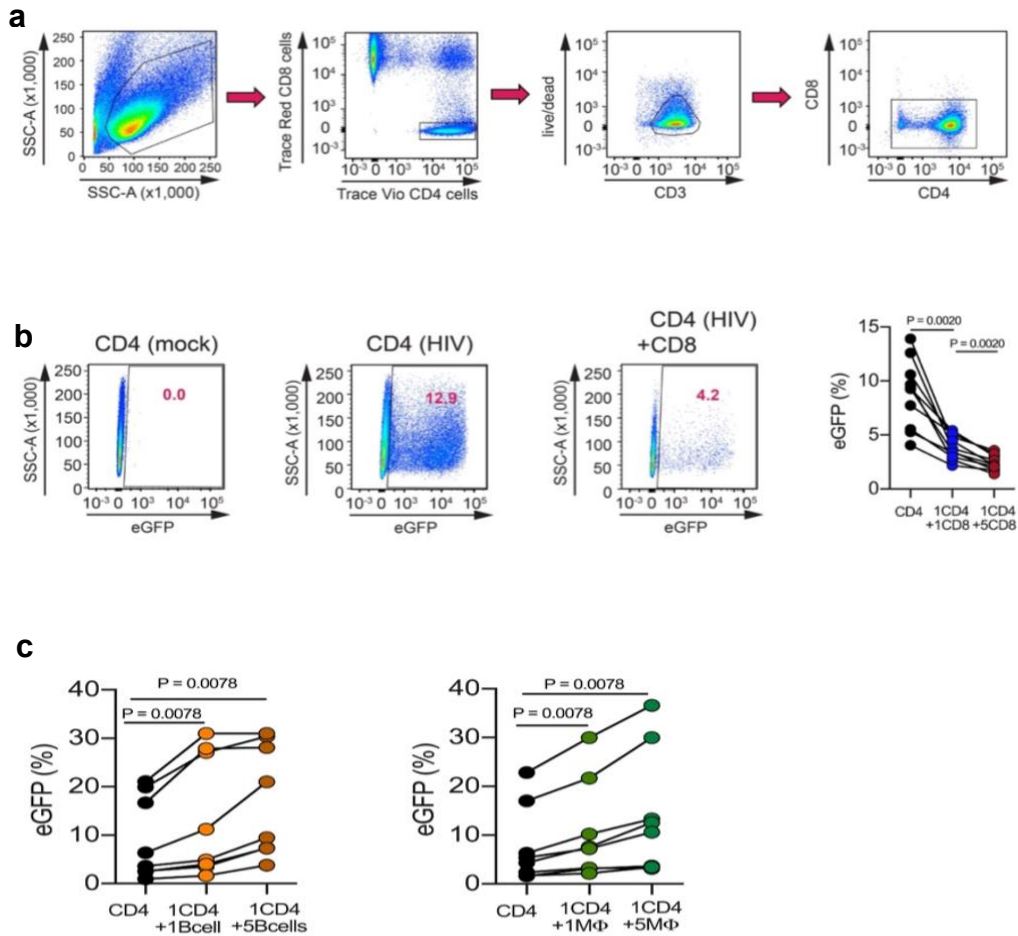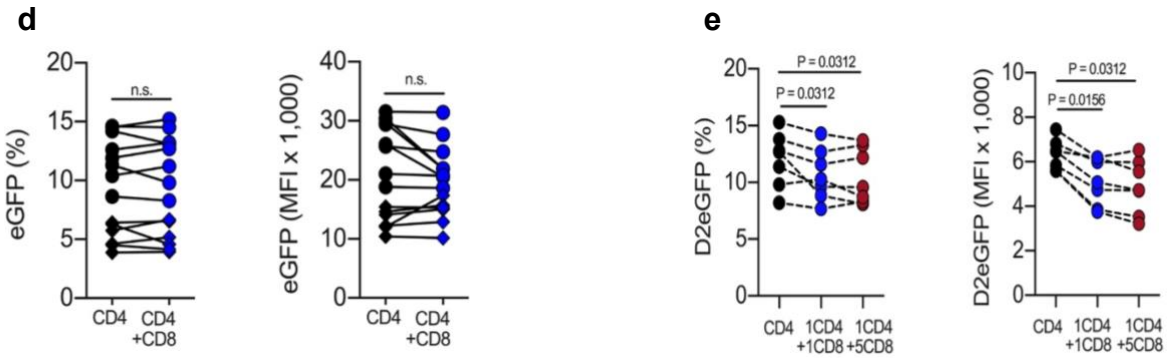
Figure 7

a) CD4/CD8 Flow Cytometry Gating Strategy

b) Representative flow plots and frequencies of uninfected (mock) and productively infected CD4+ T cells (live CD3+Vio+Red-CD8-eGFP+) from mono- or co-cultures with CD8+ T cells under multiple round of infection with NL4-3_eGFP virus (n=10)

c) Frequencies of productively infected CD4+ T cells from mono-cultures or co-cultures with activated B or monocytes cells under multiple round of infection with NL4-3_eGFP virus (n=8)

d) Frequencies and mean fluorescence intensity (MFI) of productively infected CD4+ cells from mono- or co-cultures with CD8+ T cells under single cycle of infection with NL4-3_eGFP virus with protease inhibitor Darunavir (indicated by diamonds; n=6) and with Env-defective NL4-3_eGFP complemented in trans with a dual-tropic envelope (indicated by circles; n=8)

e) Frequencies and mean fluorescence intensity (MFI) of live CD3+Vio+Red-CD8-D2eGFP+ cells from mono- or co-cultures with CD8+ T cells under single cycle of infection with NL4-3_D2eGFP (n=7).

Statistical analysis was conducted with Wilcoxon matched-pairs signed rank test and significance was attributed at p<0.05

Virus-independent CD8+ T cell-mediated immunomodulatory mechanisms on the CD4+ T cells activation and proliferation contribute to the suppression of HIV replication

Strong evidences suggest that HIV transcription is promoted in activated CD4+ T cells as compared to resting CD4+ T cells (137). Therefore, we hypothesized that CD8+ T cells could mediate the previously observed HIV suppression by modulating infected CD4+ T cells proliferation and activation levels. To this end, we decided to analyze the levels of HLA-DR, a classical activation marker, and of HLA-E (found upregulated upon *in vitro* stimulation in all memory CD4+ T subsets by our group, data not shown) by using our multiple round infection assay. Indeed, we found that

CD8+ T cells down-modulated activation markers on CD4+ T cells in both productively infected (eGFP+) and non-productively infected and uninfected (eGFP-) cells, as well as in the matched uninfected controls (mock) (Figure 8a, 8b) Experiments performed under single cycle of infection gave us the same results (Figure 8c1, 8c2).

In addition, as shown in Figure 8d, the levels of CD4+ T cells proliferation, measured as mean fold change in Cell Trace Violet MFI, were significantly reduced in the presence of CD8+ T cells limited to non-productively infected, uninfected (eGFP-) and in matched uninfected controls (mock) CD4+ T cells in the multiple round setting.

By contrast, CD8+ T cells also inhibited significantly the proliferation of productively infected CD4+ T cells (eGFP+) under single cycle infection conditions. (p=0.0027; Figure e1 and p=0.0156; Figure e2) Altogether, these findings suggest that the CD4+ T cell activation and proliferation mitigation mediated by CD8+ T cells might be a virus-independent mechanism, as strongly indicated by the immunomodulatory effect of CD8+ T cells also in the uninfected control populations, never been exposed to virus. Moreover, by affecting the proliferation and activation levels of the CD4+ T cells, CD8+ T cells could lead them to a more resting state, possibly promoting the reduction of virus replication.
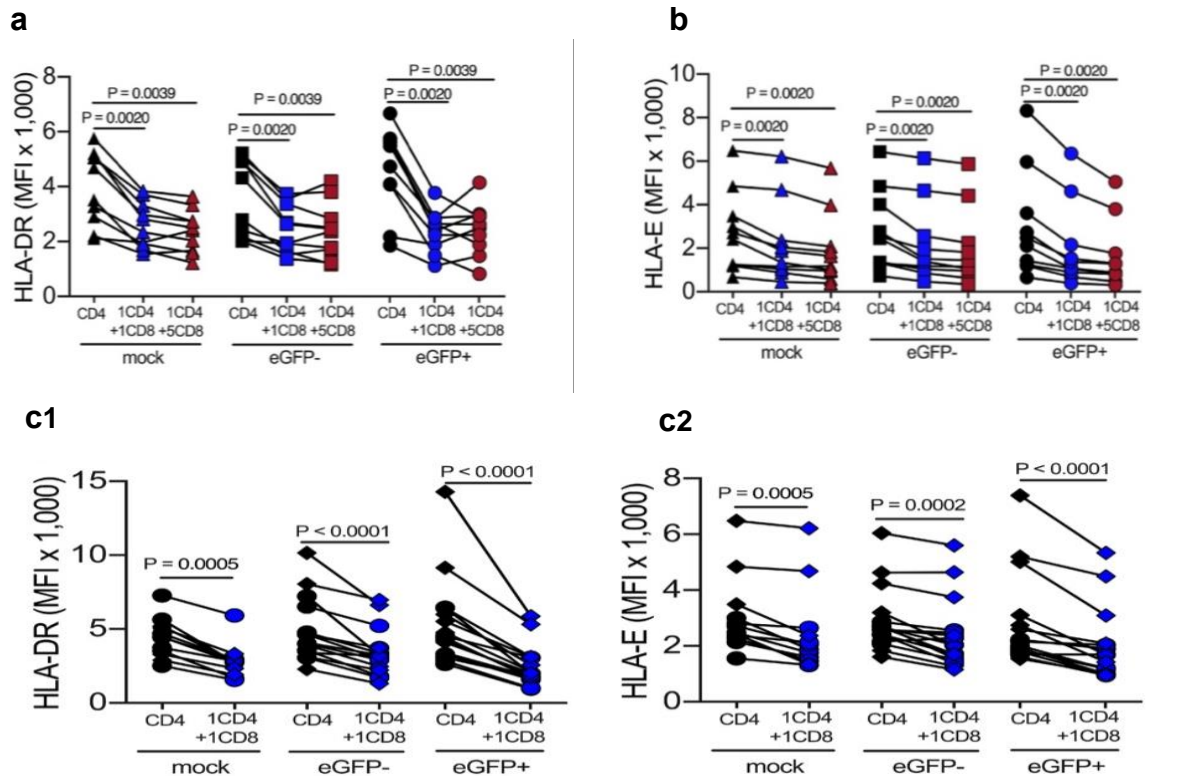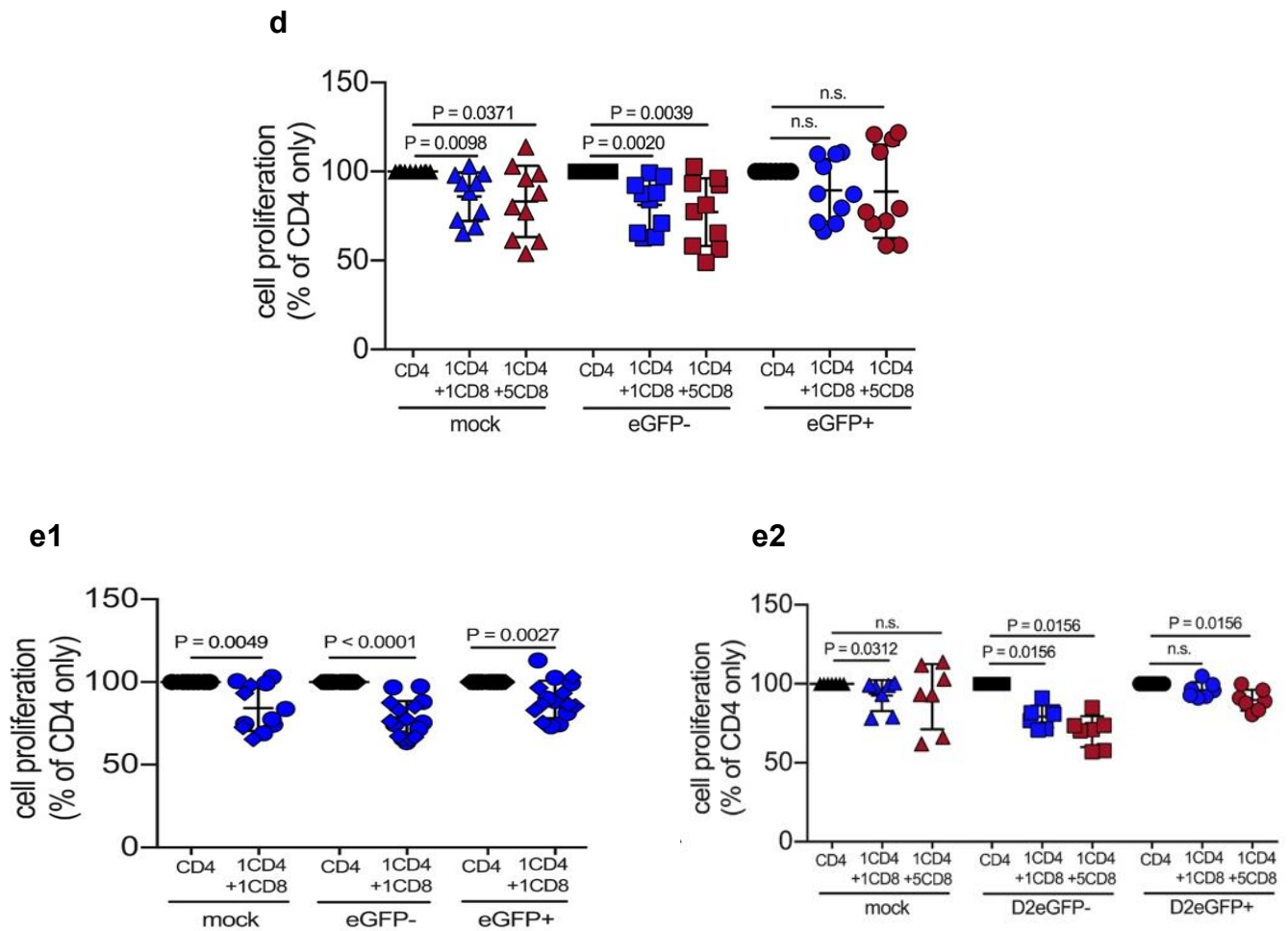
Figure 8

a) Mean fluorescence intensity (MFI) of HLA-DR for mock (uninfected), eGFP- and eGFP+ CD4+ T cells from mono- or co-cultures with CD8+ T cells under multiple round of infection with NL4-3_eGFP virus (n=10)

b) Mean fluorescence intensity (MFI) of HLA-E for mock (uninfected), eGFP- and eGFP+ CD4+ T cells from mono- or co-cultures with CD8+ T cells under multiple round of infection with NL4-3_eGFP virus (n=10)

c) c1 and c2: Mean fluorescence intensity (MFI) of HLA-DR and HLA-E for mock (uninfected), eGFP- and eGFP+ CD4+ T cells from mono- or co-cultures with CD8+ T cells under single cycle of infection with NL4-3_eGFP virus with protease inhibitor Darunavir (indicated by diamonds; n=6) and with Env-defective NL4-3_eGFP complemented in trans with a dual-tropic envelope (indicated by circles; n = 8).

d)Fold change in CellTrace Violet MFI (relative to a CD4+ T cells alone, followed by a f(x) = 1/x transformation) for mock (uninfected), eGFP- and eGFP+ CD4+ T cells from mono- or co-cultures with CD8+ T cells under multiple round infection setting with NL4-3_eGFP virus (n=10)

e) e1: Fold change in CellTrace Violet MFI (relative to a CD4+ T cells alone, followed by a f(x) = 1/x transformation) for mock (uninfected), eGFP- and eGFP+ CD4+ T cells from mono- or co-cultures with CD8+ T cells under single cycle infection with NL4-3_eGFP virus treated with protease inhibitor Darunavir (indicated by diamonds; n = 6) and with replication-incompetent Env-defective NL4-3_eGFP complemented in trans with a dual-tropic envelope are indicated by circles (n = 8).

e2: Fold change in CellTrace Violet MFI (relative to a CD4+ T cells alone, followed by a f(x) = 1/x transformation) for mock (uninfected), eGFP- and eGFP+ CD4+ T cells from mono- or co-cultures with CD8+ T cells under single cycle infection with with Env-defective NL4-3_D2eGFP virus (n=7)

Statistical analysis was conducted with Wilcoxon matched-pairs signed rank test and significance was attributed at p<0.05

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CD8+ T cell control HIV replication through a non-cytolytic, MHC-independent, and partially mediated by soluble factors, activity

MHC-I restricted, virus-specific cytotoxic CD8+ T cells (CTLs) control HIV replication through the recognition and killing of productively infected CD4+ T cells (138,139), although their number in healthy subjects is really low (140,141). Moreover, the exact contribution of CTL activity in vivo remains incompletely understood. Possible non-cytolytic mechanisms of CD8+ T cells in virus suppression have been also suggested. Overall, these considerations led us to examine the CD8+ T cell-mediated effects on the survival of infected CD4+ T cells, by measuring with the amine-reactive dye method the frequency of live CD4+ T cells and the frequency of virus-induced cell death relative to uninfected cells in the control (mock infection).

Interestingly, the results in Figure 9a and 9b show that CD8+ T cells protected target cells from virus cytopathic effect in a dose dependent manner as the increase frequency of live CD4+ and the decrease frequency of virus-induced cell death demonstrated.

In order to determine if HIV inhibition activity mediated by CD8+ T cells is also an MHC-based T cell immunoregulatory response, an anti-pan HLA-ABC blocking mAb W6/32 (20 μg/ml) or an anti-HLA-E mAb 3D12 (20 μg/ml) as well as their isotype control antibodies were added to the CD4/CD8 co-cultures. We found that HLA-ABC and HLA-E blockage did not restore HIV expression in the co-cultures (Figure 9c). In addition, we performed a heterologous co-culture system, mismatching CD4+ and CD8+ from different donors. Our results indicated that the heterologous CD8+ T cells didn't fail in mediating HIV suppression (Figure 9d). Altogether, these data support the hypothesis that CD8+ mediated virus suppression is MHC-I and MHC-E independent.

To understand if the antiviral effect of CD8+ T cells is dependent on cell-to-cell contact or on soluble factors release, we physically separated effector (CD8) and target (CD4) cell populations in a transwell (0.4 μm) cell culture system. We obtained a mean suppression of 19% at 1:1 ratios and 31% at 5:1 ratios (E:T) in the transwell system, while a 56% at 1:1 ratio and 73% at 5:1 ratio (E:T) in the cell-to-cell contact system (Figure 9e). These findings highlighted a partial role of the soluble factors release in the CD8+ T cell-mediated suppression of HIV production, that conversely

seems to require direct contact between CD8+ T cells and CD4+ infected cells for reaching the predominant effect.
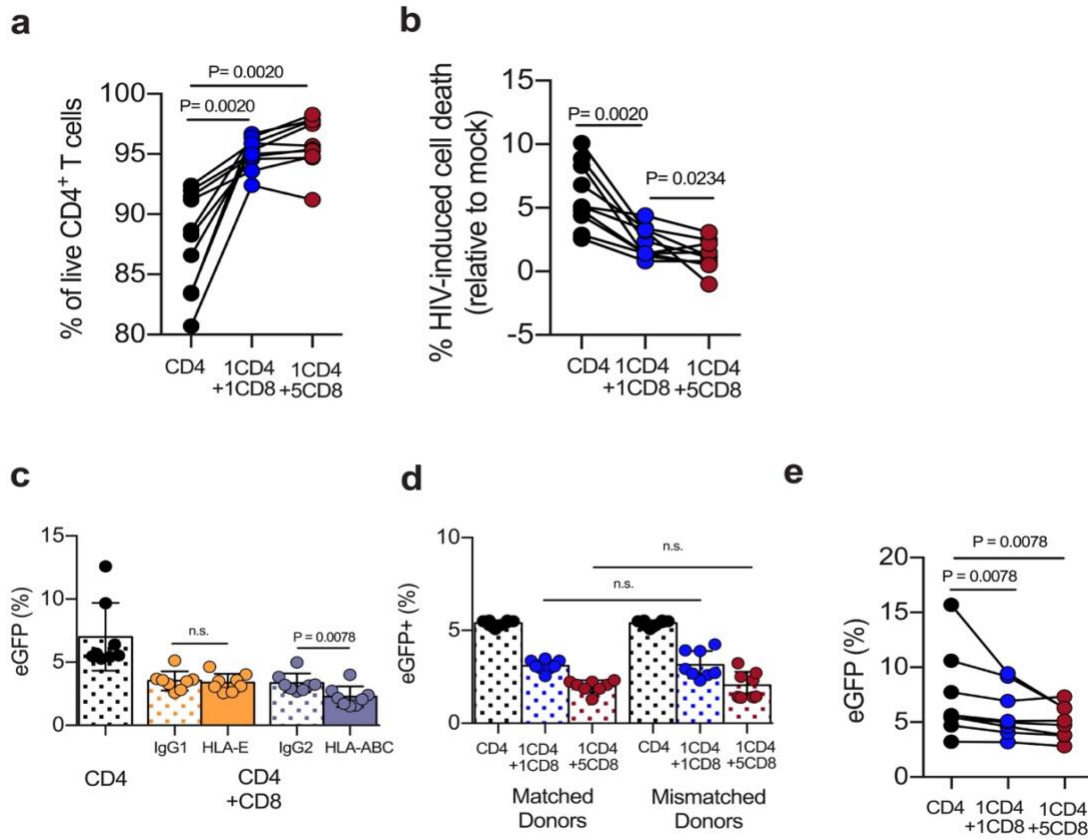

Figure 9
a) Frequencies of live CD4+ T cells (live CD3+Vio+Red-Cells) from infected mono- or co-cultures with CD8+ T cells under multiple round of infection with NL4-3_eGFP virus (n=10)

b) Frequencies of HIV-induced cell death (relative to mock) under multiple round of infection with NL4-3_eGFP virus (n=10)

c) Frequencies of productively infected CD4+ T cells from mono- or co-cultures with CD8+ T cells after anti-pan HLA-I blocking mAb W6/32 and/or anti-HLA-E blocking mAb 3D12 under multiple round of infection with NL4-3_eGFP virus (n=8) (Isotype controls are included)

d) Frequencies of productively infected CD4+ T cells from mono- or co-cultures with autologous (matched donors) or heterologous (mismatched donors) CD8+ T cells under multiple round of infection with NL4-3_eGFP virus (n=8)

e) Frequencies of productively infected CD4+ T cells from mono- or co-cultures with CD8+ T cells in a transwell assay (0.4μm) under multiple round of infection with NL4-3_eGFP virus, in which CD4+ T cells are located in the top chamber and CD8+ T cells in the bottom chamber (n=8).

Statistical analysis was conducted with Wilcoxon matched-pairs signed rank test and significance was attributed at p<0.05

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CD8+ T cell mediate viral suppression by increasing the frequency of non-productively infected CD4+ T cells

We next decided to test whether CD8+ T cells had an impact on the proviral landscape constituted by CD4+ T HIV-infected cells. To this end, eGFP- and eGFP+ CD4+ T cells from CD4/CD8 co-cultures and from CD4+ mono-cultures were sorted three days post a single cycle infection (Figure 10a, 10b), using a replicant-competent NL4-3_eGFP virus with the addition of the protease inhibitor Darunavir. When we looked at the amount of integrated HIV-DNA, we detected a significant increase in the frequency of CD4+ T cells carrying integrated HIV-DNA in the co-cultures compared to the mono-cultures alone (p=0.0078 for eGFP-, p=0.0078 for eGFP+ Figure 11a). The reactivation of sorted eGFP- CD4+ T cells with CD3/CD28 beads stimulation three days post infection (in the presence of Dolutegravir and Darunavir) from mono- and co-cultures confirmed that CD8+ T cells increase the frequency of non-productively infected CD4+ T cells (eGFP-), as shown by the frequencies of inducible provirus in Figure 11b (p=0.0078).

Finally, the levels of HIV transcripts on a per-integrated-provirus basis, calculated as copy numbers of cells-associated multiply-spliced Tat-Rev HIV-RNA per copy numbers of integrated HIV-DNA per cell, were reduced in the presence of CD8+ T cells (56%, p=0.0078 for eGFP-, and 54%, p=0.0156 for eGFP+, Figure 11c). These results suggest that CD8+ T cells, by promoting a transcriptional silencing, selectively might favor the survival of non-productively infected CD4+ T cells, that produce HIV with low efficiency on a per-infected-cell basis, thus possibly encouraging the establishment of HIV latency.
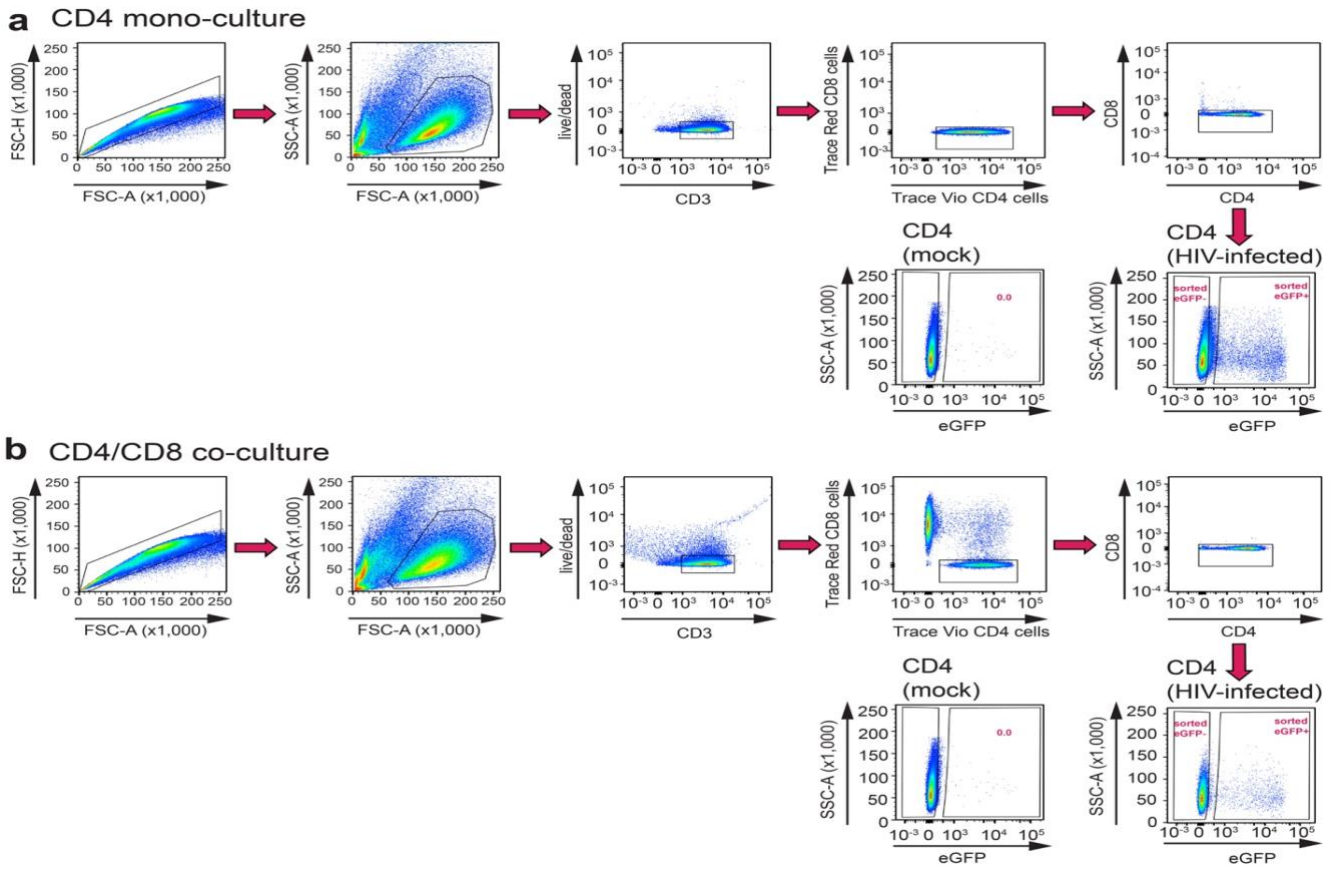
Figure 10

FACS-sorted eGFP+ and eGFP- CD4+ T cells from mono- (10a) and co-cultures (10b) with CD8+ T cells at 1:1 E:T ratio 3 days post-infection under single cycle condition.
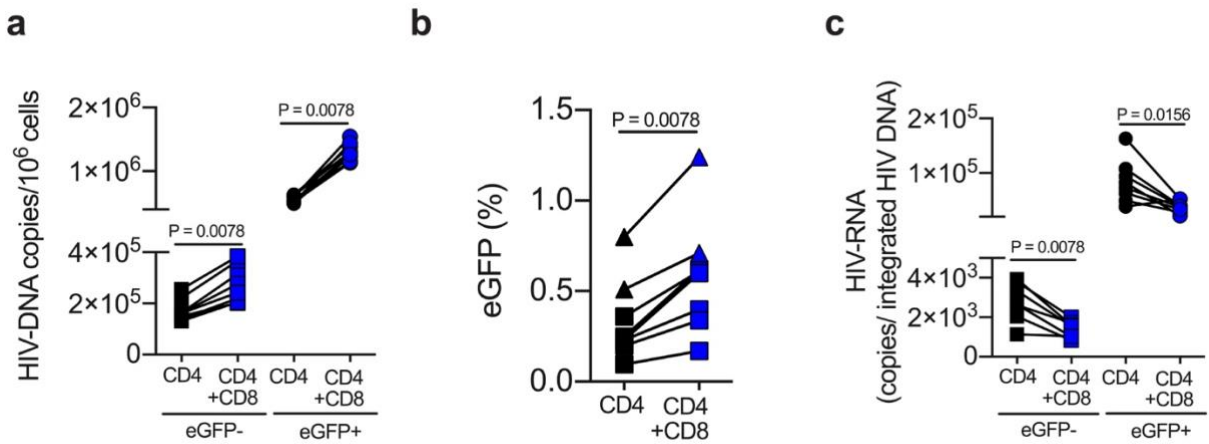
Figure 11

All *in vitro* infections were conducted using the NL4-3_eGFP virus under single cycle condition with protease inhibitor Darunavir, with the exception of 2 donors infected with the Env-defective NL4-3_eGFP in the inducible provirus experiment (indicated by triangle symbol).

a) Integrated HIV-DNA copies/10^6 cells for sorted eGFP- and eGFP+ CD4+ T cells from mono- or co-cultures with CD8+ T cells under single cycle condition using the NL4-3_eGFP virus with protease inhibitor Darunavir (n=8).

b) Frequency of eGFP+ cells 24 hours post-reactivation of sorted eGFP- CD4+ T cells.

c) Levels of HIV transcripts on a per integrated-provirus-basis using the NL4-3_eGFP virus under single cycle condition with protease inhibitor Darunavir (n=8)

Statistical analysis was conducted with Wilcoxon matched-pairs signed rank test and significance was attributed at $p < 0.05$

Secretome profile analysis reveals the CD8+ T cell-mediated suppressive activity on infected CD4+ T cells

To identify potential soluble inhibitor factors secreted by CD8+ T cells, responsible for the transcriptional suppression previously observed in the infected CD4+ T cells, we tested 274 soluble factors using a cytokine array. Comparing the supernatants from the mono-cultures of CD4+ T cells with the supernatants only from the co-cultures at 5:1 ratio (E:T) and keeping the number of cells per well constant in the mono- and co-cultures allowed us to discriminate the upregulated cytokines in the presence of CD8+ T cells.

As shown in Table 1, 18 of the tested 274 soluble factors were higher in the 5:1 (E:T) supernatants than the CD4 mono-culture supernatants. They included T helper 2 (Th2) related factors (IL-5, IL-9, IL-13, IL-13 R alpha 2 and IL-33 receptor), TNFR-related proteins (GITR ligand, GITR and TRAIL-R4) and grown factors members (G-CSF, M-CSFR and PDGFR).

Table 1. Differentially expressed soluble factors between 1CD4/5CD8 and CD4 T cells

Soluble factor	Description	n fold-increase	P-value
IL-13 R alpha 2	Th2-type cytokine receptor	180	0.005
MMP-9	Matrix metalloproteinase-9	85.8	0.02
IL-9	Th2-type cytokine	59.5	0.0009
MMP-3	Matrix metalloproteinase-3	31.2	0.05
SDF-1 beta	CXCR4 ligand	22.3	0.04
IL-18 R beta (AcPL)	Th1-type cytokine receptor	19.8	0.008
CSF1R/M-CSFR	Member of the CSF1/PDGF receptor family of tyrosine-protein kinases	18.1	0.02
PDGFR alpha	Member of the CSF1/PDGF receptor family of tyrosine-protein kinases	17.3	0.03
IL-1 R2	IL-1β Inhibitor	8.9	0.05*
TIE-1	Anti-apoptotic (via PI3K-AKT pathway)	7.5	0.05*
G-CSF	Modulates T-cell function toward a type-2 response in mice/ T-regulatory in humans	4.5	0.03*
IL-5	Th2-type cytokine	3.4	0.04*
IL-1 R4 (ST2), IL-33 R	Th2-type cytokine receptor	3.1	0.05*
TRAIL-R4	Anti-apoptotic (protects against TRAIL-Mediated Apoptosis)	3.1	0.04*
ICAM-1	Adhesion molecule	2.3	0.03
GITR	Anti-inflammatory and anti-apoptotic	1.94	0.02
GITR Ligand	Anti-inflammatory and anti-apoptotic	1.72	0.01
IL-13	Th2-type cytokine	1.6	0.04

Significance was attributed at $p < 0.05$ using paired t-test. Asterisks (*) indicate statistical significance ($p < 0.05$) using batch t test (n=6).

CD8₊ T cell-mediated virus suppression changes the transcriptome profile of infected CD4₊ T cells.

We analyzed the transcriptome profile of HIV non-productively infected and productively infected CD4$^+$ T cells alone or when co-cultured with CD8₊ T cells. These two subsets were sorted three days post infection under single cycle infection assay with Darunavir. A Principal component analysis (PCA) conducted on our samples, by comparing the 500 genes with the largest possible variance across all samples, revealed a transcriptome profile distribution based on three factors: the inter donor variability, the viral production and the presence of CD8₊ T cells. As the PCA plots show in Figure 12a, along the first principal component axis (PC#1) male donors are clearly separated from female donors (with the 35% of variance), along the second component axis (PC#2) it is possible to distinguish between the eGFP₊ CD4₊ T cells and eGFP₋ CD4₊ T cells (with the 26% of variance), independently of donor variability. Finally, along the third component axis (PC#3) the eGFP₋ and eGFP₊ CD4₊ T cells populations from co-cultures clustered away from the eGFP₋ and eGFP₊ CD4₊ T cells subsets derived from mono-cultures. These results highlight the ability of CD8₊ T cells to change the transcriptome profile on both productively and non-productively infected CD4₊ T cells.

To gain insights on the differentially expressed genes in the sorted eGFP₋ and eGFP₊ CD4₊ T cells in the presence of CD8₊ T cells, we compared mono and co-cultures in terms of RNA-seq differential expression, using a DESeq2 package (fold change threshold >1.5 or <0.67; padj <0.05). Following these criteria, we found that the eGFP₋ CD4₊ T subsets had approximately in common 50% of the differentially expressed genes (DEGs) with eGFP₊. In addition, 480 downregulated and only 213 upregulated genes transcripts were detected in response to CD8₊ T cells (Figure 12b) These data evidence a suppressive effect mediated by CD8₊ T cells on the overall transcriptional activity of productively and non-productively HIV-1 infected CD4₊ T cells.

We next decided to use a gene set enrichment analysis (GSEA) as a valuable tool to better determine which canonical signaling pathways are affected in the co-culture CD4/CD8 compared to the mono-cultures. Focusing on 217 canonical pathways derived from the BioCarta database, we obtained a significant reduction of several gene expression pathways related to cell death including CD95/Fas, Tumor necrosis factor receptor 1 (TNFR1), Bcl-2 associated death promoter (BAD) and Caspase in the presence of CD8+ T cells. Moreover, same results were observed for

pathways associated with CD4+ T cell differentiation such as Th1/Th2 response, chemokine receptors (NKT pathway), and cell polarization (DC pathway); cytokine and inflammatory response such as IL-10, ILR1, IFN (TID) and NF-kb pathways; and CD4+ T cell proliferation and DNA repair such as ARF, ATR and BRCA, G1, G2, and Ras pathway (Figure 12c). Interestingly, we also found that the presence of CD8+ T cells is associated with a significant upregulation of transcriptional regulator genes related to the Th2 pathway of CD4+ T helper differentiation (GATA3, padj=0.014 for eGFP+; and GFI1, padj=4.37x10-6 for eGFP- and padj=0.0014 for eGFP-; Figure 12d).

These results are consistent with our hypothesis of the non-cytolytic role of CD8+ T cells in the suppression of HIV expression, also suggesting that these cells might act by promoting a downmodulation of CD4+ T cells activation and a Th2 differentiation.
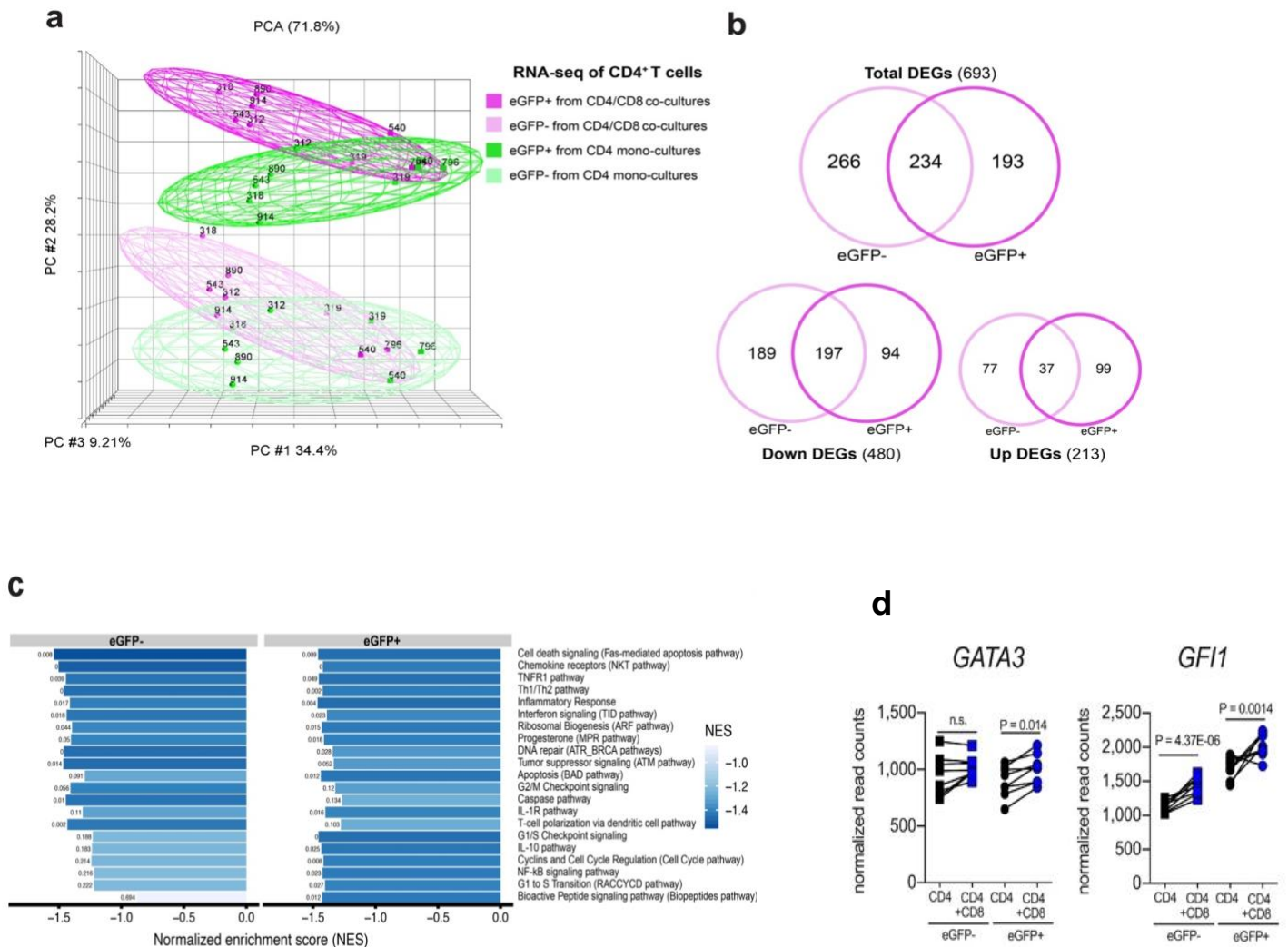
Figure 12

a) Principal component analysis (PCA) of RNA seq data derived from sorted eGFP- and eGFP+ CD4+ T cells from mono- and co-cultures (n=8) in a single cycle condition with protease inhibitor Darunavir. Female donors are indicated by circles, male donors by squares.

b) Venn diagrams representing differentially expressed genes (DEGs) in eGFP- and eGFP+ CD4+ T cells in response to CD8+ T cells

c) Barplot shows the pathways significantly downregulated in eGFP- and eGFP+ CD4+ T cells by the CD8+ T cells

d) Effects of CD8+ T cells on GATA3 and GFI1 transcriptional regulator genes.

Discussion

Numerous lines of evidence suggest that CD8+ lymphocytes exert potent antiviral effects during the HIV pathogenesis. In particular, their cytotoxic activity, responsible for control of viremia during the earlier stages of the infection, has been long described. It has been proposed that CD8+ T cells also use non-cytolytic mechanisms to control HIV infection. Recently, CD8+ T cells were shown to be required in order to maintain viral suppression also under ART, suggesting that these cells might contribute to persistence of HIV latently-infected CD4+ T cells and pose a barrier to HIV cure (125). Assessing the relative contributions of the CD8+ T cell-mediated suppression of virus production could be crucial for exploring new immunotherapeutic approaches, such as therapeutic vaccines and clinical interventions, to reduce viral reservoirs in HIV-infected individuals.

In the current study, we developed a dual color CD4/CD8 co-culture assay to examine the non-cytolytic activity of autologous TCR-activated CD8+ T cells from HIV-negative healthy donors in modulating *ex vivo* infection of CD4+ T cells. This strategy allowed us to distinguish both T cell subsets following co-culture and analyze the eGFP expression in infected CD4+ T cells, considering the HIV Nef-mediated downmodulation of CD4 in infected cells (142).

By using a replication-competent NL4-3_eGFP virus in the experimental setup, we demonstrated that autologous TCR-activated CD8+ T cells from healthy donors significantly suppressed HIV-eGFP expression in a dose dependent manner, inhibiting viral spread during the cellular culture. Furthermore, co-cultures of infected CD4+ T cells with activated B cells or Monocytes, under the culture conditions described above, revealed a specific, cell-type effect of CD8+ T cells in inhibiting HIV expression, as indicated from higher frequencies of productively infected CD4+ cells in the presence of B cells or Monocytes, in contrast to the effect observed with CD8+ T cells. Since CD8+ T cells may block HIV/SIV spread from cell-to-cell via CCR5-binding chemokines (i.e., MIP1a/CCL3, MIP-1b/CCL4, and RANTES/CCL5) (93), we then assessed if these cells exert their antiviral effects by blocking viral entry or post-entry, using viruses limited to a single cycle of infection: by blocking the production of mature viral particles from the replication-competent NL4-3_eGFP virus with the protease inhibitor Darunavir or by infecting CD4+ T cells with a replication-incompetent, Envelope (Env)-defective NL4-3_eGFP complemented in trans with a

dual tropic Env. In both cases, we didn't observe significant change in the levels of eGFP expression in the presence of CD8$_+$ T cells, probably due to the enhanced half-life of 26 hours of the eGFP protein.

Based on these findings, we decided to modify the replication-incompetent, Env-defective NL4-3_eGFP construct by adding a short-lived, destabilized version of the eGFP reporter gene, under the control of the viral LTR, to better understand the process that regulates the CD8$_+$ T cell-mediated transcriptional silencing. This change led to a significant decrease of the D2eGFP+ frequencies and mean fluorescence intensity (MFI), demonstrating that CD8$_+$ T cells are not just able to inhibit virus spread, but also to act directly on HIV LTR-driven gene expression.

Consistently with the previous results, we also confirmed that CD8$_+$ T cells significantly reduced the levels of HIV-RNA transcripts on a per-integrated-provirus basis similarly in both productively and non-productively infected CD4$_+$ T cells (56%, p=0.0078 for eGFP$_-$, and 54%, p=0.0156 for eGFP$_+$; Figure 11c). Since no differences were recorded between eGFP$_+$ and eGFP$_-$ subsets in terms of CD8$_+$ T cell-mediated transcriptional inhibition, we proposed that CD8$_+$ T cell-mediated HIV suppression could reflect a virus-independent mechanism.

The reduced levels of activation and proliferation, found in both productively and non-productively infected CD4$_+$ T cells as well as in the matched uninfected control (mock infection), allowed us to confirm that the virus itself is not responsible for driving the CD8$_+$ T cell-mediated inhibition activity. Of note, similar results were observed not only under multiple round of infection, but also with single cycle infection assays. Since HIV transcription is generally more effective in activated CD4$_+$ T cells compared to resting CD4$_+$ T cells (114), our findings suggest a novel immunoregulatory, non-cytolytic mechanism of HIV replication silencing, by which CD8$_+$ T cells lead CD4$_+$ T cells to a more resting state.

It is known that MHC-I restricted virus-specific cytotoxic CD8$_+$ T cells (CTLs) control HIV replication through the recognition and killing of productively infected CD4$_+$ T cells (138,139). However, their presence in healthy donors is extremely rare. Therefore, we investigated whether the mechanism of virus suppression mediated by TCR-activated CD8$_+$ T cells from HIV-negative healthy donors was most likely non-cytolytic. To this end, we measured with the amine-reactive dye method the frequency of live CD4$_+$ T cells and the frequency of virus induced cell death relative to uninfected cells in the control (mock infection) and we found that the presence of CD8$_+$

T cells promoted CD4+ T cell survival, strongly supporting our hypothesized CD8+ T cell-mediated protection against virus cytopathic effect.

We also demonstrated that this non-cytolytic activity was independent from the MHC-based T cell response: in fact, after treating CD4/CD8 co-cultures with anti-pan HLA-ABC and anti-HLA-E blocking mAb and, furthermore, mismatching CD4+ and CD8+ T cells from different donors in a heterologous co-culture system, HIV expression wasn't restored in co-cultures.

To further determine if the obtained viral suppressive effect of CD8+ T cells was associated with cell-to-cell contact, we physically separated CD4+ and CD8+ T cells using a transwell (0.4 μm) cell culture system. Surprisingly, the generated results indicated that soluble factors are partially responsible for the CD8+ T cell-mediated suppression of HIV production, while cell-to-cell contact seems to mediate the most potent suppressive effect.

Since the experiments described above highlight that CD8+ T cell-mediated transcriptional silencing is at least partially mediated by soluble factors, we decided to analyze the secretome profile of our CD4/CD8 co-cultures using a global approach of cytokine array. Additionally, previous reports demonstrated that CD8+ T cells could exert their antiviral effect through the production of chemokines, although it hasn't been yet identified a potential soluble factor responsible of the HIV inhibition activity mediated by these effector cells.

Interestingly, the secretome profile identified a preferential enrichment for Th2 cytokines and anti-inflammatory mediators.

These findings are also consistent with the analyzed transcriptome profile of eGFP- and eGFP+ CD4+ sorted subsets from mono- and co-cultures three days post infection in a single cycle condition with Darunavir. Overall, these data showed that co-culture with activated CD8+ T cells has a profound effect on both productively and non-productively HIV-infected CD4+ T cells, resulting in a general reduction of the transcriptional activity of these cells. In particular, we observed significant attenuation of several signaling pathways related to cell death, inflammation and proliferation, but a significant upregulation of transcriptional regulator genes associated with the Th2 pathway of CD4+ T helper differentiation (GATA3).

Altogether, our results suggest that activated CD8+ T cells modulate the level of CD4+ T cell proliferation and activation, possibly promoting a Th2 and an anti-inflammatory differentiation, through a virus- and MHC-independent mechanism.

In the setting of HIV infection, we demonstrated that the non-cytolytic antiviral activity of CD8+ T cells strongly inhibit HIV replication, by silencing the LTR-driven viral transcription in infected CD4+ T cells. Based on these evidences, we could hypothesize that all CD8+ T cells antiviral cytotoxic as well as non-cytolytic mechanisms might play a critical role in keeping the level of plasma viremia to a minimum, in absence of antiretroviral therapy, in the elite controller HIV-infected population.

Importantly, we observed that the HIV transcriptional silencing in infected CD4+ T cells, in presence of activated CD8+ T cells, is not associated with a decline of the copy numbers of integrated HIV-DNA or of the frequencies of inducible provirus.

Therefore, CD8+ T cells, by increasing the number and the survival of latently-infected cells, might be involved in the establishment and the maintenance of the reservoir under antiretroviral therapy and represent a major obstacle to HIV eradication.

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