Mechanism of CD8 T+ cell-mediated control of HIV-1 infection in HIV controllers

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So Youn Shin

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Abbreviations

Ad5: adenovirus type 5 vector

AGM: African green monkey

AID : animal infectious dose

AIDS: Acquired immunodeficiency syndrome

ANRS: Agence Nationale de Recherche sur le Sida et le hépatites

ART: antiretroviral therapy

AZT: zidovudine

CAF: CD8 antiviral factor

CEA: commissariat á l'énergie atomique

ConA: concanavalin A

CTL : Cytotoxic T lymphocyte

DC: dendritic cells

DNA: deoxyribonucleic acid

ELISPOT : Enzyme-linekd immunosorbent spot

ELISA: enzyme-linked immunosorbent assay

ESN: exposed seronegative

EU : exposed uninfected

FCS: fetal calf serum

GFP: Aequorea Victoria green-fluorescent protein

gp41: glycoprotein 41

HAART: Highly active antiretroviral therapy

Hem: hemophilia

Het: heterosexual

HIC: HIV controllers

HIV: human immunodeficiency virus

HLA: human leukocyte antigen

HVTN: HIV vaccine trial network

IDU: injection drug user

IFN: Interferon

IgA: immunoglobulin A

IL: Interleukin

ir: intrarectal

iv: intravenous

LIF: leukemia inhibiting factor

LTNP : Long Term Nonprogressor

LTR: long terminal repeat

MHC: major histocompatibility complex

MIP: macrophage inflammatory protein

MMS: man who has sex with man

m.o.i.: multiplicity of infection

NIH: national institutes of health

NK cell: natural killer cell

pDC: plasmatoid dendritic cells

PBMC: peripheral blood mononuclear cell

PCR: polymerase chain reaction

PD-1: programmed death -1

PHA: phytohemagglutinin A

p.i.: post infection

PVL: plasma viral load

RANTES: regulated upon activation normal T cells expressed and secreted

RNA: ribonucleic acid

SDF-1: stromal cell-derived factor -1

SFC: spot-forming cells

SIC: SIV controllers

SIV: simian immunodeficiency virus

SM; sooty mangabey

SR HIC: strong responder HIV controller

TCID: tissue culture infection dose

TNF: tumor necrosis factor

WR HIC: weak responder HIV controller

3TC : lamivudine

Abstract

Mechanism of CD8 T cell-mediated control of HIV-1 infection in HIV controllers

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(Directed by Professor June Myung Kim)

"HIV controllers (HICs)" are a rare group of HIV infected individuals who have a spontaneous and durable control of the virus at an undetectable level without antiretroviral treatment. Understanding the mechanism of HIV-1 control in these individuals could give us a valuable scientific background in developing novel vaccine strategies and immune therapies. This spontaneous viral control in HICs is usually associated to strong and functional HIV-specific CD8+ T cell responses. It has been recently published that *ex vivo* $CD8^+$ T cells from HICs were able to efficiently suppress HIV-1 infection in autologous $CD4^+$ T cells, suggesting a central role of $CD8^+$ T cell responses in the control of HIV-1 infection *in vivo*.

To further characterize the HIV suppressive capacity of $CD8^+$ T cells in HICs, the study has been extended investigating 19 HICs. Most of the HICs showed strong HIV suppressive capacity by *ex vivo* $CD8^+$ T cells (strong responder HICs) confirming the previous results published. Suppressive capacity in strong responder HICs was stable over time and broad. Importantly, the $CD8^+$ T cell mediated

suppressive capacity in HICs correlated strongly with the frequency of Gag-specific $CD8^+$ T cells. Furthermore, five HICs who had relatively poor HIV-suppressive $CD8^+$ T cell capacities were identified (weak responder HICs). Among them, at least three had highly replicative viruses suggesting that there might be another virus control mechanism playing a role in these individuals.

My results on HIV controllers support the value of CD8+ T cell suppression assay for measuring the effective anti-viral CD8+ T cell response since it reflects the concurrence of specificity, magnitude, and quality of response. For this, I have developed a rapid, and convenient form of the viral suppression assay based on the intracellular detection of p24 by flowcytometry. This assay was directly applied to a collaborative study which was to investigate the effect of antigen sensitivity of CD8+ T cells on the magnitude of HIV-suppressive capacity.

On the other axis, I investigated the possibility of an animal model for understanding the mechanism of durable virus control in HIV controllers by characterization of a subset of cynomolgus macaques spontaneously controlling SIVmac251 Infection. Unlike HICs, the CD8+ T cell mediated anti-viral capacity doesn't seem to be different from that observed in viremic or ART animals. In fact, the capacity of CD8+ T cell *ex vivo* to suppress SIV infection doesn't seem to be related to either the route of infection or the viral loads. These results suggest that, differently from what is believed to occur in HIC, the antiviral capacity of circulating CD8+ T cells in the SIV controllers is not determinant to their capacity to spontaneously control the virus *in vivo*. Identification of the mechanisms of virus control in SICs despite weak antiviral capacity of blood CD8 T cells might provide us with some clues for better understanding the control of HIV infection in HICs.

In conclusion, *ex vivo* capacity of CD8+ T cells to suppress HIV-1 infection is peculiar to HIV controllers (HIC) and the suppressive capacity correlated strongly with the frequency of Gag-specific CD8+ T cells; however, some HICs are able to tightly control the virus with relatively poor HIV-1 suppressive CD8+ T cell capacity suggesting other possible mechanisms. I have developed a rapid and

convenient from of viral suppression assay which measure the effective anti-viral CD8+ T cell response. Unlike in HIV controllers, antiviral capacity of circulating CD8+ T cells is not determinant in the spontaneous control of viremia observed in the early phase of chronic infection in SIV controllers.

Key words: HIV suppression, CD8+ T cells, HIV controllers, SIV

Mechanism of CD8+ T cell-mediated control of HIV-1 infection in HIV controllers

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I. Introduction

Twenty-five years since the discovery of human immunodeficiency virus (HIV) as the cause of acquired immunodeficiency syndrome (AIDS), the pandemic has not been controlled despite of the considerable amount of world wide effort. The global percentage of people living with HIV seems to be stabilized in 21st century but at an unacceptably high level; furthermore, the overall number of people living with HIV has increased ever more as a result of the ongoing number of newly infected individuals and increased life expectancy of infected individuals due to antiretroviral therapy. Since the introduction of highly active antiretroviral therapy (HAART), the mortality and morbidity caused by HIV infection has been significantly decreased; however, there have been other emerging problems such as drug resistance, drug-related complications, and the lack of coverage in resource limiting regions due to the high cost of drugs.

Back to the basics – A lesson from the failure of STEP trial

The ultimate control of the HIV epidemics could be accomplished by preventing newly developed infections. In this regards, there have been constant efforts to develop an effective HIV vaccine. However, such efforts to develop an effective anti-HIV vaccine, either preventive or therapeutic, have not yielded the ultimate fruitfulness. The recent failure of STEP trial – a phase 2b 'proof-of-concept' efficacy study testing an adenovirus type 5 vector (Ad5) expressing the HIV Gag, Pol, Nef proteins by the HIV Vaccine Trials Network (HVTN) and Merck & Co.- caused a major set back in the HIV vaccine researching field. The vaccine neither prevented infection nor had an impact on early plasma virus levels in the vaccine recipients compared with the placebo recipients; moreover, there were a greater number of HIV-1 infections occurred in vaccinees than in placebo recipients in subjects with pre-existing Ad5-specific neutralizing antibody titres [1,2,3]. Furthermore, the post hoc analysis of STEP data revealed that, there was an increased incidence of HIV in men who had pre-existing Ad5-specific neutralizing antibodies and who were uncircumcised. [1, 3] After these disappointing results of the STEP trial, there has been a shift towards fundamental research to better understand the host-viral interaction in HIV infection and to search for correlates of protection against HIV.

Models for understanding the correlates of protection against HIV infection

1. Protection against infection

A. Exposed-uninfected individuals (EU)

A very nice model to investigate the correlates of protection against HIV infection is exposed-uninfected individuals. They are naturally resistant to infection remaining uninfected despite of frequent exposure to HIV-1. There is not yet a clear explanation for the low susceptibility to infection; however, a number of probable protective factors have been reported so far including virus characteristics, levels of plasma viral load (PVL), host genes variants, and mucosal and systemic HIV-1 specific cellular and humoral responses. The possible protective factors for the low susceptibility of HIV infection in these individuals are listed below (Table 1).

Table1. Possible protective factors for the low susceptibility in infection in exposed

uninfected individuals[4]

Genetic

- Reduced or lack of CCR5 expression
- Genes closely related to HLA class I A2 alleles

Immune

- HIV-specific CD4+ T cells
- HIV-specific CD8+ CTL
- CD8+ cell noncytotoxic antiviral response
- Increased NK cell function
- Increased RANTES level in the genital tract
- Reduced susceptibility of PBMC to infection
- Reduced immune activation
- Serum neutralizing antibodies against cellular components (e.g. HLA and CD4)[b]
- IgA antibodies react to CCR5 in sera, saliva, and genital fluids [b]
- Anti-HIV neutralizing IgA antibodies in serum and vaginal fluids [b]
- Cervicovaginal anti-gp41 antibodies that block transcytosis [b]

Other

- Exposure to killed virus or viral antigens
- Low viral load of partner genital fluid and blood

[a]CTL, Cytotoxic T lymphocytes; IgA immunoglobulin A.

[b]These observations were made on a small number of exposed seronegative individuals (ESN) and need to be further substantiated

B. Placental barrier – Mother to child transmission

Another example of natural protection is the placental barrier. Even in the absence of antiretroviral treatment, 90% of fetus seems to be protected from in utero transmission of HIV-1.[5-7] Several studies have reported that cytokines and chemokines in placental environment are involved in the natural protection of the fetus against mother to child transmission. [8-10] The principal placental cytokines known to have different effects on HIV-1 replication are as follows: leukaemia inhibiting factor (LIF),

interferon (IFN)-ɣ , interleukin (IL)-16, regulated upon activation normal T cells expressed and secreted (RANTES) and stromal cell-derived factor-1 (SDF-1) inhibit replication; tumour necrosis factor (TNF)-α and IL-8 increase replication; and IL-10 can both inhibit and increase replication depending on the HIV-1 cell targets.[11]

2. Protection against disease

A. Natural host of SIV infection - African non human primates

There are two simian models of SIV infection: the non-pathogenic SIV infection in African natural host species, and the pathogenic SIV-infection of non-natural host rhesus macaques. SIV infection is typically non-pathogenic in African species of monkeys, in particular sooty mangabey (SM) and African green monkeys (AGMs). Despite high levels of virus replication and limited antiviral CD8+ T-cell responses, SIV infection is typically non-pathogenic in African species of monkeys, suggesting that direct consequences of virus replication alone cannot account for the progressive CD4+ T-cell depletion in AIDS. Understanding the host-viral interaction and the mechanisms of protection against disease progression in these natural hosts of SIV infection could give us a key insight to the correlates of protection in HIV infection. While the final mechanism underlying the non-pathogenic SIV infection in African natural host species is still largely unknown, there are three main and consistent immunological characteristics in these individuals such as: i) preservation of peripheral CD4+ T cell homeostasis; ii) lower level of immune activation, iii) lower expression of CCR5 on CD4+ T cells.[12]

B. Elite controllers

Where there is a nonpathogenic model of SIV infection in African nonhuman primates, there is a very nice model to investigate the correlates of natural protection in HIV-infected humans from developing immunodeficiency called “HIV controllers” or “elite controllers”. HIV controllers are a rare group of HIV infected individuals who have a spontaneous and durable control of the virus at an undetectable level without antiretroviral treatment. Understanding the virus control mechanism in these

individuals could give us a valuable scientific background in developing novel vaccine strategies and immune therapies. My study is focused on understanding the virus control mechanisms in this perculiar population. They are explained more thoroughly in the following section.

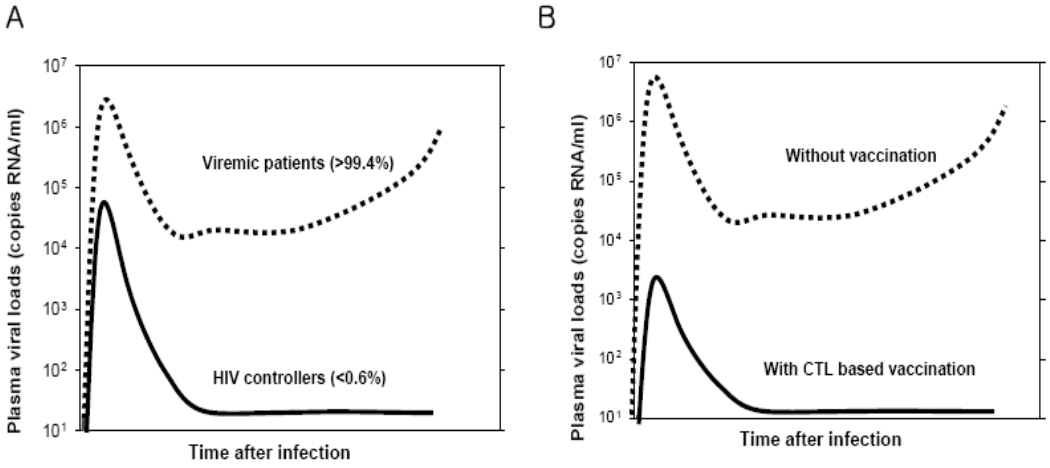
HIV controllers: A rare group of individuals who spontaneously control the virus

The majority of HIV-infected individuals without antiretroviral therapy show evidences of ongoing viral replication and eventually progressive immune deterioration. However, there are a very rare (~0.3%) group of population who shows a spontaneous and durable confinement of virus[13, 14]. They are called "HIV Controllers" (HIC) and defined as HIV infected individuals having undetectable viremia by standard assays (<50 copies RNA/ml) for prolonged period (>10 years) in the absence of therapy [13, 14]. (Figure 1) Differently from long-term non-progressors (LTNP), who are only defined on an immunological basis and could have highly variable viral loads, HIV controllers are characterized by consistently undetectable plasma HIV-1 RNA and very low and stable amounts of viral DNA in their peripheral blood mononuclear cells (PBMCs), often below one RNA copy per millimeter.[15] (Table 2) Recently, there has been a shift of concept in approaching the HIV vaccine development: From « sterilizing », « preventing » to « controlling » the virus using T cell mediated immune response. [16] The main idea is that by controlling viremia under a certain level, immune deterioration and secondary transmission could be reduced to further prevent AIDS progression and HIV epidemic expansion. (Figure 1) For this, a through understanding of T cell-mediated efficacy in control of HIV is a necessity. However, as we could deduce from the disappointing results of STEP trial, our understanding of T cell–mediated efficacy in control of HIV remains largely unknown. This new approach is to somewhat mimic the clinical course of HIV controllers. Identification of the efficient mechanism of HIV-1 control in these individuals, especially one that is related to T cell mediated suppression of the virus, may provide valuable information for developing novel vaccine strategies and immune therapies.

Table 2. Long Term Nonprogressors (LTNP) vs. HIV controllers[17]

	Long Term Nonprogressors (LTNP)	**HIV controllers (HIC)**
Definition	Immunologic: CD4+ T-cell count	Virologic: plasma
Frequency among HIV-1 infected patients	5%	<1%
Level of viral RNA in plasma	Variable	Undetectable
Level of viral DNA in PBMC	Variable but lower than in progressors	Extremely low
Disease progression in cohorts	Yes	Extremely rare

Figure1. A successful CTL based vaccine mimics the clinical course of a HIV controller.



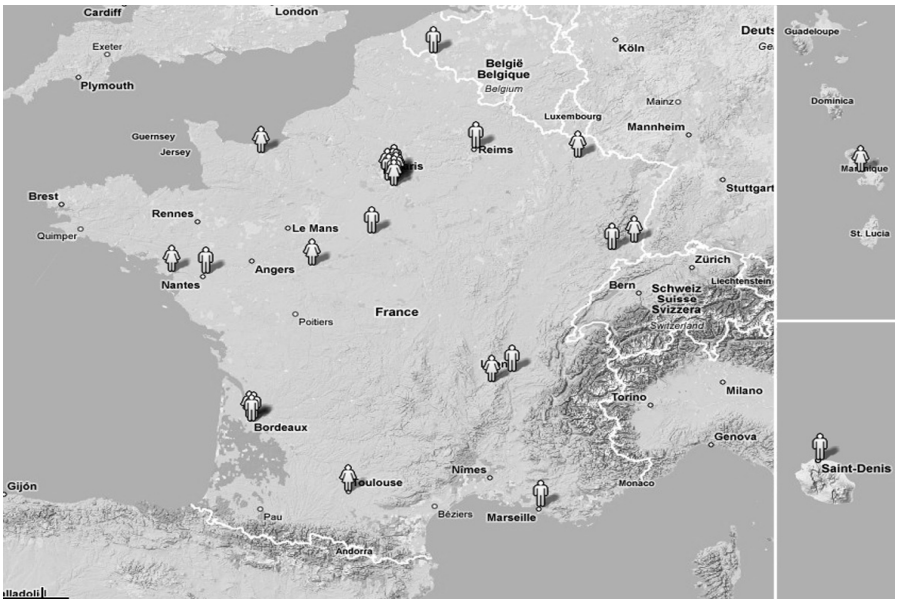
Clinical course of **A.** most untreated HIV infected individuals and HIV controllers **B.** The goal of successful CTL-based vaccine. **A.** Most antiretroviral untreated HIV infected individuals have continuous viral replication and eventually develop profound immunodeficiency and progress to AIDS; however, there is a rare subpopulation (<0.6%) of HIV-infected patients who has a spontaneous and durable confinement of virus. **B.** The goal of a successful CTL-based vaccine is to reduce HIV-1 replication to a level that reduces or eliminates secondary transmission and further destruction of immune system. This new approach is to somewhat mimic the clinical course of HIV controllers showing in **A.**

Observatoire National des "HIV Controllers" – EP 36 study group

The obvious interest to unveil the mechanism in which HIV controllers are able to achieve such a striking control of infection is driving important international efforts to identify, assemble and study larger population of this exceedingly rare group of individuals. In France, a consortium of research groups constituted by immunologists, virologists, geneticists, epidemiologists and clinicians (ANRS-EP36), coordinated by Dr. Olivier Lambotte and Pr. Jean François Delfraissy (CHU Kremlin-Bicêtre), was established in 2005 for this purpose. Initially 15 HIV controllers were identified among more than 2200 infected patients (CHU Kremlin-Bicetre and SEROCO cohort) [13]. Since 2006, a nationwide monitoring program has been established to further

investigate the subject. Up until now, more than 80 HIC have been recruited so far. (Figure2)

Figure2. Observatoire National ANRS des "HIV Controllers in France



Since 2006, a nationwide monitoring program for HIV controllers has been established in France, and after 2 years of recruitment, ~80 HIV controllers have been recruited (median age when diagnosis: 29 years old, 42% female, 87% Caucasians, median years of follow up 19 years, median CD4 count: 751 CD4/mm3 [IQR: 581-950], Subtype B predominance)[18]

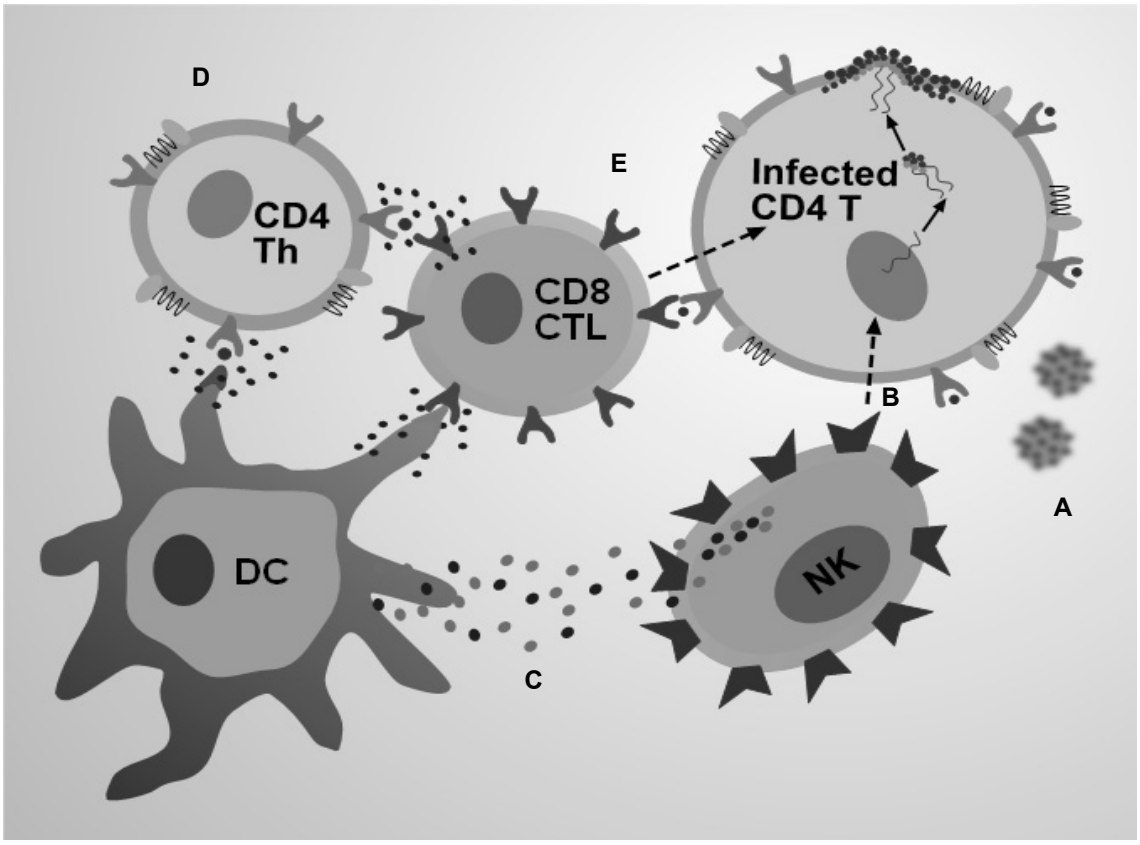
The ANRS-EP36 study group has investigated several hypotheses concerning the efficient mechanism of virus confinement in HIV controllers such as the presence of defective viruses [19], a reduced susceptibility of the cells to HIV-1 [13], the role of immune response, including neutralizing antibodies and CD4+ and CD8+ T cell responses [13, 20] and the role of innate immunity. Results from the ANRS-EP36 and other groups have revealed that at least some HICs are infected with fully replication competent HIV-1 [19, 21], indicating that infection with HIV-1 variants with attenuated replicative capacity cannot be a general factor accounting for undetectable viremia in HIV controllers. Furthermore, $CD4^+$ T cells from HIV controllers are found to be highly susceptible to HIV-1 *in vitro* infection, discarding, therefore, that an intrinsic

resistance of CD4+ T cells to HIV-1 could contribute to the HIV controller status [13, 21]. Neutralizing antibodies do not appear to be involved in protection [22];however, an effective multifunctional HIV-specific $CD8^+$ T cell response is thought to be central to viral control in these individuals[13, 23, 24] . The role of innate immunity is currently under investigation. There are also other important contributions by various research groups trying to unveil the underlying mechanisms of viral confinement in these individuals. The mechanism of viral containment in HIV controllers is yet largely unknown; however, what have been suggested as possible mechanisms for virus control in HIV controllers are listed below. (Table 3), (Figure 3)

Table3. Potential virus control mechanisms in HIV controllers[17, 25]

Probably determining	Evidences
Adaptive immune response	
HIV-specific CD8+ T-cell cytotoxic response	Enriched in certain class I HLA alleles (B57, B27), CD8+ T cells produce multiple cytokines and/or proliferate in response to HIV peptides
HIV-specific CD4+ T-cells	CD4+ T cells express high amount of HIV-specific IL-2 and interferon-γ in response to HIV peptides
Adaptive immune response	
Natural Killer cells	Enriched for certain NK cell receptors that are involved in regulating the NK cell function
Plasmacytoid dendritic cells (pDCs)	Number and/or function are high in some HICs
Immunoregulations	
T regulatory cells (Tregs)	Tregs low in some HICs, particularly in lyphoid tissues
Defective viruses	Viruses containing mutations and/or deletions in key regulatory or accessory genes are found in some HICs
	Mutations induced by CD8+ T cell pressure leading to reduced viral fitness
Probably not determining	
Innate restriction factors	
Lesser susceptibility to infection	
Neutralizing antibodies	
Antiviral soluble factors (β-chemokines, CAF)	

Figure3. Potential mechanism possibly contributing to the spontaneous control of viremia in HIV controllers[17]



A. Although HICs harbour fully infectious HIV-1 strains, infection with attenuated viruses might explain HIC status in some particular cases. Although not yet clear in HICs, indirect evidences suggest that NK cells might also contribute to the control of viremia in these individuals. These cells might do so by **B.** directly eliminating infected cells, or **C.** by providing the optimal cytokine environment ot develop an effective adaptive responses. **D.** CD4+ T helper function is preserved in HICs and might be important for the maintenance of the HIV-specific CD8+ T cell responses. **E.** HIV-specific CD8+ T cells from HICs are highly functional and cytotoxic and are probably a major driving force in the control of HiV-1, at least, in the long term. On the contrary, other mechanisms such as reduced CD4+ T cell susceptibility to infection or neutralizing antibodies do not seem to widely contribute to HIV control in HICs. Still, the susceptibility to HIV infection of other target cells (i.e. macrophages or dendritic cells) and the role of other antibodies' activities (e.g. antibody-dependent cell cytotoxicity) need to be assessed.

Study of the mechanism of natural protection in HIV controllers: Why is CD8+ T cell mediated antiviral capacity important?

There are many evidences suggesting that CD8+ T cells play a pivotal role in control of HIV replication. For example, it is observed that a marked decline of HIV plasma viremia followed after a peak viremia of ~10^7 HIV RNA copies/ml in acute HIV infection is temporally associated with the appearance of HIV-specific CD8+ cytotoxic T lymphocyte (CTL) responses[26, 27], suggesting that CTLs may be responsible for controlling viremia in the early stage of infection. Furthermore, there is more direct evidence suggested by SIV infected macaques models. With the infusions of CD8-specific monoclonal antibodies in animals with chronic SIV infection, it resulted in increased viral load in the macaques that controlled viruses. [28]. In fact, there are special characteristics of CD8+ T cells in HIV controllers suggesting that these cells might play an important role in the control of viremia. The $CD8^+$ T cell specific response is strong in most HIV controllers and high frequencies of HIV-specific CD8+ T cells despite very low levels of viral antigen are found in these individuals[13]. HIV-specific CD8+ T cells from HIV controllers are highly capable to proliferate and produce perforin.[24] In addition, HIV-specific CD8+ T cells from HIV controllers can, after stimulation with their cognate antigen, generate a multifunctional response that includes degranulation and chemokine and cytokine secretion (IFN-γ, MIP-1ß, TNF-α, interleukin-2, and/or CD 107a)[23]. Some HLA-Bw4 haplotypes (i.e. B27, B57) are known to be associated with virus control in HIV controllers. These alleles are strongly enriched among the different cohorts of HIV controllers (~60% of HIV controllers in our group bear the HLA-B57 allele and ~25% the HLA-B27) compared to noncontrollers (7-9 for both alleles) suggesting an important role of class I-restricted CD8+ T cells [13, 29, 30] However, although these observations strongly support the hypothesis that the HIV-specific CD8+ T cell response is a dominant factor driving the containment of HIV in HIV controllers, a direct link between the immune response and the control of viremia was lacking.

The research group that I worked with has recently made an important contribution in this regard. It was shown that purified *ex vivo* CD8+ T cells (in the absence of further stimulation) from most HIV controllers, as apposed to CD8+ T cells from viremic subjects, were able to efficiently control HIV-1 superinfection of autologous CD4+ T

cells[13] (Figure 4a). This result suggested that this antiviral activity is likely to be functional *in vivo* and, therefore, that CD8+ T cells circulating in the blood of HIV controllers are suitably prepared to control HIV-1 infection. Furthermore, this anti-HIV capacity of CD8+ T cells was not mediated by soluble inhibitory factors but is due to the elimination of infected CD4+ cells suggesting a cytotoxic mechanism of HIV suppression(Figure 4b, c). These cells expressed the activation marker HLA-DR but not CD38 [13] (Figure 5). This unique phenotype differentiates HIV-specific CD8+ T cells from HIV controllers from those of non-controllers and is likely related to their potential to expand upon antigenic stimulation [13]. The constitutive antiviral capacity of CD8+ T cells could account for the control of viral replication in HIV controllers; however, the mechanism in which how it is working remains to be defined.[13] The clarification of the mechanism underlying control of HIV infection in HIV controllers and the characterization of the antiviral capacity of CD8+ T cells from these individuals may provide important information for the development of new immunotherapies and vaccine strategies.

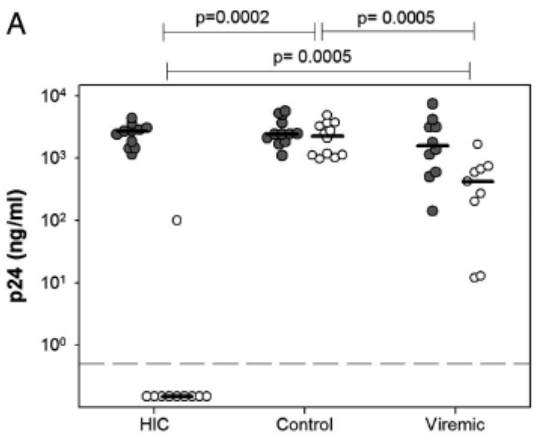
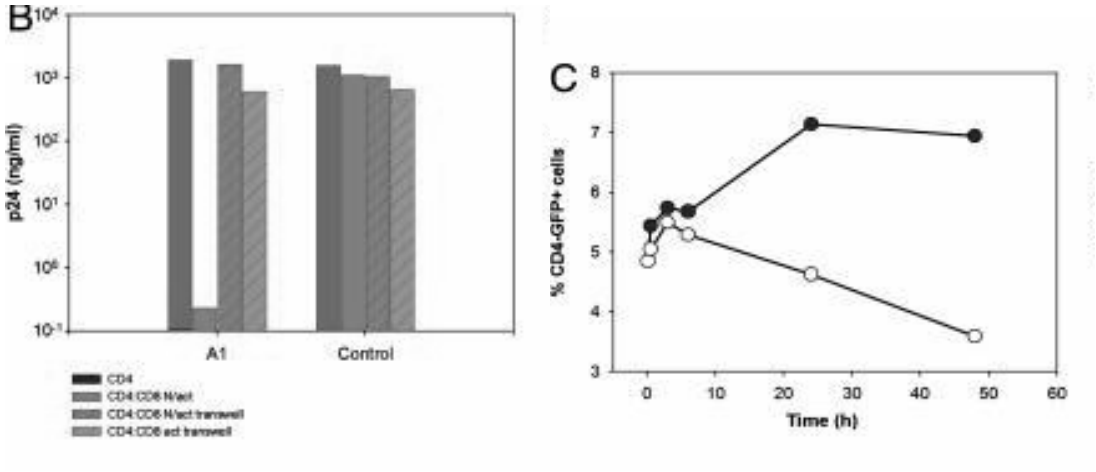


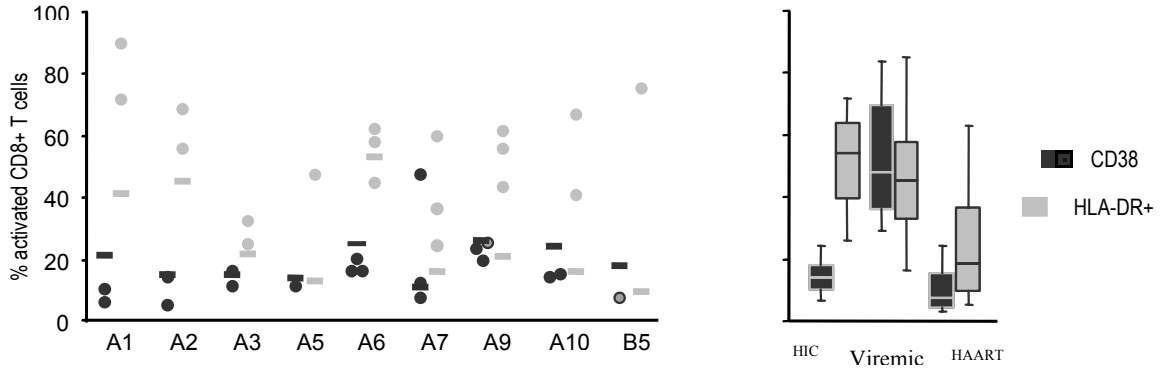
Figure4. HIC shows a potent CD8+ T cell mediated anti-HIV capacity [13]



(a) HIV-1 in vitro infection assays were done with cells from HIC, uninfected donors (control), or HIV viremic individuals. PHA-activated CD4+ T cells were infected with the replicative HIV-1 BaL in the absence (gray) or presence (white) of autologous unstimulated CD8+ T cells (1:1 ratio). Circles represent the average (n=3 independent infections) peak p24 values for each studied individual. Horizontal lines indicate median values for each group. Statistical differences in CD8+ T cell-mediated inhibition between groups are indicated above the graph. (b) CD4+ T cells from HIC and from uninfected donors were infected with replicative HIV-1 BaL. CD4+ T cells cultured alone are shown as reference (gray bars). Autogous unstimulated CD8+ T cells were added directly to the CD4+ T cell culture (blue solid bars) or to Transwell inserts that were placed in the CD4+ T cell-containing well (blue hatched bars). Autologous PHA-stimulated CD8+ T cells were added to Transwell (orange hatched bars). The ratio of CD8+ to CD4+ T cells was 1:1. One representative experiment with HIC (subject A1) and one uninfected control is shown. Results are peak p24 levels. (c) CD4+ T cells from HIC were infected with a HIV-1 vesicular stomatitis virus glycoprotein pseudotype bearing the GFP reporter gene (filled circles). Forty-eight hours later, autologous unstimulated CD8+ T cells were added to half of the CD4+ T cells at a 1;1 ratio (open circles). At the indicated time points, an aliquot of each cell suspension was labeled with anti-CD4 antibodies and the quantity of double-positive CD4+GFP+ was assessed by flow cytometry. One representative experiment with HIC (subject A7) is shown.

Figure5. Differentiation and activation status of HIV-specific CD8+ T cells from HIC in comparison with viremic and HAART subjects.

A B



A. Frequencies of CD38 (black) and HLA-DR (gray) expression on total CD8+ T cells (horizontal bars) and HIV-specific CD8+ T cells (circles) in nine HIC. **B.** Comparison of CD38 (black) and HLA-DR (gray) expression (median, 25^{th}-75^{th} and 10^{th}-90^{th} percentiles) on HIV-specific CD8+ T cells in HIC, viremic, and HAART groups. Statistical differences between groups are indicated above the bars.

With these scientific evidences, I have decided to characterize the peculiarity of HIV controllers and find out the mechanisms underlying HIV-specific CD8+ T cell mediated HIV suppressive capacity in these individuals. As for the issue of perculiarity of HIV controllers, there are two questions remained to be explored:

(i) whether the spontaneous anti-HIV activity of the $CD8^+$ T cells from HIV controllers was the consequence (rather than the cause) of controlled viremia; ii) whether HIV controllers are actually a peculiar group of population or they are just at one extreme end of the evolution of infection in the distribution of infected patients. iii) and whether the HIV-specific CD8+ T cell mediated HIV suppressive capacity is consistently found in all HIV controllers in a larger study

In order to address these questions, our group is analyzing the anti-viral capacity of $CD8^+$ T cells *ex vivo* from:

(i) a group of HIV-1 infected patients treated with antiretroviral therapy and with undetectable viral loads (ART group);

(ii) through a collaboration with the ANRS ALT cohort, a group of individuals with apparent non-progression to disease despite being infected for long periods (>15 years) of time but with detectable, albeit usually weak, viral loads.

(iii) expanded number of patients in help with the ANRS-EP36 national HIV controller monitoring program.

Furthermore, through a collaboration with the ANRS PRIMO cohort, a national wide cohort of patients with primary HIV infection started in 1996 (744 individuals so far), individuals with the feature of HIV conrollers are currently under investigation.

In this study, I have focused on characterizing the mechanisms of viral confinement in HIV controllers in a larger number of HIV controllers with the help of ANRS-EP 36 HIV controller monitoring program. Furthermore, to overcome the obstacles of HIV controller study such as their rarity, lack of information on the early disease course, and lower feasibility to obtain tissue samples, I have tried to investigate the possibility of setting up an animal model for studying HIV controllers using SIVmac239 infected cynomolgous macaques.

II. Hypothesis and Objectives

The main objective of this study is to characterize the mechanisms of spontaneous control of HIV-1 replication by the CD8+ T cells from HIV controllers. In addition, a group of infected macaques have been analyzed in order to determine whether the mechanisms that account for viral control in SIV controllers are the equivalent to those in their human counterparts hoping that they may represent an accurate animal model for the HIV controllers.

III. Materials and Methods (common)

1. Primary cell culture

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation. From freshly isolated PBMCs CD4+ and CD8+ T lymphocytes were isolated via positive selection and negative selection with antibodies coated magnetic beads (Miltenyi Biotec, Paris, France) respectively. CD4+ T lymphocytes were activated with phytohaemagglutinin A (PHA) at 2µg/ml in the presence of IL-2 (Chiron, France) at 100units/ml whereas CD8+ T lymphocytes were left in culture without any cytokines or chemokines for 72 hours. The culture medium was RPMI 1640 containing 10% FCS and penicillin/streptomycin (100 U/ml).

2. Productive infection in vitro

CD4+ T cells (10^5) were super-infected with HIV-BAL (R5) at m.o.i. $10^{-3.6}$ in triplicate in 96-well plates with a spinoculation protocol [31]. For some experiments SIVagm.Gril, SIVmac251, and HIV-2.SBL or autologous primary viruses were used for infection. For coculture, 10^5 CD4+ T cells were mixed with 10^5 CD8+ T cells (CD8:CD4 ratio 1:1) at the moment of infection. After infection the cells were washed and cultured for 14 days. HIV-1 replication was monitored every 3-4 days in supernatants by p24 ELISA (Zeptometrix, Gentaur, France). For the experiment of macaques, SIVmac replication was monitored by p27 ELISA (Zeptometrix, Gentaur, France). Infectivity assays were carried out in the presence of 100 IU/ml IL-2. We have previously shown that the presence of this cytokine during the infectivity assays did not affect the suppressive capacity of unstimulated CD8+ T cells[13].

3. Intracellular p24 assay

Activated CD4+ lymphocytes ($5*10^4$) were super-infected *in vitro* with HIV-1 BAL (R5) as described above. Various dilutions of virus (m.o.i $10^{-1.6}$ to $10^{-2.6}$) were used in parallel to yield a similar infection level in each individual experiment. CD4+ T cells were cultured in the absence or presence of non-stimulated CD8+ T cells (CD8:CD4 ration 1:1)

72 hours after the infection, cells were harvested and stained with CD4-ECD (SFCI12T4D11) and CD8-PC5 (B9.11). Cells were then permeabilized (Cytofix/Cytoperm Fixation and permeabilization kit, BD bioscience) and stained with KC57-FITC (FH190-1-1) to determine the intracellular production of HIV antigen. Antibodies were from Beckman Coulter. Flow cytometric analysis was performed using Cytomix FC500 with CXP Acquisition software (Beckman Coulter).

4. Viral isolation from peripheral blood CD4+ T cells

Between 2 to $5*10^6$ CD4+ T cells from each patient were activated with PHA and IL-2 as described above. Viral production in culture supernatants was monitored

for 28 days by p24 ELISA. When required, CD4+ T cells were re-activated at day 10 with CD8-depleted PHA-preactivated allogeneic PBMCs, PHA and IL-2. Virus-containing supernatants from CD4 T cell cultures were titrated on mixed PHA-activated CD4+ T cells from two blood donors.

5. **ELISPOT assay**

Interferon (IFN)-γ secretion by HIV-specific CD8+ T cells was quantified with an ELISPOT assay using appropriate stimuli [32]. We used a set of 124 peptides corresponding to known optimal CTL epitope from the HIV-1 Env, Gag, Pol and Nef proteins or controls (NIH HIV Molecular Immunology Database: http://www.hiv.lanl.gov/content/immunology/index.html). The peptides were synthesized by Neosystem (Strasbourg, France). They were used at a final concentration of 2µg/ml. IFN-γ spot-forming cells (SFCs) were counted with a KS-ELISPOT system (Carl Zeiss Vision) and expressed as SFCs/10^6 PBMC after subtracting the background. Wells were considered positive if they contained at least 50 SFC/10^6 PBMC and exhibited at least two times the background level.

6. **HIV DNA quantification**

Total DNA was extracted from whole blood using a QIAamp DNA minikit (QIAGEN), according to the manufacturer's instruction. HIV-1 DNA was then quantified by real-time PCR (LTR amplification-ANRS)[33]. Four PCRs testing one microgram of total DNA for each of them were performed per extract for this ultra-sensitive assay (threshold; 10 copies/million leukocytes)[34]

IV. Projects and Results

1. Study of the mechanism of natural protection in HIV controllers

Project 1: Study of the mechanism of natural protection in HIV controllers

A. Brief introduction

CD8+ T cells can at least partially control HIV [27, 28, 35]. Some HLA class I molecules, and particularly alleles B27 and B57, have been linked to better control of HIV infection [29, 30, 36]. The presence of Gag-specific CD8+ T cells, and the breadth of their specificities, have also been linked to low HIV viremia [37-39]. One of the most compelling indications of the pressure exerted by CD8+ T cell responses is the emergence of variants that escape recognition by these cells [40-44]. However, most HIV-infected individuals have uncontrolled viremia and eventually progress to AIDS despite strong CD8+ T cell responses.

Rare individuals called “HIV controllers” (HICs) spontaneously and durably control HIV infection in the absence of therapy, possibly illustrating what truly effective CD8+ T cell responses can achieve [17, 25]. HICs have extremely low and stable amounts of viral DNA in their peripheral blood mononuclear cells (PBMC)

[14] and undetectable plasma viral load [15]. The protective HLA alleles B27 and B57 are overrepresented among these individuals [13, 22, 29, 30, 45]Despite very low levels of antigen in blood[13], most but not all HICs have high frequencies of HIV-specific CD8+ T cells that preferentially target the viral Gag protein [45-47]. Studies of CD8+ T cell responses in HICs have revealed important characteristics of functional HIV-specific CD8+ T cells in HIV infection. Contrary to cells from viremic individuals, HIV-specific CD8+ T cells from HICs can, upon stimulation with their cognate antigen, proliferate and generate a multifunctional response that includes degranulation and chemokine/cytokine secretion [23, 24, 48]. This could be related to a peculiar activation phenotype of these cells [13] and to constitutive telomerase activity that protects them against senescence [49]. However, how much of this is the cause and how much the consequence of viral control and low-level immune activation remains to be determined. It has been recently shown that CD8+ T cells from most HICs are endowed with a striking capacity to suppress HIV infection ex vivo[13], a property that is likely to be relevant in vivo. To further characterize this HIV suppressive activity I have extended the study to a larger group of 19 HICs and evaluated the relationship between this activity and HIV-specific CD8+ T cell responses.

B. Objectives

To further investigate the mechanism of virus control in a larger group of HIV controllers, and in particular the HIV suppressive capacity of CD8+ T cells

C. Study subjects and Methods

1) Study subjects

Nineteen patients diagnosed with HIV-1 infection at least 10 years previously who had never received antiretroviral treatment and in whom more than 90% of plasma HIV RNA assays gave values below 400 copies/ml were studied (Table 4): eight have been described elsewhere[13], and 11 were newly recruited from the ANRS EP36 national monitoring program on HIV controllers.

The subjects were serologically HLA-typed by complement-mediated lymphocytotoxicity testing (InGen One Lambda, InGen - Technopolis - 91380 Chilly Mazarin). All had very weak and stable DNA load (Table 4). All the subjects gave their written informed consent.

ontrollers included in the study.

	Subtype of infecting virus	HLA	Median CD4 count [first-last] (cells/ml)	Viral follow up (years)	Median RNA VL (copies/ml)	VL Blips (50-400 copies/ml)[3]	HIV DNA (log copies/10^6 cells)
	B	A2/*A74*/**B27**/**B57**	1041 [775-1528]	11	<50	**7**/16	NA
	B	A2/A29/**B27**/**B57**	844 [1107-522]	15	<50	0/13	NA
	NA	A1/A2/B44/**B57**	632 [1151-632]	10	<50	**4**/13	1.67
1	B	A1/A2/B51/**B57**	839 [1217-785]	10	<50	**9**/18	1.62
	B	A2/B44/**B57**	613 [601-408]	11	<50	**7**/18	1.77
	B	A3/A23/B7/**B57**	709 [599-493]	6	<50	**1**/12	0.5
	B	A2/**B27**/**B57**	958 [1290-849]	12	<50	**1**/15	0.5
	B	A2/A32/**B27**/B60	597 [602-649]	10	<50	0/15	1.67
	B	A2/A68/B14/**B57**	890 [1000-937]	12	<50	**0(2)**/16	1.31
	B	A3/A30/**B27**/B51	640 [883-330]	11	<50	**2**/20	1.04
	B	A3/A30/**B57**/B63	1035 [960-1033]	11	<50	0/11	1.04
	NA	A2/A31/B39/**B57**	896 [665-877]	11	<50	**3(1)**/16	1.04
	B	A2/B7/**B57**	1194 [601-1196]	11	<50	0/8	1.94
	B	A3/A30/B7/**B57**	504 [390-556]	11	<50	**2**/19	1.28
	B	A24/A32/**B27**/B62	976 [1107-1142]	9	<50	0/17	2.08
	B	A3/A29/B35/B44	727 [653-828]	11	<100	0/21	1.64
1	NA	A43/A74/**B57**/B72	771 [902-773]	9	<50	0/11	0.5
	NA	A11/A30/B13/**B27**	800 [800-717]	10	<50	0/9	NA
	A2	A2/A68/B60/B14	928 [790-808]	12	<50	0/10	1.57

of their CD8+ T cells (log p24 decrease, Figure 1C). Double line separates strong and weak responders
ug use; MMS, male-male sex
inations with detection limit <50 copies/ml. VL >400 RNA copies/ml are indicated between brackets

2) HIV DNA quantification (p. 22)

3) Primary cell culture (p. 20)

4) Productive infection in vitro (p. 20)

5) Intracellular p24 assay (p. 20)

6) Viral isolation from peripheral blood CD4+ T cells (p. 21)

7) ELISPOT assay (p. 21)

8) Depletion of HIV-specific CD8+ T cells

Depletion of CD8+ T cells producing IFN-γ upon stimulation with HIV peptides was performed with an IFN- γ secretion assay enrichment kit (Miltenyi Biotec) as recommended by the manufacturer. Briefly, purified CD8+ T cells were stimulated for 6h with appropriate pools of specific HIV-peptides. Subsequently, the cells were labeled (5min at 4℃) with an IFN- γ catch reagent that attached to the cell surface of all leukocytes. The cells were then incubated for 45min at 37℃ to allow IFN- γ secretion. The secreted IFN- γ was captured by the IFN- γ catch reagent on the positive, secreting cells. These cells were subsequently labeled with a second IFN- γ

specific Ab conjugated to R-PE. The IFN- γ secreting cells were magnetically labeled with anti-PE magnetic beads and depleted by magnetic field separation. Purity of the depleted fraction was evaluated by flow cytometry.

9) Antibodies

The following Abs were used: CD8-ECD or –PC5(clone B9.11), CD3-PC5(UCHT1), CD45RO-ECD(UCHL1), HLA-DR-ECD (Immu-357), and CD38-FITC (T16), all from Beckman Coulter; and CD27-FITC(M-T271) from BD Bioscience

10) Pentamer staining and phenotyping

HIV-specific CD8+ T cells were identified by using soluble PE- or allophycocyanin-labeled peptide-HLA class I multimers (Proimmune; Beckman Coulter Immunomics). The following epitopes were used: the HLA-A*0201-restricted peptide ligands SLYNTVATL (Gag 77-85) and ILKEPVHGV (Pol 476-484), the A*0301-resticted peptide ligands RLRPGGKKK (Gag20-28) and QVPLRPMTYK (Nef 73-82), the B*2705-restricted peptide ligand KRWIILGLNK (Gag 263-272), and the B*5701-restricted peptide ligands KAFSPEVIPMF (Gag 162-172), TSTLQEQIGW (Gag240-249), and QASQDVKNW (Gag 308-316). PBMC were incubated with pentamers (1μ g/ml) for 30min and then with relavant Abs for 15min. Cells were washed in Cell Wash (BD Biosciences) plus 1%BSA, incubated for 10min with FACS lysing solution (BD Biosciences). After washing, cells were fixed in 1% paraformaldehyde for flow cytometry with an Epics XL (Beckman Coulter) or a FACSCanto flow cytometer (BD Biosciences) and analyzed with RXP software (Beckman Coulter).

11) Proliferation assay

The proliferative capacity of HIV-specific CD8+ T cells was evaluated by

flow cytometry. PBMC were stained with 0.35μM CFSE (Molecular Probes) for 10min at 37℃, and after washing they were stimulated for 5 days with 2μg/ml peptide or medium alone. After labeling with pentamer, anti-CD8, and anti-CD3 Abs, PBMC were fixed in 1% paraformaldehyde for flow cytometry as described above.

12) Statistical anayses

All values throughout the text are means ± standard deviation. P values were calculated with the rank sum test. Correlations were identified by simple linear regression analysis and Spearman's rank correlation test. SigmaStat 3.5 software was used (Systat Software Inc-SSI, CA).

D. Results

1) Unstimulated CD8+ T cells from most HICs have strong HIV suppressive capacity

In a previous study it has been reported that undetectable viremia in 9 out of 10 HICs was associated with a remarkably strong capacity of their circulating CD8+ T cells to control in vitro HIV-1 infection of autologous CD4+ T cells[13]. In order to extend this observation, I have used the same viral suppression assay to assess the ex vivo anti-HIV capacity of CD8+ T cells from 19 HICs, 11 of whom were newly recruited for this study and 8 were retested (Table 4). Viral replication was readily detected in the supernatants of purified CD4+ T cells from all 19 HICs after PHA activation and challenge with HIV-1 BaL (Fig. 6A). A marked reduction in HIV-1 infection (undetectable in 8 HICs) was generally observed when autologous unstimulated CD8+ T cells from HICs were added to the culture (Fig. 6A). The associated CD8+ T cell-mediated decrease in the level of HIV proteins was due to the absence of infected CD4+ T cells in the co-culture (Fig. 6B). As a whole, the HIV-suppressive capacity of CD8+ T cells from HICs (2.79 ± 1.31 log p24 decrease, CD8:CD4 vs CD4) was much stronger than that of cells both from viremic individuals (0.82 ± 0.53 log p24 decrease, CD8:CD4 vs CD4), confirming the previous results[13], and from HAART-treated individuals with undetectable viral load (0.62 ± 0.63 log

p24 decrease, CD8:CD4 vs CD4) (Fig. 6C). In particular, CD8+ T cells from 14 of the 19 HICs suppressed HIV far more strongly (log p24 decrease > 2) than cells from both viremic and treated individuals (Fig. 6C). These subjects are referred to below as strong responder HICs. Longitudinal analysis (more than 12 months) of CD8+ T cell antiviral activity in five strong responder HICs included in the previous study suggested that this HIV suppressive capacity is a stable characteristic (table 5). In contrast, here we identified five "weak responder" HICs whose CD8+ T cells could not efficiently control HIV infection of autologous CD4+ T cells (log p24 decrease < 2) (Fig. 6B,C): the HIV-suppressive capacity of these subjects' CD8+ T cells was no stronger than that of viremic or HAART-treated patients (Fig. 6C). We have reported that susceptibility of CD4+ T cells from HICs to in vitro HIV infection was not different than that of cells from healthy blood donors[13], and no significant differences were found either between weak responder and strong responder HICs (p=0.331) (Fig. 6A)

To determine whether the weak HIV-suppressive activity observed in certain HICs was due to our use of a laboratory-adapted HIV strain, I analyzed the capacity of nonstimulated CD8+ T cells from weak responders A13 and A19 and from strong responder A21 to suppress superinfection of their own CD4+ T cells by autologous viruses previously obtained in primary culture of these individuals' cells. CD8+ T cells from strong responder A21 equally controlled CD4+ T cell superinfection by HIV-BaL and by autologous virus (Fig 6D). In contrast, the weak CD8-mediated HIV suppression in subject A13 was not improved when his autologous virus was used to challenge his CD4+ T cells (0.01 vs 0.16 log p24 decrease with HIV –BaL and the autolgous virus, respectively) (Fig 6D). CD8+ T cells from weak responder A19 showed a strong capacity to inhibit infection by autologous viruses (0.33 vs 1.76 log p24 decrease for HIV BaL and autologous virus infection, respectively) (Fig 6D), although the level of suppression did not reach that observed in strong responders. Interestingly, while most HICs were infected by subtype B viruses, subject A19 was infected by HIV-1 subtype A2 (Table 4). Therefore, although the use of nonautologous viruses might lead to an underestimation of the HIV-suppressive

activity of CD8+ T cells, it was unlikely to explain the differences observed between weak and strong responders.

Figure6. Potent CD8+ T cell mediated HIV suppressive capacity in HIV controllers (a) PHA-activated CD4+ T cells were infected, in the absence (filled) or presence (white) of autologous unstimulated CD8+ T cells (1:1 ratio), with replicative HIV-1 BaL. Circles represent the average (n=3 independent infections) peak p24 values detected in culture supernatants for each individual. Horizontal lines indicate median values. (b) PHA-activated CD4+ T cells from HICs A6 and A13 were superinfected with HIV-1 Bal and left alone (central panels) or co-cultured with autologous unstimulated CD8+ T cells (right panels). Three days later the level of infection was determined by quantifying intracellular p24 on $CD8^{neg}$ cells. (c) The HIV suppressive capacity of CD8+ T cells, as determined by the log fold decrease in the level of secreted p24 (CD4 vs CD4:CD8 cell cultures), was compared in the 19 HICs (circles), 13 chronically HIV-infected subjects with viremia >7000 copies/ml (triangles), and 8 HAART-treated patients with virologic control (plasma HIV RNA <50 copies/ml) for more than 23 months (squares). Horizontal lines indicate median values. (d) p24 production in culture sypernatants (mean±Sd, n=3) at the peak of viral replication after superinfection of CD4+ T cells from HIV controllers with equivalent infectious doses (moi of $10^{-3.6}$) of HIV-1 Bal or filtered supernatants containing autologous HIC viruses. CD4+ T cells were cultured alone (filled bar) or in the presence of non prestimulated CD8= T cells (open bars).

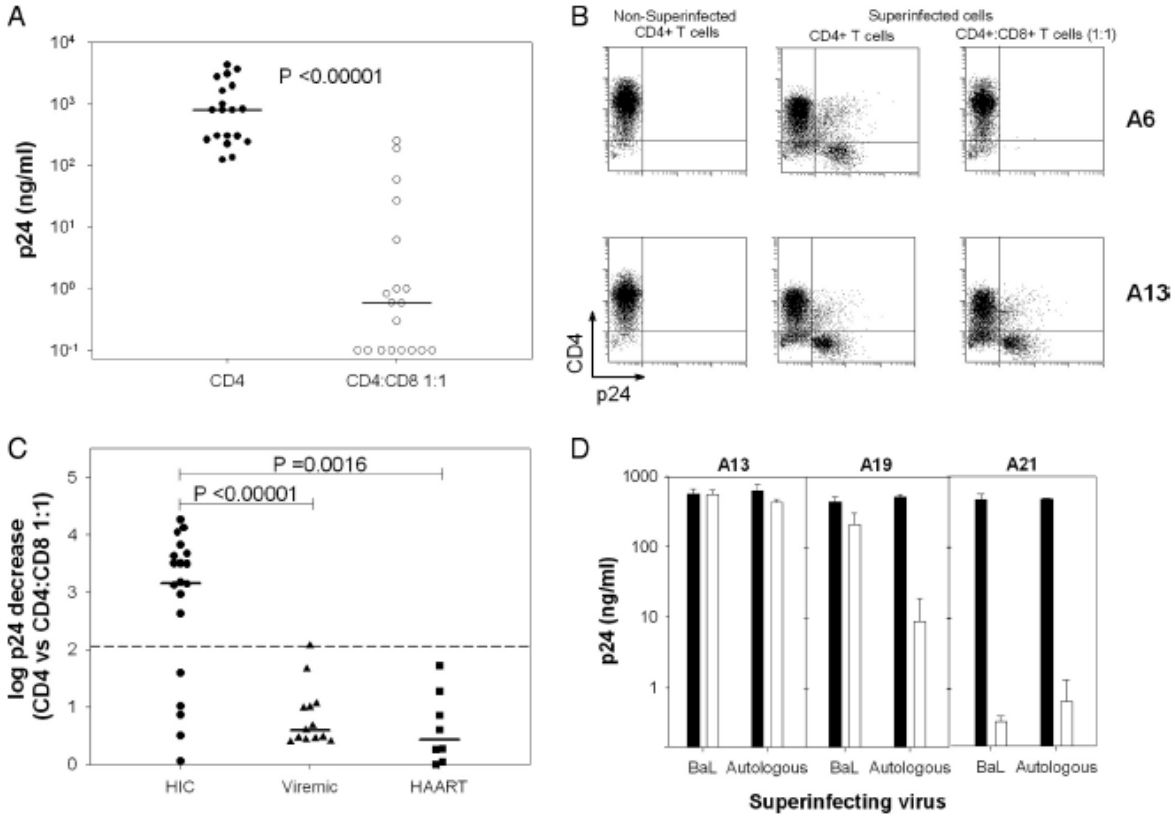


Table5. log p24 decrease (CD4 vs CD4:CD8 1:1) during follow up (>12 months) of HICs

HIC	n^a	Median Value	First Sample		Last Sample	
A1	7	3.5	Sept 2005	4.3	May 2008	3.5
A3	3	3.1	Jan 2006	3.1	Jan 2007	3.3
A4	5	3.8	Jan 2005	4.2	Apr 2008	3.5
A6	7	3.8	Sept 2005	4.3	Nov 2007	3.8
B5	7	3.5	Oct 2005	1.2	May 2008	3.5

[1] number of blood samples analysed

2) CD8-mediated HIV suppressive capacity in HICs correlates with the frequency of IFN-ɣ producing cells

To examine whether the difference between strong and weak responder HICs was associated with a difference in HIV-specific CD8+ T cell responses, I have determined the frequency of IFN-ɣ -secreting CD8+ T cells upon stimulation with

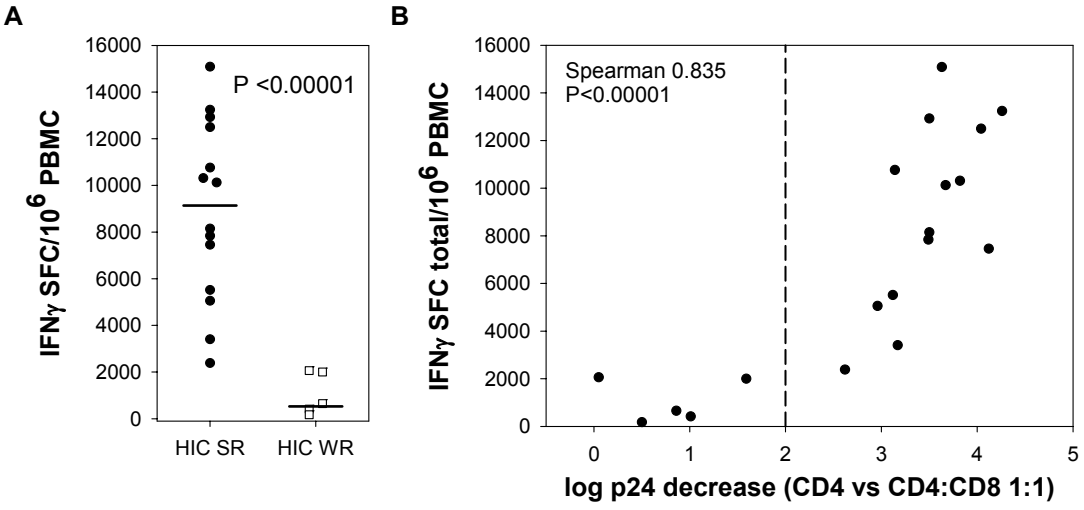
appropriate HLA-defined optimal HIV-1 Env, Gag, Pol and Nef peptides in an Elispot assay. The numbers of IFN-ɣ -secreting cells were heterogeneous (Fig. 7A), in agreement with recent reports [45, 47]. The highest frequencies of HIV-specific CD8+ T cells were observed in strong responders (8517 ± 4038 SFCs/10^6 PBMC vs 1058 ± 903 SFCs/10^6 PBMC in weak responders HICs, p=0.0014) (Fig. 7A). The frequency of HIV-specific CD8+ T cells in the strong responder HICs was not significantly different, as a whole, from that observed in chronically viremic patients ([13]and not shown). The magnitude of the CD8+ T cell response in weak responder HICs was similar to that in HAART-treated patients ([32]and not shown).

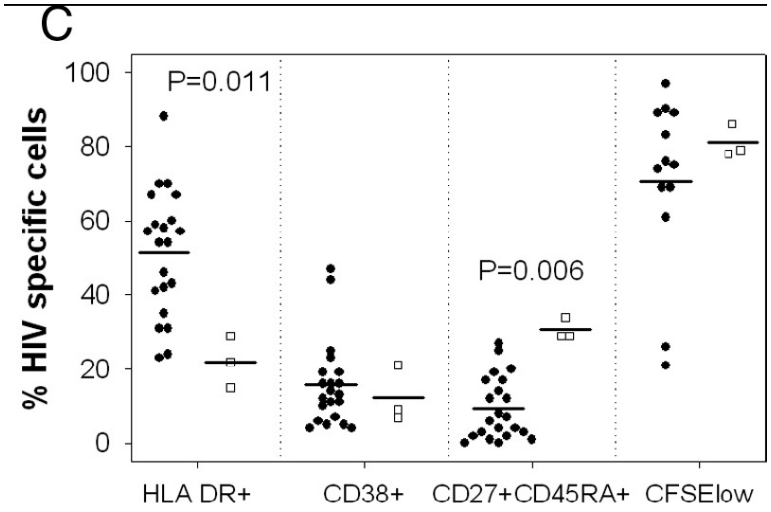
Interestingly, I have found a strong correlation between the frequency of IFN ɣ -producing CD8+ T cells upon peptide stimulation and the HIV-suppressive capacity of unstimulated CD8+ T cells from the same HIC (Spearman 0.835, p<0.00001) (Fig. 7B). This supports the possibility that the *ex vivo* anti-HIV activity of CD8+ T cells from HICs is driven by HIV-specific cells, in keeping with an MHC class-I-mediated mechanism[13]. This correlation further distinguished strong and weak responder HICs (Fig. 7B).

We explored whether differences could also be observed between strong and weak responder HICs at the phenotypical level of their HIV-specific CD8+ T cells. Due to the low frequency of these cells in weak responders, we could perform these analyses only in three of them. HIV-specific CD8+ T cells from strong responder HICs possessed a discordant activation phenotype with high expression of the activation marker HLA-DR associated with a low CD38 expression (Fig. 7*C*), in keeping with our previous study[13]. In contrast, the expression of both activation markers was low in the cells from weak responders (Fig. 7*C*), a phenotype that is found in HAART subjects[13]. HIV-specific CD8+ T cells from both strong and weak responders had high proliferative potential (Fig. 7*C*), which is a hallmark of a high-quality HIV-specific CD8+ T cell response in HICs [24]. Interestingly, we found in weak responders an increase of a subpopulation of HIV-specific CD8+ T cells characterized by the coexpression of CD27 and CD45RA (Fig. 7*C*). It has been recently reported that this

subpopulation is characteristically abundant in HIV patients treated during acute primary HIV infection and may represent a stable quiescent long-term memory pool.[50]

Figure7. Correlation between CD8-mediated HIV suppressive capacity and the frequency of IFN-γproducing cells in HICs **A.** Frequencies of HIV-specific IFNγ-secreting CD8+ T cells in strong responder HICs (log p24 decrease > 2) (SR) and in weak responder HICs (log p24 decrease < 2) (WR). An average of 41 ± 9 peptides were tested in each subject, depending on the results of HLA typing. Each symbol corresponds to the sum of spot-forming cells (SFC) per 10^6 PBMC obtained with individual peptides described as being restricted by HLA antigens. Horizontal lines are median values for each group. **B.** Correlation between the HIV suppressive capacity of CD8+ T cells from HICs (log p24 decrease as shown in figure 6C) and their frequency of IFN -producing CD8+ T cells upon HIV peptide stimulation. Each circle represents one HIC. Vertical dashed line separates weak responder and strong responder HICs. **C.** Percentage of HIV-specific cells (based on HIV multimer and CD8 expression) from strong and weak responder HICs that express *ex vivo* HLA-DR and CD38, coexpressed CD27 and CD45RA, or proliferated (and lost CFSE labeling) after 5 days of peptide stimulation. Each symbol represents one specificity for one HIC. Horozontal lines are mean values for each group.





3) CD8-mediated HIV suppressive capacity in HICs correlates strongly with the magnitude of gag-specific CD8+ T cell responses

The response to Gag contributed most (average 51.8%) to the total HIV-specific CD8+ T cell response (Fig. 8A). In strong responder HICs the contribution of the Gag response was 56.8% on average. The contribution of the Gag response was in average 37.9% in weak responder HICs (p=0.14). Responses to Nef peptides also contributed significantly to the overall CD8+ T cell response in HICs (average 31.8% overall and 31.2% in the strong responders) (Fig. 8A). The contributions of Env and Pol responses were much smaller (7.9% and 8.7% respectively in the whole HIC population) (Fig. 8A).

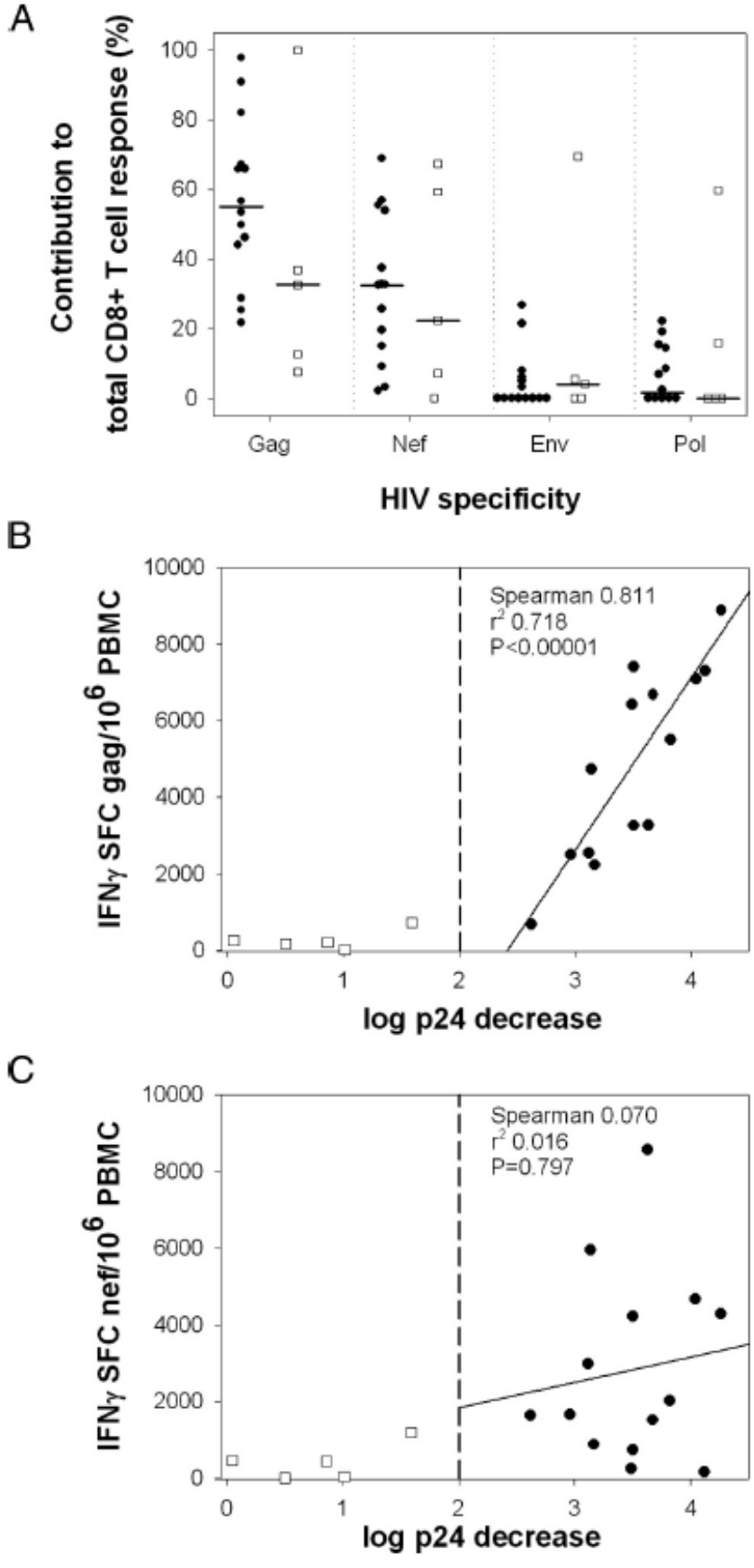
The contributions of the responses to the different HIV proteins were not different in HICs than in viremic patients (not shown), although a tendency was observed to a greater contribution of Gag responses in strong responder HICs than in viremics (average, 38%; p =0.08). The magnitude of the Gag response was higher in HICs (3682±2969 SFCs/106 PBMC) than in viremics (1703±2061 SFCs/106 PBMC, p

=0.05). In contrast, Gag responses contributed less and Nef responses more to the total HIV-specific CD8+ T cell response in HAART-treated patients (14.3% and 60.6% of Gag and Nef responses) than in HICs (p =0.014 and p =0.025,respectively).

We then examined the influence of the specificity of HIC CD8+ T cells on the efficiency of HIV suppression. The correlation between the HIV-suppressive capacity of nonstimulated CD8+ T cells and the frequency of IFN-ɣ -producing CD8+ T cells upon peptide stimulation was strongest for Gag peptides (Spearman 0.907, p= 0.00001) (Fig. 8*B*). This correlation was unlikely to be due to a bias for HLA-B57-restricted Gag responses since in the 13 individuals carrying this HLA allele, HIV-specific CD8+ T cell responses targeting HLA-B57 restricted Gag epitopes represented, on average, 26±12% of their total response. Other responses were either restricted by HLA-B57 but not directed at Gag (19±18%) or restricted by other alleles and directed at Gag (28±28% of the response) or at other proteins (27± 20%). As mentioned, Nef was also a main target of the HIV-specific CD8+ response; however, only a weak correlation was found with the magnitude of Nef responses (Spearman 0.473, p = 0.040) (Fig. 8*C*). Furthermore, this correlation with Nef responses was completely lost when weak responder HICs were excluded from the analyses (Spearman 0.070, p = 0.797). Most interestingly, in the group of strong responder HICs, the CD8+ T cell anti-HIV capacity still correlated more tightly with the magnitude of Gag responses (Spearman 0.812, p =0.00001) than with the total frequency of IFN-ɣ -producing CD8+ T cells (Spearman 0.634, p= 0.007). Overall these results suggest that the numbers of CD8+ T cells responding to Gag epitopes influence the capacity of CD8+ T cells from HICs to suppress HIV infection of autologous CD4+ T cells. To evaluate more directly the impact of Gag responses in the HIV-suppressive activity of CD8+ T cells from strong responder HICs, we first tried to compare the HIV-suppressive capacity of FACSAria-sorted pentamer-positive cell fractions. Unfortunately, and despite a fairly good viability, functionality of these cells was compromised. Hence, we compared the relative weight of Gag and Nef responses by assessing the HIV-suppressive capacity of CD8+ T cell fractions depleted of either one response or the other. These experiments were performed with

cells from three HICs (A3, A6, and A11) with similar numbers of HIV-specific cells (11,270, 12,473, and 12,612 SFC/106 PBMC, respectively) and a contribution of the Gag response to the total HIV-CD8+ T cell response close to 50% (49.8%, 63.0%, and 55.3%, respectively). CD8+ T cells isolated from HICs were stimulated with 1) a pool of all the optimal HIV-1 peptides that were recognized in individual ELISPOT assays (not shown); (2) a pool of Gag peptides only; (3) a pool of Nef peptides only. As shown in Fig. 9*A*, the suppression of HIV infection observed when autologous unstimulated CD8+ T cells from strong responder HICs were added to CD4+T cell cultures was lost when the CD8+ T cells that produced IFN-ɣ upon stimulation with the complete pool of recognized HIV peptides were removed. In the case of A3, both the cell fractions depleted of Gag-specific or Nef-specific CD8+ T cells retained strong HIVsuppressive capacity (Fig. 9*B*). For A11, depletion of Gag-specific cells caused the nearly complete loss of HIV-suppressive capacity, whereas depletion of Nef-specific cells had no effect (Fig. 9*B*). For A6, the depletion of Gag-specific cells also caused a strong loss of HIV-suppressive capacity (Fig. 9*B*). Removal of Nef-specific cells occasioned a more modest loss of HIV-suppressive capacity. In summary, although the respective contributions of Gag and Nef responses were difficult to quantify precisely, Gag-specific CD8+ T cells seemed to strongly contribute to the HIV-suppressive capacity of CD8+ T cells in all three strong responder HICs evaluated, in agreement with the correlations described above. In contrast, the contribution of Nef responses was more variable.

Figure8. CD8-mediated HIV suppressive capacity in HICs correlates strongly with the magnitude of gag-specific CD8+ T cell responses **(a)** Percentage of the HIV-specific CD8+ T cell response that was due to CD8+ T cells secreting IFN upon stimulation with Gag, Nef, Env and Pol peptides. Each symbol represents one HIC. Circles represent strong responders and squares weak responders. Horizontal dashed lines are mean values for each group. (b,c) Correlation between the HIV suppressive capacity of CD8+ T cells from HICs and their frequency of IFNγ-producing CD8+ T cells upon stimulation with Gag and Nef peptides, respectively. Each circle represents one HIC. Vertical dashed line separates weak responder and strong responder HICs.


A
Contribution to total CD8+ T cell response (%)
100
80
60
40
20
0
Gag
Nef
Env
Pol
HIV specificity
B
IFNγ SFC gag/10^6 PBMC
10000
8000
6000
4000
2000
0
Spearman 0.811
r² 0.718
P<0.00001
0
1
2
3
4
log p24 decrease
C
IFNγ SFC nef/10^6 PBMC
10000
8000
6000
4000
2000
0
Spearman 0.070
r² 0.016
P=0.797
0
1
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log p24 decrease

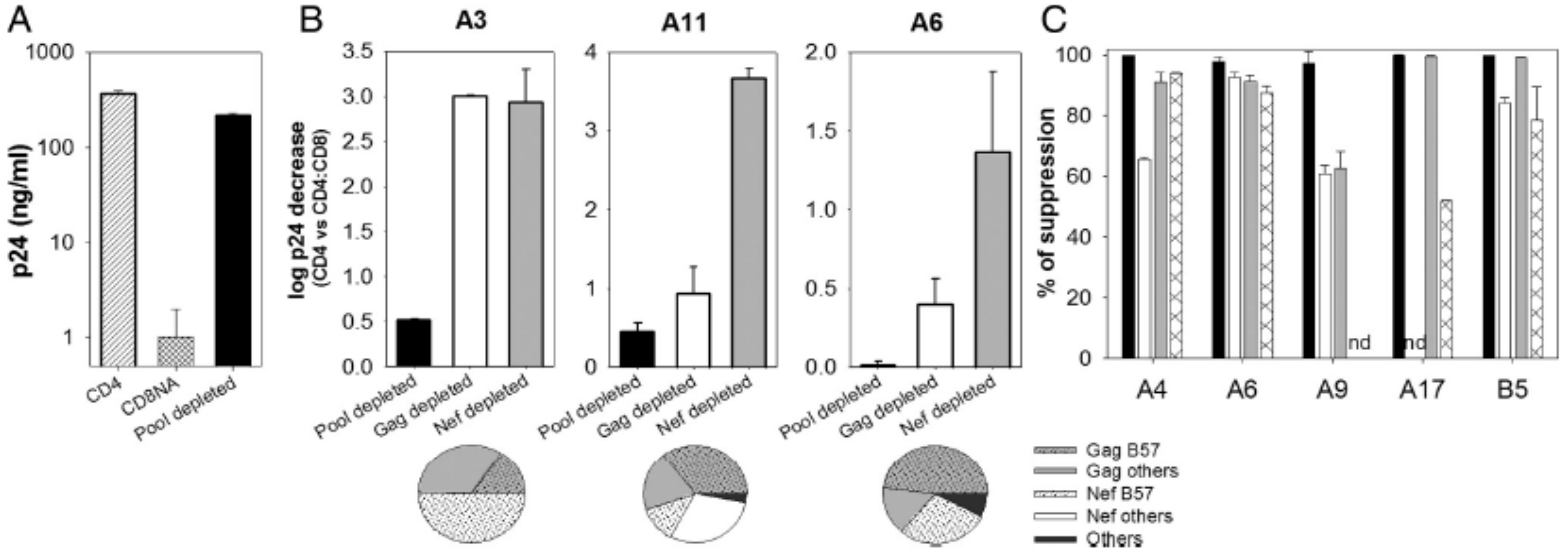


Figure 9. Gag-specific CD8+ T cell seemed to strongly contribute to the HIV-suppressive capacity in SR HICs whereas the contribution of Nef response was more variable. **A.** P24 production in culutre supernatants (mean±SD, n=3) at the peak of viral replication after superinfection of CD4+ T cells from A6 with HIV-1 Bal. CD4+ T cells were cultured alone or in the presence (CD4/CD8 of 1:1) of non-prestimulated CD8+ T cells or CD8+ T cells depleted of HIV-specific CD8+ T cells. These results are representative of experiments with 5 HICs. **B.** HIV-suppressive capacity of CD8+ T cells (mean±SD, n=3), as determined by the log fold decrease in the level of secreted p24 (CD4 vs CD4/CD8, 1:1 cell cultures), after depletion of HIV-specific cells (black bars), Gag-specific (open bars), or Nef specific (gray bars) cell fractions. Pie chart at the bottom represent the relative contribution of Gag and Nef responses to the total HIV-sepcific CD8+ T cell response. **C.** CD4+ T cell from HIV controllers were infected with repicative HIV-1.BaL (black bar), SIVagm.Gril (open bars), SIVmac.251(gray bars), or HIV-2.SBL (pattened bars) and cultured alone or with autologous unstiumlated CD8+ T cells. Viral replication was monitored by p24 or p27 ELISA on culture supernatants. Bars indicate the level of suppression at the peak of viral replication when CD8+ T cells were present in the culture (mean±SD, n=3). n.d. Experiment not done.

4) CD8-mediated HIV suppressive capacity in strong responder HICs is broad

We have already reported a broad capacity of CD8+ T cells from strong responder HICs to effectively control superinfection by different HIV-1 subtypes [13]. Interestingly, CD8+ T cells from strong responder HICs also partially suppressed infection of CD4+ T cells by other human lentiviruses such as HIV-2, SIVmac, and SIVagm (Fig. 9*C*). At least some of the HIV-1 epitopes recognized by HIV-specific CD8+ T cells from strong responders HICs were conserved in the other lentiviruses used in our experiments (not shown). In accordance with a MHC-mediated mechanism, the capacity to suppress SIV infection was totally lost when CD8+ T cells were separated from autologous CD4+ T cells by semipermeable membranes (not shown), as was shown in the case of HIV-1[13].

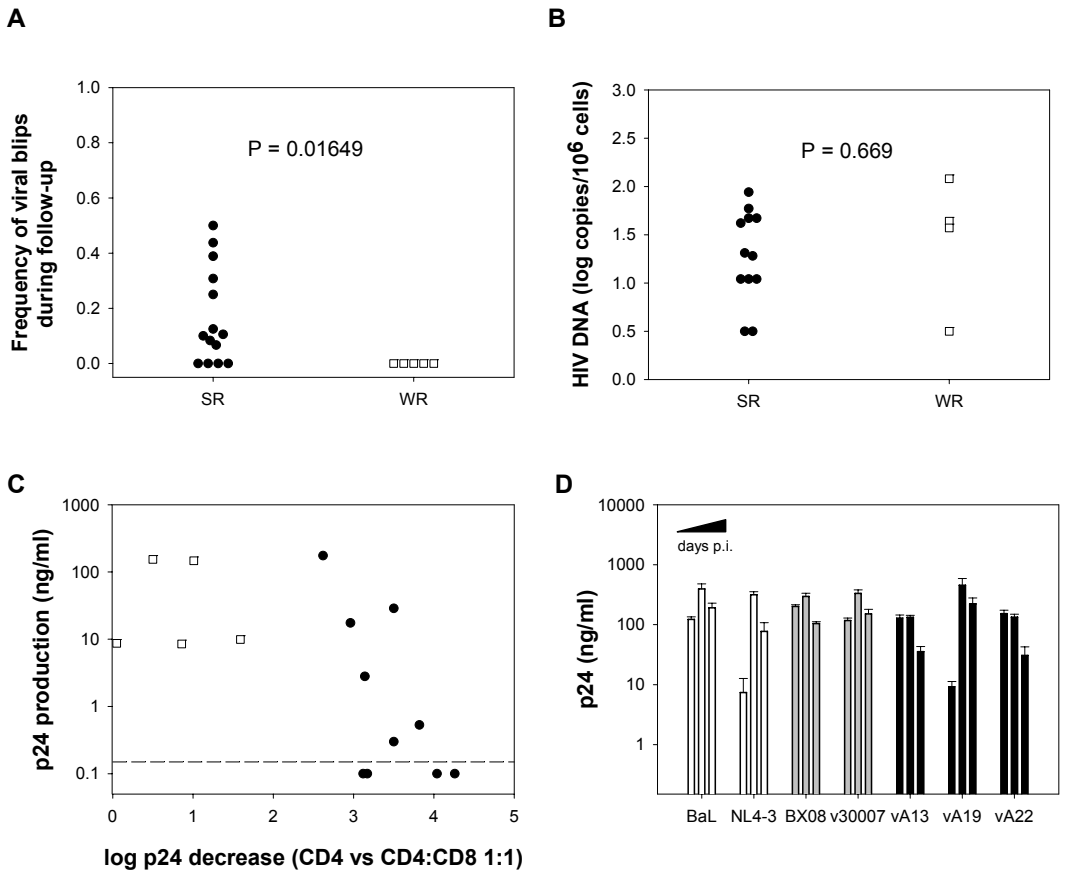
5) Weak responder HICs carry infectious replicative viruses

A recent report by Hatano et al suggest that low level viral replication is on going in most HICs [51]. I have thus examined whether differences between strong responder and weak responder HICs might exist at a virological level. Ultrasensitive viral load tests were not available for this study. However, given the long documented virological follow-up of the patients in the study we had access to multiple RNA viral load determinations for all HICs (Table 4). The length of the follow-up and the number of viral load determinations were similar for strong responder and weak responder HICs (P=0.309 and P=0.515 respectively, Table 4). Interestingly, historical plasma viral load results showed that small blips of viral RNA were more frequently detected during follow-up among strong responder HICs than among HICs with weak CD8+ T cell responses, who appeared to control HIV infection more tightly (P=0.016, Figure 10A and Table I).

HIV-1 DNA level in blood cells, which is a stable parameter that gives an estimation of the HIV-1 reservoir size [52], was available for most HICs (Table 4). Despite the differences in the frequency of viral RNA blips mentioned above, proviral DNA levels were very low in all the HICs, regardless of the strength of their CD8+ T cell responses (figure 10B). We then investigated whether autologous viral replication

might be activated upon stimulation of CD4+ T cells from HICs. Surprisingly, replication-competent viruses were more readily detected in the supernatants of activated CD4+ T cells from weak responders than from strong responders (Fig. 10C). Moreover, autologous virus production upon CD4+ T cell stimulation correlated negatively with the HIV-suppressive capacity of CD8+ T cells (Spearman -0.635, p=0.01). I obtained enough autologous viruses from weak responder HICs A13, A19 and A22 to test their infectivity. These viruses were able to spread and infect heterologous CD4+ T cells as efficiently as other lab-adapted strains and primary isolates (Fig. 10D). Their titers (6.1, 5.6 and 5.5 TCID50/ml for vA13, vA19 and vA22 respectively) were also similar (5.4 TCID50/ml for both BaL and NL4.3, and 6.1 TCID50/ml for v30007). Therefore, at least some HICs with weak CD8+ T cell responses carry highly replicative in vitro viruses. This is in agreement with recent reports showing that defective or attenuated viruses do not generally account for the control of viral replication in HICs[19, 21, 53].

Figure10. Weak responder HICs are infected with fully replicative virus and controls HIV-1 tightly without strong CD8+ T cell reponses. **A.** Frequency of viral load determinations with values above 50 HIV RNA copies per ml of plasma during follow up and **B.** total HIV-DNA in blood cells at inclusion, for strong responder HICs (SR, circles) and weak responder HICs (WR, squares). Each symbol represents one HIC. **C.** Correlation between peak p24 production detected in the supernatant of 10^5 CD4+ T cells from weak responder (squares) and strong responders HICs (circles) upon PHA stimulation (mean of three values) and HIV-suppressive capacity of CD8+ T cells from 16 HICs. Each symbol represents one HIC. The dashed line represents the background level. **D.** Kinetics of viral replication (3, 7 and 10 days post-infection) after infection (1.2 ng of p24/10^6 cells) of CD4+ T cells from a single healthy blood donor. Viruses from subjects A13 and A19 were obtained at 10 days and 14 days, respectively, of culture of PHA-activated CD4+ T cells. Viruses from A22 were obtained after 8 days of culture of PHA-activated CD4+ T cells and 5 additional days of culture in the presence of heterologous PHA-activated CD4+ T cells. White bars represent lab-adapted viruses, gray bars primary isolates and black bars HIC-derived viruses. The mean and standard deviation are shown (n=3).



E. Conclusion

Our results confirmed that a strong CD8+ T cell HIV-suppressive capacity is characteristic of most HIC, although the identification of weak-responder HIC suggests that host mechanisms unrelated to CD8+ T cell response may also contribute to restrain HIV infection. Directly relevant to the development of effective T cell-based vaccines, our results underline the importance of Gag responses in the antiviral potency of CD8+ T cells from strong-responder HIC.

2. Implementation of a rapid Viral “Suppression” Assay for measuring the antiviral capacity of CD8+ T cells.

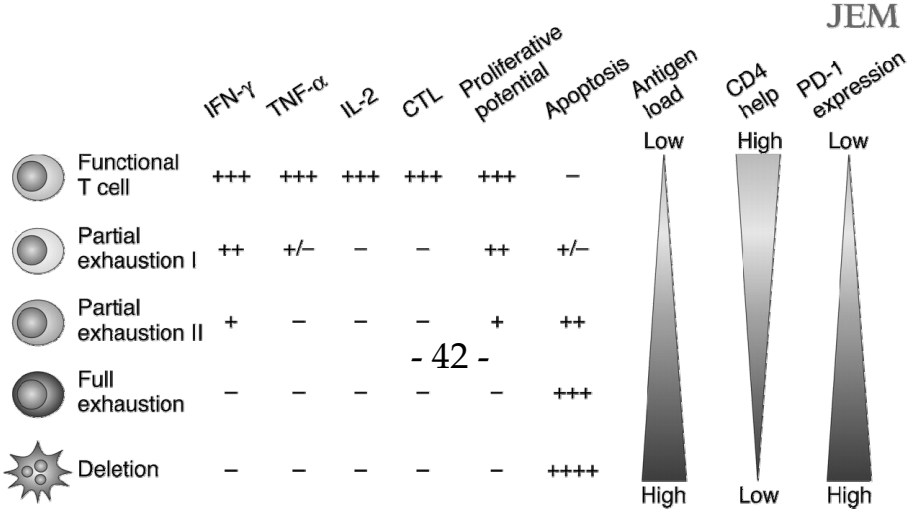
Project 2: Implementation of a rapid Viral “Suppression” Assay for measuring the antiviral capacity of CD8+ T cells.

A. Brief introduction

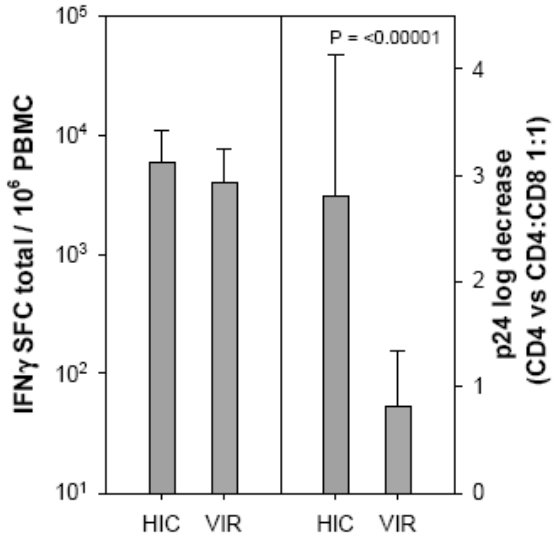
Current methods used to asses HIV specific CD8+ T cell responses such as quantitative ELISPOT assays or intracellular cytokine staining might not accurately measure the function of HIV-specific CD8+ T cells; furthermore, their convenience to evaluate vaccine-elicited cellular immune responses has been challenged. As it is mentioned above, recent Merck STEP HIV vaccine trial failed to provide any protection against HIV despite the induction of significant immune responses as determined by qualitative ELISPOT assay which measures the number of IFN- γ producing cells. [54] As you can see below, secretion of IFN- γ is rather preserved until the cells are quite exhausted which perhaps results in no quantitative difference in the number of IFN- γ producing cells between viremic and HIV controllers.[55] (Figure 11a, b)

Figure 11. Qualitative ELISPOT assay might not accurately measure the function of HIV-specific CD8+ T cells (a) T cell exhaustion during chronic viral infections. Virus-specific CD8+ T cells posses multiple functions including production of IFN-γ, TNF-α, IL-2, cytotoxicity, antigen-driven proliferation, and resistance to apoptosis. During chronic infections, functions can be exhausted. Exhaustion represents a spectrum from mild (Partial exhaustion I: little IL-2 and poor TNF-α and cytotoxicity) to moderate (Partial exhaustion II: modestly defective IFN-γ, cytotoxicity, and little IL-2 or TNF-α) to severe (Full exhaustion: lack of IFN-γ, TNF-α, IL-2, and cytotoxicity). Finally, physical deletion (apoptosis) of T cells occurs. [55] (b) HIV-1 specific CD8+ T cell effector function is better reflected by viral "suppression" assay. Whereas there is no difference in the number of IFN- γ producing cells between HICs and viremic patients, a clear difference was observed in the CD8+ T cell mediated HIV suppression as reflected by the decrease in the production of p24 (ELISA) when superinfected CD4+ T cells were cultured in presence of autologous CD8+ T cells

(a)



(b)



It has been previously shown that there is a significant difference in the CD8+ T cell mediated suppressive capacity of HIV infection between HIV controllers and viremic individuals.[13] Thus, suppressive capacity of the CD8+ T cells in controlling HIV-1 infection could be used as a surrogate marker in evaluating efficacy of novel vaccine in vaccine trials to differentiate efficient CD8+ T cell responses with inefficient responses. In this regard, a convenient standardized virus "neutralization" assay, which measures the capacity of $CD8^+$ T cell mediated HIV suppression quantitatively, will therefore be useful for this aim.

B. Objectives

To develop a rapid, and convenient form of the viral suppression assay

C. Study subjects and Methods

1) Obtaining human peripheral blood samples

50ml of peripheral blood samples were obtained by venipuncture from 16 HIV controllers (patients diagnosed with HIV-1 infection at least 10 years previously who had never received antiretroviral treatment and in whom more than 90% of plasma HIV RNA assays gave values below 400 copies/ml), 9 HAART-naïve Viremic patients, and HIV-seronegative healthy donors. All the subjects gave their written informed cons

2) Isolation and culture of Primary Cells (p.20)

3) Intracellular p24 assay

Activated CD4+ lymphocytes ($5x10^4$) were superinfected in vitro with HIV-1 BaL (R5) with a spinoculation protocol[31]. Various dilutions of virus (m.o.i. $10^{-1.6}$ to $10^{-2.6}$) were used in parallel, in order to obtain similar levels of infection in each individual experiment. CD4+ T cells were culture in the presence or absence of unstimulated CD8+ T cells (CD8:CD4 ratio 1:1). 72 hours after infection, cells were harvested and stained with CD4-ECD (SFCI12T4D11) and CD8-PC5 (B9.11). Cells were then permeabilized (Cytofix/Cytoperm Fixation and permeablization kit, BD bioscience) and stained with KC57-FITC (FH190-1-1) to detect intracellular HIV antigens. Antibodies were from Beckman Coulter. Flow cytometry was performed with Cytomix FC500 and CXP acquisition software (Beckman Coulter).

4) Viral suppression assay using ELISA p24

CD4+ T cells (10^5) were super-infected with HIV-1 BaL (R5) in triplicate at an m.o.i. of $10^{-3.6}$ in 96-well plates with a spinoculation protocol[31]. For coculture, 10^5 CD4+ T cells were mixed with 10^5 CD8+ T cells (CD8:CD4 ratio 1:1) at the moment of infection. After challenge the cells were washed and cultured for 17 days. Viral replication was monitored every 3-4 days in supernatants by p24 ELISA (Zetometrix, Gentaur, France).

5) Statistical analyses

All values throughout the text are means ± standard deviation. P values were calculated with the rank sum test. Correlations were identified by simple linear regression analysis and Spearman's rank correlation test. SPSS 12 (SPSS Inc.,

Chicago Illinois, USA) and SigmaStat 3.5 software was used (Systat Software Inc-SSI, CA).

D. Results

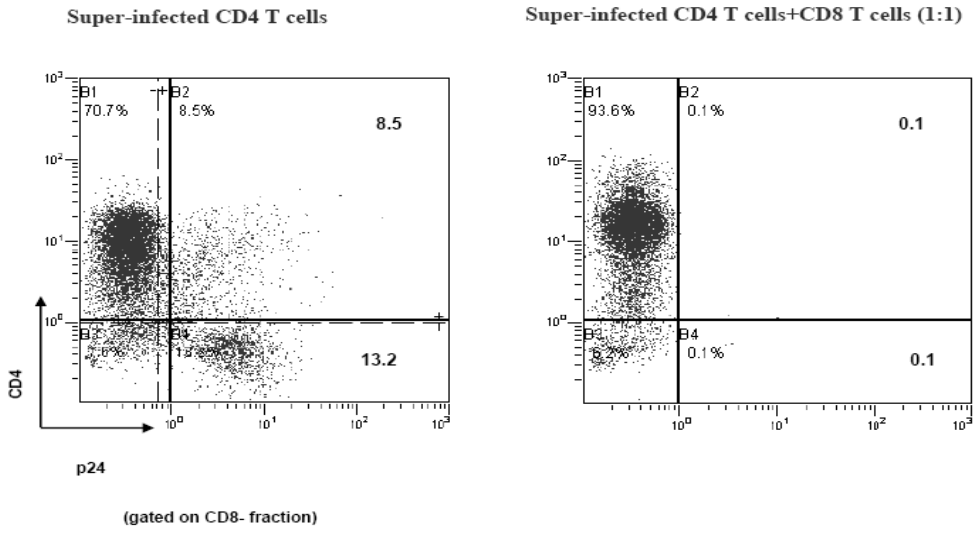
1) Viral suppression assay can directly show the elimination of HIV infected CD4+ T cells

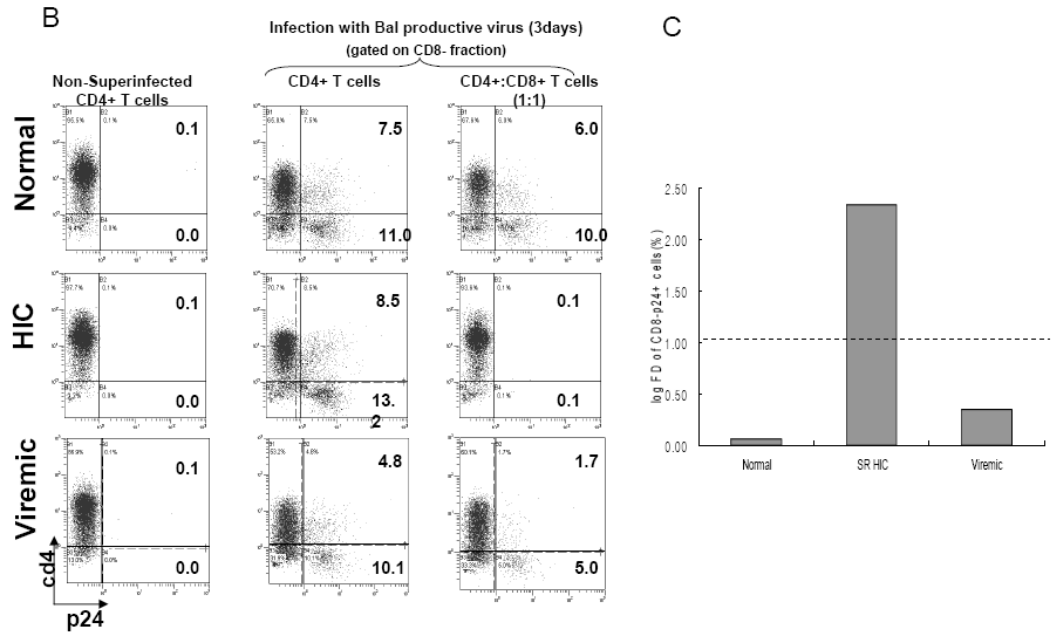
Current standard assays used to measure the HIV-specific CTL responses such as ELISPOT assays and intracellular cytokine staining are not able to measure the CTL function to suppress HIV-1 infection directly. Furthermore, the quantitative measurement yielded by such assays does not correlate to the control of HIV-1 *in vivo*. However, with the intracellular p24 assay, I could directly show the ultimate CTL function by showing the elimination of super-infected CD4+ T cells when autologous *ex vivo* CD8+ T cells were added in culture. In this study, when the *ex vivo* CD8+ T cells were added to the superinfected autologous CD4+ T cells from one HIV controller, a striking elimination of HIV-infected cells was observed (Figure 12A). This elimination of superinfected CD4+ T cells was due to the cytotoxic killing of the infected cells (data not shown). Whereas suppression of HIV-1 infected cells by *ex vivo* autologous CD8+ T cells were clearly observed in elite controllers, this phenomenon was not observed in HIV^- health blood donors and partial elimination of HIV-infected cells was observed with viremic patients. (Figure 12B) Here, we decided to express the CD8+ T cell mediated HIV suppressive capacity measured with intracellular p24 assay as logarhythmic fold decrease of CD8-p24+ cells(%) of superinfected CD4+ T cells, when ex vivo CD8+ T cells were added in the culture. (Figure12C).

Figure12. Intracellular p24 assay- Read out **A.** This is an example of results obtained for one of the HIV controllers. Cells were gated on the $CD8^{neg}$ fraction to avoid the effect of CD4+ downregulation in HIV infected cells. A clear suppression of infection in superinfected CD4+ T cells was obserserved in the presence of CD8+ T cells. **B.**

This is an example of intracellular p24 assay results from one healthy donor, one HIV controller and one viremic patient. While CD4+ T cells were infected at the same level, after adding *ex vivo* CD8+ T cells, the difference in the level of suppression in each group of patients are clearly seen. Whereas in HIV controllers, there was nearly complete suppression of infection, such suppressive capacity was not observed in viremic patients or healthy donors. **C.** CD8+ T cell mediated HIV suppressive capacity was expressed as a logarhythmic fold decrease of CD8-p24+ cells (%). In this regard, figure 12B was replotted as follows.

A





2) Optimization of viral suppression assay parameters

The main purpose of viral suppression assay to detect and measure strong and efficient CD8+ T cell mediated viral suppressive capacity quantitatively. As previously reported, I have assumed that antiviral capacity larger than 2 log suppression in ELISA p24 assay was strong and efficient, which is mostly seen in "strong responder HICs" (ref: project 1). Likewise, we have observed antiviral capacity greater than 1 log fold decrease in intracellular p24 assay was mostly observed in strong responder HICs. However, HIV suppressive capacity by CD8+ T cells is somewhat modulated by level of infection in the superinfected CD4+ T cells since the results are obtained once at 72 hour post infection. For example, when the level of infection is too low, CD8+ T cell mediated HIV suppressive capacity might be overestimated. Likewise, when the level of infection is too high, suppressive capacity might be underestimated [56]. To decide the range of optimal level of superinfection, various viral dilutions were used when the superinfection was done. CD8+ T cell mediated HIV suppressive capacity measured concurrently from intracellular p24 assay and ELISA p24 assay were compared. When the level of super infection was very low (<8% of CD8-p24+ cells), there tend to be an error that weak responder in ELISA p24 assay seem to be a strong responder. However, when the

level of superinfection was very high (>32% of CD8- p24+cells), there tend to an error that strong responder in ELISA p24 assay seem to be a weak responder. (Table 6) Based on these findings, I have decided that the optimal level of superinfection should be 8~32% of CD8-p24 cells. To obtain the optimal level of superinfection, I decided to use at least 2 different viral dilution when making the superinfection. Furthermore, I have reduced the cells per well to $5*10^4$ cells, in which condition gave us a consistant results when compared with $1*10^5$ cells/well condition. To evaluate the well to well variability, I have used a duplicated well system for each condition. However, since the variability was very low, we decided to use single set for each condition to save the samples and materials used.

Table6. The range of optimal level of superinfection. HIV suppressive capacity by CD8+ T cells can be modulated by level of infection in the superinfected CD4+ T cells. To decide the optimal superinfection level in the autologous CD4+ T cells, series of experiements with different infection level of CD4+ T cells were compared with matched simultaneously done virus suppression assay results using p24 ELISA assay. When the level of super infection was very low (<8% of CD8-p24+ cells), there tend to be an error that weak responder in ELISA p24 assay (<2 log p24 suppression) seem to be a strong responder (≥1 log FD CD8-p24+ cells). However, when the level of superinfection was very high (>32% of CD8- p24+cells), there tend to an error that strong responder in ELISA p24 assay (≥2 log p24 suppression) seem to be a weak responder (<1 log FD CD8-p24+ cells). Based on these findings, we have decided that the optimal level of superinfection should be 8~32% of CD8-p24 cells.

Infection level (% of CD8-p24+ cells)	number of experiments	[1]SR→WR	[2]WR→SR	Total disaccordance
<4	4	1	1	2
4~8	14	0	2	2
8~12	8	0	0	0
12~16	4	0	0	0
16~20	7	1	0	1*
20~24	9	0	0	0
24~28	8	0	0	0
28~32	3	0	0	0
>32	2	2	0	2

[1]SR→WR: an error that strong responder in ELISA p24 assay (≥2 log p24 suppression) was estimated to be a weak responder (<1 log FD CD8-p24+ cells); [2]SR→WR: an error which weak responder in ELISA p24 assay (<2 log p24 suppression) was estimated be a strong responder (≥ 1 log FD CD8-p24+ cells). 1*: This individual was a viremic patient with acceptionally high suppressive capacity noted in ELISA p24 (log p24 supression =2.13). This patient was estimated as weak-responder in Intracellular p24 assay (log FD of CD8-p24+ cells(%) =0.80)

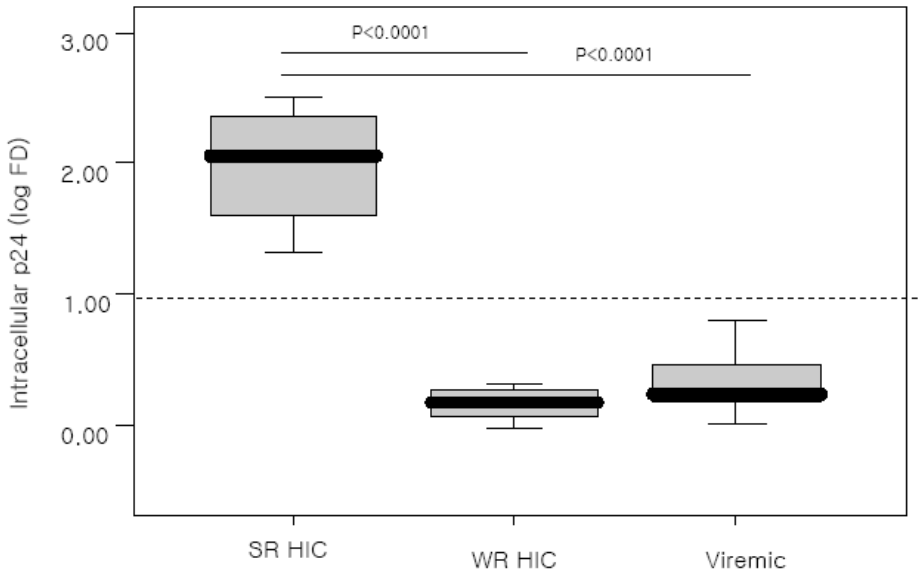
3) Intracellular p24 assay can rapidly distinguish the effective HIV-1 specific CD8+ T cell function

In this study, CD8+ T cell mediated HIV suppressive capacity was measured and expressed as logarhythmic fold decrease of CD8-p24+ cells(%) when the *ex vivo* CD8+ T cells were added in the culture of superinfected autologous CD4+ T cells. Twelve unduplicated results from 6 strong responder HIV controllers, 10 results from 5 weak responder HIV controllers, and 10 results from 8 viremic patients which meet the optimal superinfection level criteria were compared. As a whole, HIV suppressive capacity of CD8+ T cells from SR HICs (2.01±0.05 log fold decrease of CD8-p24+ cells, CD8:CD4 vs CD4) was much stronger than that of cells both from viremic individuals (0.31±0.23 log fold decrease of CD8-p24+ cells, CD8:CD4 vs CD4) and WR HICs (0.16±0.11 log fold decrease of CD8-p24+ cells, CD8:CD4 vs CD4), confirming the previous results obtained from ELISA p24 assay.[13] (ref. project 1) In intracellular p24 assay, all of the SR HICs showed statistically significant higher suppressive capacity more than 1 log suppression of fold decrease; whereas , none of the viremics showed suppressive capacity more than 1 log suppression of fold decrease. (Figure13A). The results obtained from intracellular p24 assay was strongly

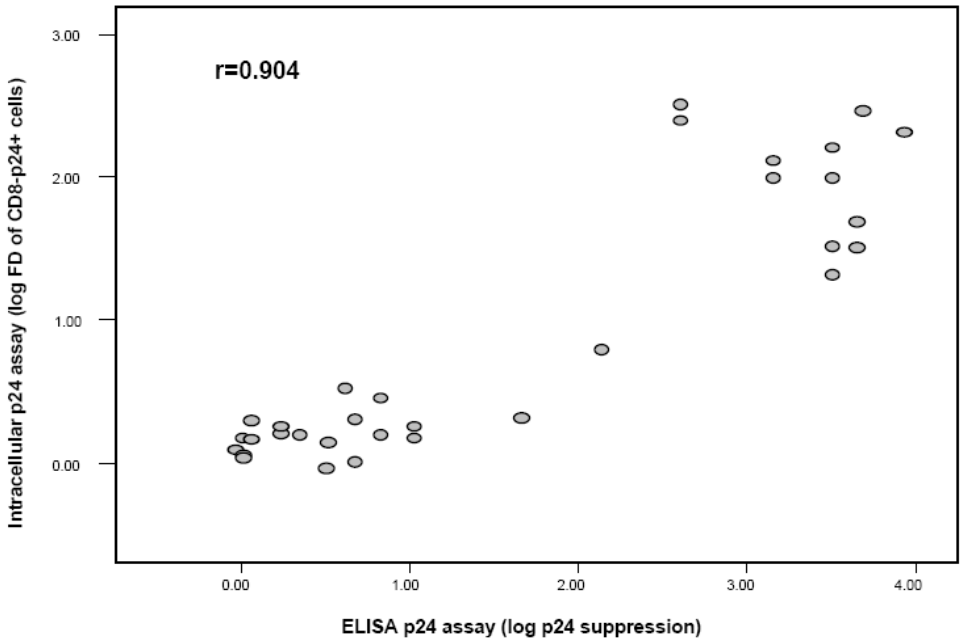
correlated with the results obtained from ELISA p24 assay (Pearson 0.901, P<0.00001). (Figure 13B)

Figure13. Intracellular p24 assay can rapidly distinguish the effective HIV-1 specific CD8+ T cell function **A.** The HIV suppressive capacity of CD8+ T cells measured by intracellular p24 assay, as determined by the log fold decrease of the CD8-P24+ cells (CD4 vs CD4:CD8 cell cultures), was compared in the 12 sets of experiments with Strong responder HICs, 10 with chronically HIV-infected subjects with viremia >7000 copies/ml, and 10 with Weak responder HICs were compaired. The CD8+ T cell mediated HIV suppressive capacity was significantly higher in SR HICs when compared to viremic patiens or WR HICs. **B.** The HIV suppressive capacity of CD8+ T cells measured with intracellular p24 assay (shown here in y axis) correlated strongly with that obtained from ELISA p24 assay (shown here in x axis). (r=0.904, p<0.00001)

A



B



E. Conclusion

The antiviral capacity of circulating CD8+ T cells can thus be measured in 72 hours using a small number of cells. This assay could be used as a surrogate marker in evaluating efficacy of HIV specific responses in vaccine trials.

2-1: Collaborative study: Impact of clonal avidity in the polyfunctionality and anti-viral capacity of HIV-specific CD8+ T cells

A. Brief introduction

As it is mentioned above, a number of characteristics of HIV-specific CD8+ T cells such as polyfunctionality[23], proliferative capacity[24]; and HIV suppressive activity[13] have been known as being associated with slow disease progression in HIV-1 infected patients. As for the role of functional avidity, a parameter which reflects recognition efficiency or antigen sensitivity, contrasting data have been reported. For example, it was reported by Victor Appay′s group that in addition to strong polyfunctionality and turnover, HIV specific CD8+ T cells associated with superior control of HIV-1 replication display high functional avidity[57].However, there have been a report from another group that HIV specific CD8+ T cells zith strong polyfunctionality are actually those that display low, rather than high, antigen sensitivity[58]

Virus specific CD8+ T cells differ according to their HLA restriction, targeted antigen, clonal diversity, activation status and replicative history. This heterogeneity in the nature and environment of T cells is an obvious obstacle to solve the functional complexity of the anti-HIV T cell response. In this regards, a collaborative study with Victor Appay (CHU Pitié Salpêtrière) was done in order to evaluate the impact of clonal avidity in the polyfunctionality and antiviral capacity of HIV-specific CD8+ T cells. The intracellular p24 assay that we have developed was directly applied to the study.

B. Methods

We have performed a detailed study of the interplay between T cell function attributes using a bank of HIV-specific CD8+ T cell clonotypes isolated in vitro. Primary HLA B*2705+ CD4+ T cells were purified (>99%) from freshly isolated PBMC by positive selection with antibody-coated magnetic beads. CD4+ T cells were activated with PHA and IL-2 for 3 days and infected in vitro with HIV-1 NL4.3 (X4) at a multiplicity of infection (m.o.i) of $10^{-1.8}$ with spinoculation protocol. For coculture,

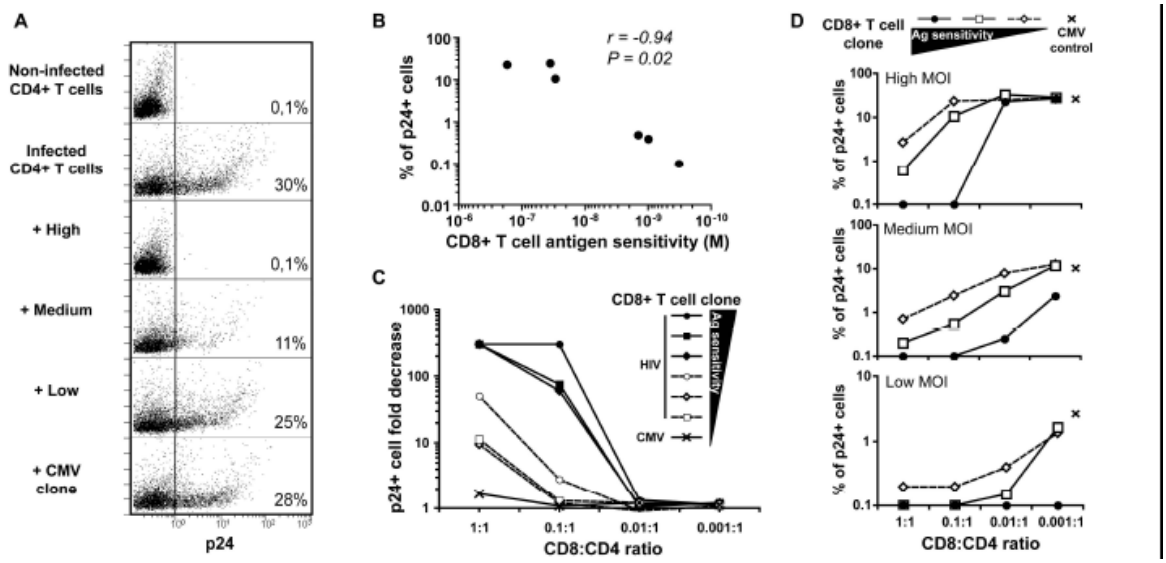
10^5 CD4+ T cells were mixed with CD8+ T cells at different ratio. Cells were harvested, permeablized and labeled with anti-p24mAb to monitor HIV-1 infection.

C. Results

We tested the capacity of KK10-specific CD8+ T cell clones to suppress HIV replication using infected HLA B*2705+ primary CD4+ T cells by intracellular p24 assay. Notably, the presence of highly sensitive CD8+ T cell clones prevented the production of p24 in the infected CD4+ T cell cultures to levels approaching those of the negative controls, i.e. in the complete absence of virus; thus, these CD8+ T cells had almost totally suppressed HIV replication in this assay (Fig. 14A). However, KK10-specific clones with lower levels of antigen sensitivity were not as effective. In fact, the presence of such clones had virtually no effect on the dynamics of HIV infection and replication (Fig. 14A). Importantly, we found an inverse correlation between the proportion of infected cells expressing intracellular p24 and the antigen sensitivity of the CD8+ T cell clones present in the cultures under equivalent conditions (Fig. 14B). In the presence of disproportionate numbers of T cell clones with lower levels of antigen sensitivity, however, apparent levels of HIV suppression could nonetheless be observed (Fig. 14C).Finally, we tested the capacity of representative clones to suppress HIV replication under conditions of decreasing viral input. Lower effector to target ratios were required for suppression of HIV replication at lower multiplicities of infection in these assays (Fig. 14D); however, under all conditions, highly sensitive clones exhibited greater potency in their ability to suppress HIV infection and replication.

Figure14. Suppression of HIV-1 infection *in vitro* by HIV-specific CD8+ T cell

clones with dfferent avidity **A**,Primary CD4+ T cells isolated from an HLA B*2705+ healthy donor and blasted with PHA were infected with the replicative HIV-1 strain NL4.3 (m.o.i. = $10^{-1.8}$) in the presence or absence of KK10-specific CD8+ T cell clones with distinct levels of antigen sensitivity or a control NV9-specific CD8+ T cell clone; effector:target (E:T) ratio 1:10. After 3 days, HIV-1 infection levels were measured using intracellular p24 staining. Numbers show the percentages of p24+ cells in the cultures. **B**, Inverse correlation between CD8+ T cell antigen sensitivity and HIV-1 infection *in vitro* (% of p24+ cells) determined using Spearman's rank test. **C**,Assessment of suppressive activity (fold decrease of p24+ cells compared to infected CD4+ T cell controls in the absence of CD8+ T cells) for CD8+ T cell clones at different E:T ratios. **D**, Measurement of p24+ cells at decreasing multiplicities of infection (MOI) ($10^{-1.8}$, $10^{-2.2}$, $10^{-3.3}$) and different E:T ratios for CD8+ T cell clones with low, medium or high levels of antigen sensitivity. $CD8^{neg}$ cells were gated for the analyses.



D. Conclusion

Highly sensitive CD8+ T cell clones display potent HIV suppressive activity.

3. Characterization of a Subset of Cynomolgus Macaque Spontaneously Controlling SIVmac251 Infection

Project 3: Characterization of a Subset of Cynomolgus Macaque Spontaneously Controlling SIVmac251 Infection

A. Brief Introduction

Studies on HICs are somewhat hampered because of their rarity, lack of information on the early disease course, and lower feasibility to obtain tissue samples. As it was mentioned above, the maintenance of large numbers of (activated) HIV-specific CD8+ T cells in HIV controllers despite the absence of detectable antigen in the periphery is puzzling, and this might be due to viral replication in deeper tissues in these individuals. An animal model, such as SIV infected macaque model could be adapted to solve some of the unanswered questions in HICs.

David Watkins' group recently identified, among animals challenged with SIVmac239 virus, a group of SIV controllers that had less than 500 viral copies/ml during a followed up of 1-5 years [28]. These SIV controllers (SICs) share some characteristics with HICs such as over-representation of certain MHC class I molecules which are believed to be associated with the control of virus; Mamu B*17(human equivalent of HLA-B57) and Mamu B*08(human equivalent of HLA-B27) [59], and strong SIV-specific CD8+ T cell responses [28]. Interestingly, the CD8+ T cells from these animals were reported to have antiviral activity *ex vivo* [28]. One of my main objectives has been to analyze a group of infected macaques in order to determine whether the mechanisms that account for viral control in SICs are the equivalent to those in their human counterparts, and if therefore they may represent an accurate animal model for the HIV controller phenomenon.

To do so, and in collaboration with Bruno Vaslin's group (CEA, Service d'Immuno-Virologie, DSV/iMETI, IPSC, Fontenay-aux roses, France), we have studied the pathogenic model of SIVmac251 infection of cynomolgus macaques that closely resembles HIV infection in humans [60].

B. Objectives

To determine the presence of similarities in mechanism of durable virus control in SIV infected macaques and HIV infected human Controllers.

C. Study subjects and Methods

1) SIV infected cynomolgus macaques

Twenty four cynomolgus macaques are chronically infected by pathogenic SIVmac251. Among them, 12 were inoculated intravenously with 50AID50 and received either AZT, 3TC, and Indinavir post exposure (4 hours to 28 days) or placebo; 6 were inoculated intravenously with 5,000 AID50; 6 received 50AID50 intrarectally. (Table 7)

Table7. Basic characteristics of cynomolgus macaques included in the study.

Code	Exposition	Viral Dose (SIV_{mac251})	Treatment (4h-28d p.i)	CD4+ cells/µl of blood First/Last value*	Viral RNA copies/ml Median/Last value*	Viral DNA copies/10^6 PBMCs Median/Last value*
15729	iv	5000AID50		1334 / **1110** (315)	$1.8x10^4$ / **$5.3x10^4$** (226)	$2x10^4$ / $5.9x10^3$ (149)
15816	iv	5000AID50		1348 / **88** (315)	$3.1x10^5$ / **$1.8x10^5$** (226)	$1.8x10^4$ / $1.3x10^4$ (149)
16834	iv	5000AID50		1606 / **990** (315)	$2.3x10^4$ / **<60** (226)	$2.2x10^4$ / $2.5x10^3$ (149)
20555	iv	5000AID50		1190 / **1231** (315)	$4.4x10^4$ / **<60** (273)	$1.7x10^4$ / $1.7x10^4$ (149)
20784	iv	5000AID50		951 / **238** (315)	$2.5x10^5$ / **$1.6x10^5$** (273)	$9.2x10^4$ / $1.2x10^5$ (149)
20973	iv	5000AID50		1014 / **1073** (315)	$7.3x10^5$ / **$7.2x10^5$** (273)	$2.6x10^5$ / $3.7x10^5$ (149)
15596	iv	50AID50		1869 / **1143** (315)	$3.7x10^4$ / **$3.7x10^4$** (550)	$4.4x10^4$ / $5.3x10^4$ (128)
15693	iv	50AID50		1434 / **1104** (315)	$5.1x10^3$ / **$2.2x10^4$** (550)	$1.4x10^4$ / $9.2x10^3$ (149)
20483	iv	50AID50	Placebo	911 / **608** (315)	$2.5x10^4$ / **$2.8x10^4$** (550)	$1.1x10^4$ / $1.1x10^4$ (149)
20525	iv	50AID50		1018 / **404** (315)	$2.1x10^4$ / **$4.3x10^4$** (550)	$1x10^4$ / $1.1x10^4$ (149)
20595	iv	50AID50		1075 / **439** (315)	$1.2x10^4$ / **$4.6x10^4$** (550)	$1.2x10^4$ / $7.9x10^3$ (149)
20654	iv	50AID50		1388 / **783** (315)	$8.5x10^3$ / **$6.3x10^4$** (550)	$1.5x10^4$ / $1.6x10^4$ (149)
20565	iv	50AID50	AZT :	1409 / **1663** (315)	<60 / **<60** (273)	<30 / <30 (149)
20613	iv	50AID50	2x4,5mg/kg/d	1286 / **855** (315)	$9.1x10^2$ / **$6.6x10^2$** (273)	$6.1x10^2$ / $7.6x10^2$ (149)
20625	iv	50AID50	3TC :	905 / **100** (315)	$4.1x10^3$ / **$2.6x10^4$** (273)	$2.2x10^3$ / $6x10^3$ (149)
20695	iv	50AID50	2x2,5mg/kg/d	1983 / **1789** (315)	$4.2x10^2$ / **<60** (273)	$1.3x10^3$ / $1.6x10^3$ (149)
20802	iv	50AID50	Indinavir:	394 / **215** (315)	$5.9x10^2$ / **$2x10^3$** (273)	$3.6x10^3$ / $4x10^3$ (149)
20906	iv	50AID50	2x20mg/kg/d	1055 / **619** (315)	$6.2x10^3$ / **$1.4x10^4$** (273)	$5.5x10^2$ / $4x10^3$ (149)
13170	ir	50AID50		956 / **754** (459)	<60 / **<60** (907)	$1.6x10^4$ / <30 (184)
13237	ir	50AID50		1187 / **813** (459)	<60 / **<60** (907)	$1.4x10^4$ / <30 (184)
13311	ir	50AID50		1009 / **240** (459)	$6.4x10^3$ / **$1.6x10^5$** (907)	$6.9x10^4$ / <30 (184)
13316	ir	50AID50		861 / **905** (459)	<60 / **$7x10^2$** (907)	<30 / <30 (184)
13457	ir	50AID50		1730 / **1205** (459)	$3.5x10^2$ / **60** (907)	$3.8x10^4$ / $1.8x10^3$ (184)
13523	ir	50AID50		2660 / **1511** (459)	<60 / **<60** (907)	<30 / <30 (184)
V401	None			NA		
20195	None			NA		
OBL5	None			NA		
OBAK	None			NA		
OBEN	None			NA		
OBEC5	None			NA		

iv: intravenous; ir: intrarectal; NA: non available; * In brackets the day post infection corresponding to the last available value

2) Primary cell culture

Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using BD vacutainer CPT cell preparation tubes (BD, NJ, USA). Red blood cells were eliminated by red blood cell lysis buffer (Miltenyi biotec,

Paris, France) as written in the protocol. From freshly isolated PBMCs CD4+ and CD8+ T lymphocytes were isolated via positive selection and negative selection with antibodies coated magnetic beads (Miltenyi Biotec, Paris, France) respectively. CD4+ T lymphocytes were activated with concanavalin A (conA) at 10µg/ml in the presence of IL-2 (Chiron, France) at 100units/ml whereas CD8+ T lymphocytes were left in culture without any cytokines or chemokines for 72 hours. The culture medium was RPMI 1640 containing 10% FCS and penicillin/streptomycin (100 U/ml).

3) Productive infection in vitro

CD4+ T cells (10^5) were super-infected with SIVmac251 at 1.3*10-4 m.o.i in triplicate in 96-well plates with a spinoculation protocol[31]. For coculture, 10^5 CD4+ T cells were mixed with 10^5 CD8+ T cells (CD8:CD4 ratio 1:1) at the moment of infection. After infection the cells were washed and cultured for 14 days. HIV-1 replication was monitored every 3-4 days in supernatants by p27 ELISA (Zeptometrix, Gentaur, France). Infectivity assays were carried out in the presence of 100 IU/ml IL-2.

D. Results

1) Identification of a group of SIV controllers who spontaneously controlled SIV infection

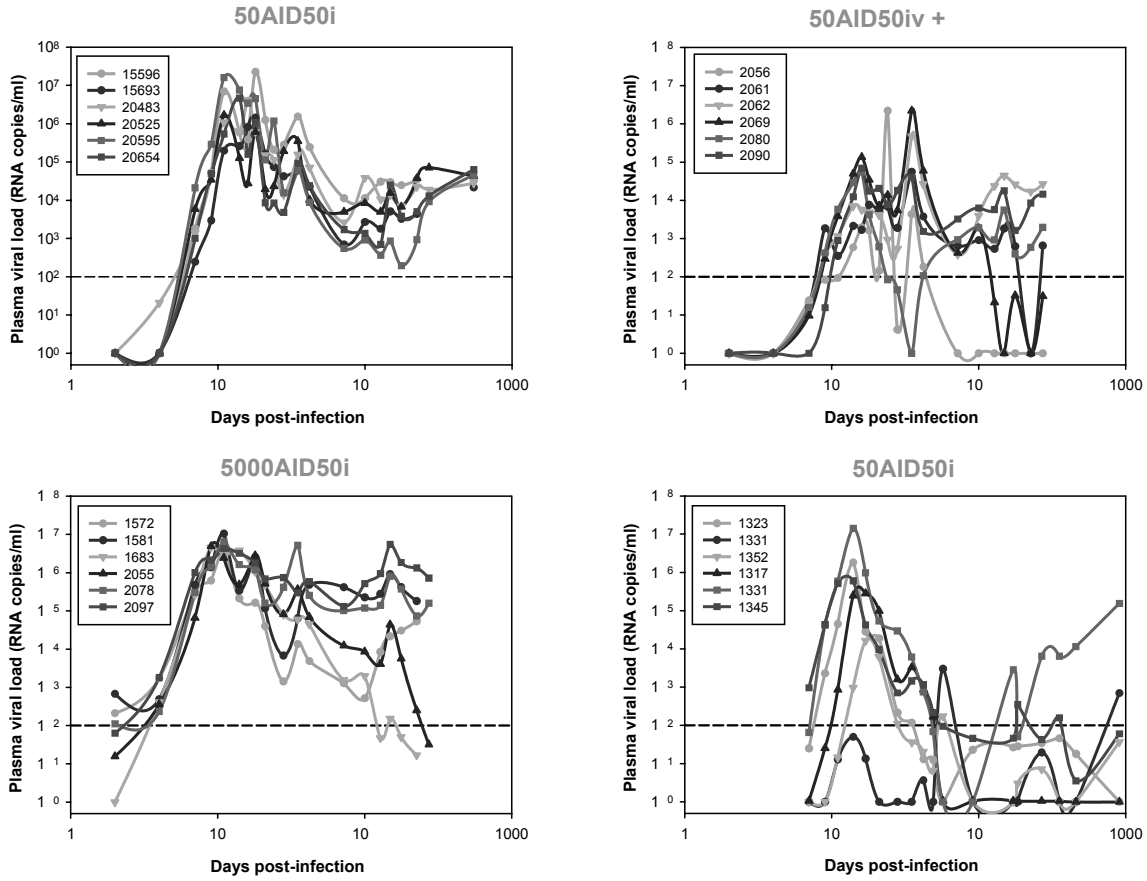
This study included 24 cynomolgus macaques chronically infected by pathogenic SIVmac251. As mentioned above, twelve were inoculated intravenously with 50AID50 and received either AZT, 3TC and Indinavir post-exposure (4 hours to 28 days) or placebo; 6 were inoculated intravenously with 5,000AID50; 6 received 50AID50 intrarectally. CD4+ T cell counts, plasma RNA and PBMC DNA viral loads were monitored closely after the inoculation. As it is clearly shown in the figure 15, different outcomes were observed for the plasma viral loads and CD4+ T cell count after the inoculation. (Figure 15 a, c) Eight of these macaques persistently controlled plasma viremia (<100 RNA copies/ml) after 9 to 18 months of infection (5/6 in

50AID50ir group, 2/6 in 50AID50iv+ART group; 1/6 in 5,000 AID50iv group) and maintained CD4+ T cell count. Among them, 5 SIV controllers from 50 AID50ir group successfully continue to suppress viral replication after 30 months. (Figure not shown). We have identified and characterized a group of cynomolgus macaques spontaneously controlling SIVmac251 infection.

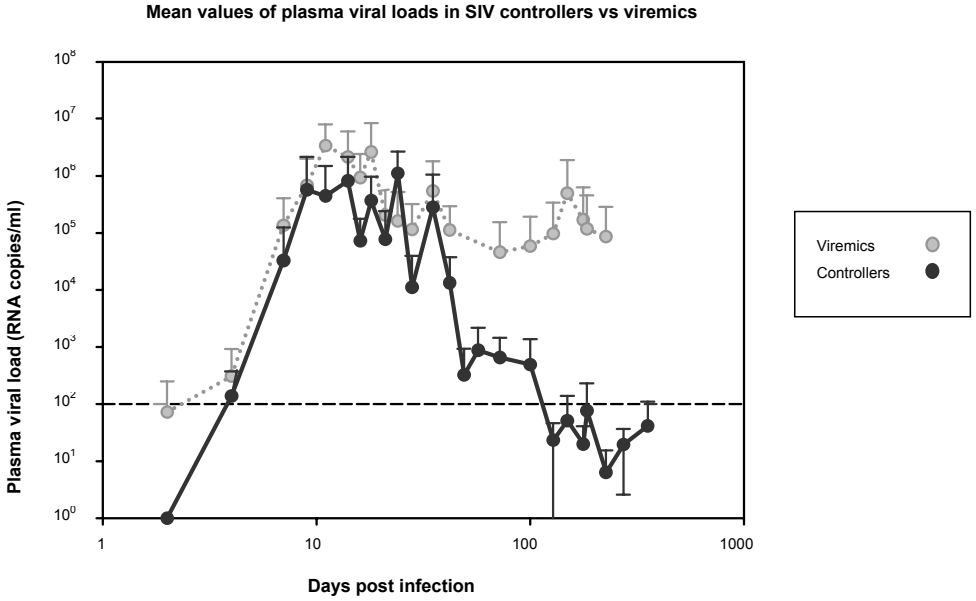
Figure15. Evolution of plasma viral loads and CD4+ T cells in 24 cynomolgous macaques infected with SIVmac251. **A.** Different outcomes were observed for the plasma viral load after inoculation of 24 cynomolgus macaques **B.** In particular, some of them (8) were able to control infection below 100 copies (in 6 the control of viremia was achieved spontaneously and 2 received ART from 4h to 28 days p.i.) **C.** The evolution of CD4+ T cells was monitored as a proportion of values on day 0. **D.**

A rapid and persistent decline in the number of CD4+ T cells was observed in the macaques with the highest viral load set points.

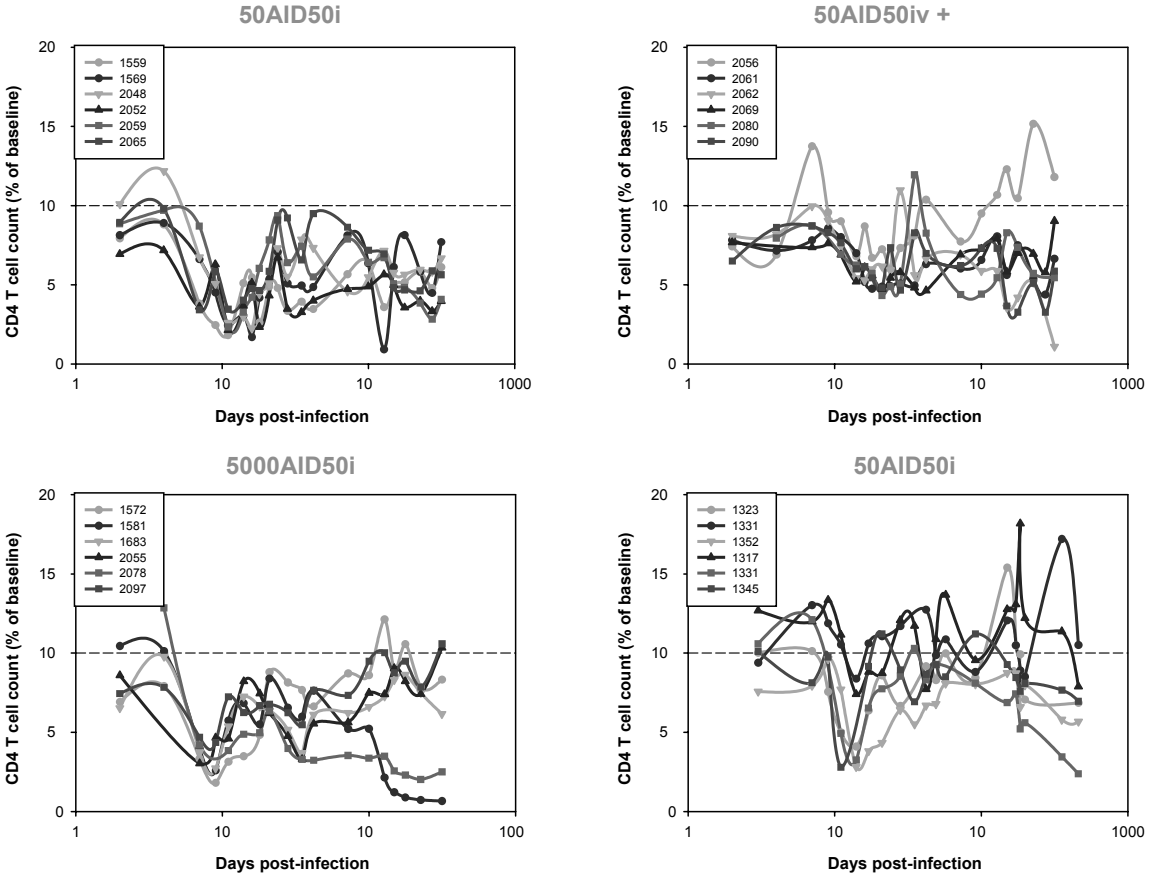
A. Plasma viral RNA loads of 24 cynomolgus macaques inoculated with SIVmac251.



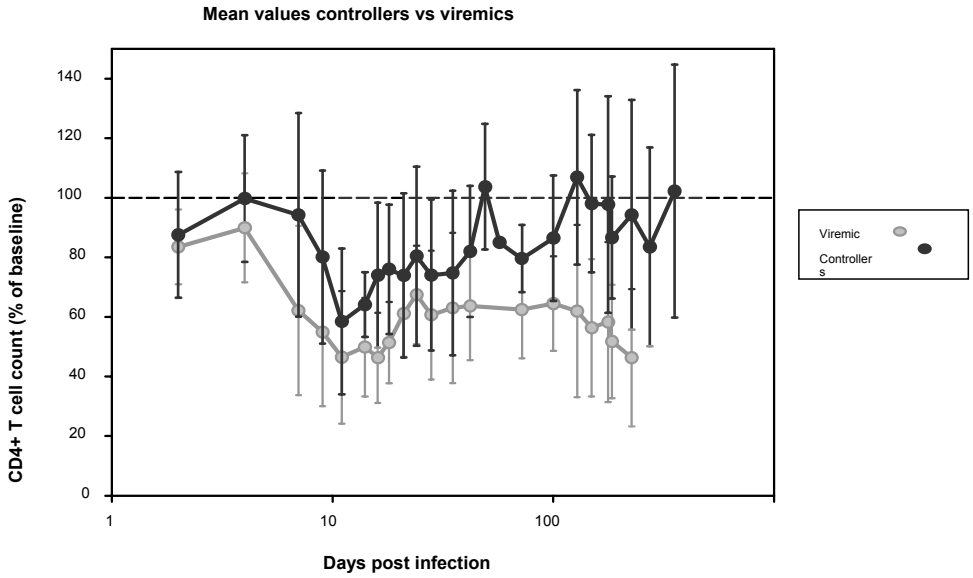
B. Mean values of plasma viral loads in SIV controllers vs viremic individuals



C. CD4+ T cell count of 24 cynomolgous macaques inoculated with SIVmac251.



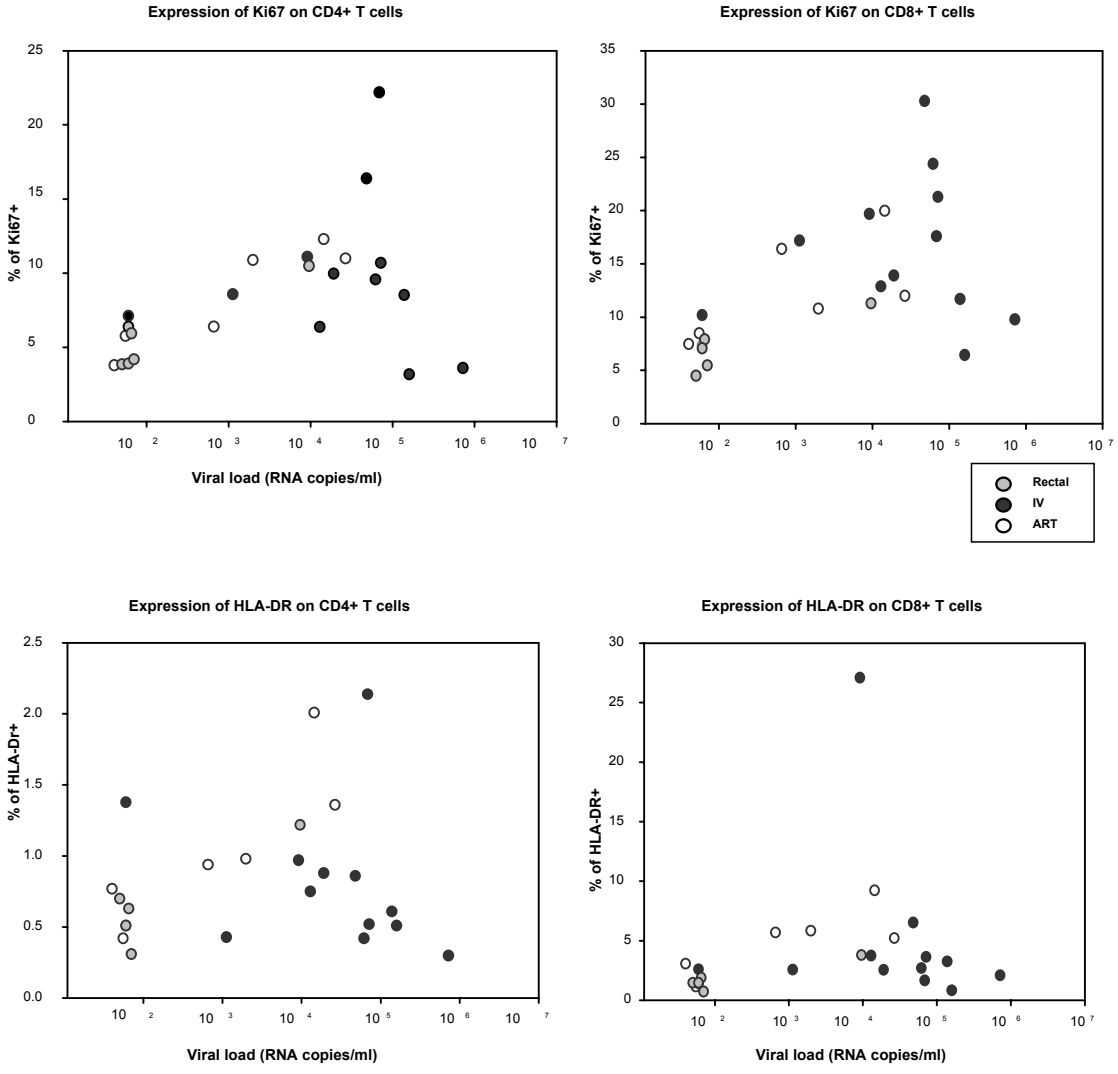
D. Mean values of CD4+ T cell count in SIV controllers vs viremic individuals



2) CD4+ and CD8+ T cells from SIV controllers showed weak immune activation profile

We have investigated the activation profile of CD4+ T cells and CD8+ T cells of SIV controllers and viremic macaques. As it is shown in figure 16, CD4+ and CD8+ T cells expressed lower levels of HLA-DR and Ki67 in controllers than in viremic macaques ($P=0.006$, $P<0.001$, $P=0.004$ for Ki67+CD4+, Ki67+CD8+ and HLA-DR+CD8+ cells respectively) suggesting weak immune activation. (Figure 16). Their CD4+ and CD8+ T cells both showed significantly lower CD69 compared to viremic macaques. ($p=0.028$, $p=0.005$, respectively) (data not shown).

Figure16. Activation status of CD4+ and CD8+ T cells. Bell-shaped distributions were observed when the levels of activation markers on the surface of CD4+ and CD8+ T cells from the different macaques were compared to the corresponding viral loads. Thus, T cells from macaques spontaneously controlling SIVmac251 infection possessed a low activation phenotype, while T cells from viremic macaques (up to 104-105 RNA copies/ml) were more activated. Low activation profiles were also found for the T cells from macaques with persistently high viremia.



3) *Ex vivo* CD8+ T cells from controllers partially suppress SIV infection and the capacity of CD8+ T cells to inhibit SIV infection is unrelated to viral load or route of infection

Ex vivo CD8+ T cell from SIV controller partially suppress SIV infection whereas unstimulated CD8+ T cells from healthy macaques showed no capacity to control SIVmac251 superinfection. (Figure 17A).Similar to their human counter-parts, this suppressive capacity was unrelated to the secretion of soluble factors. (Figure 17B). However, CD8+ T cells from SIV controllers showed capacity *ex vivo* to suppress SIV infection (1.47± 0.68 log p27 decrease CD4:CD8 1:1 vs CD4) not different to viremic animals (1.10± 0.39). (Figure 18A) This suggest that unlike in HIV controllers, potent antiviral capacity of circulating CD8+ T cells is not determinant in the spontaneous control of viremia observed in the early phase of chronic infection in this group of SIV controllers.

We have investigated whether there is a correlation between the CD8+ T cell mediated SIV suppressive capacity and their viral loads or route of infection in SIVmac251 infected cynomolgous macaques. As it is clearly shown in figure 18, the capacity of *ex vivo* CD8+ T cells to inhibit SIVmac251 infection of autologous CD4+ T cells was no different in controllers than in viremic macaques. The inhibitory capacity of CD8+ T cells was not related to the infection route either. (Figure 18)

Figure17. *Ex vivo* CD8+ T cells from controllers partially suppress SIV infection . **A.** Unstimulated CD8+ T cells from healthy macaques showed no capacity to control SIVmac251 superinfection in autologous CD4+ T cells. In contrast, viral replication was significantly reduced when CD4+ T cells from controller macaques were superinfected and co-cultured with autologous unstimulated CD8+ T cells. **B.** These CD8+ T cells required to be in contact with the CD4+ T cells in order to inhibit SIVmac251 infection

A. **B.**

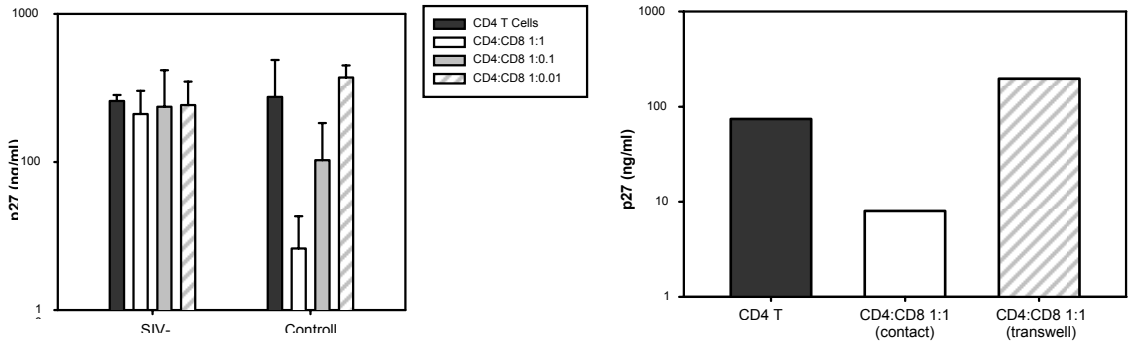
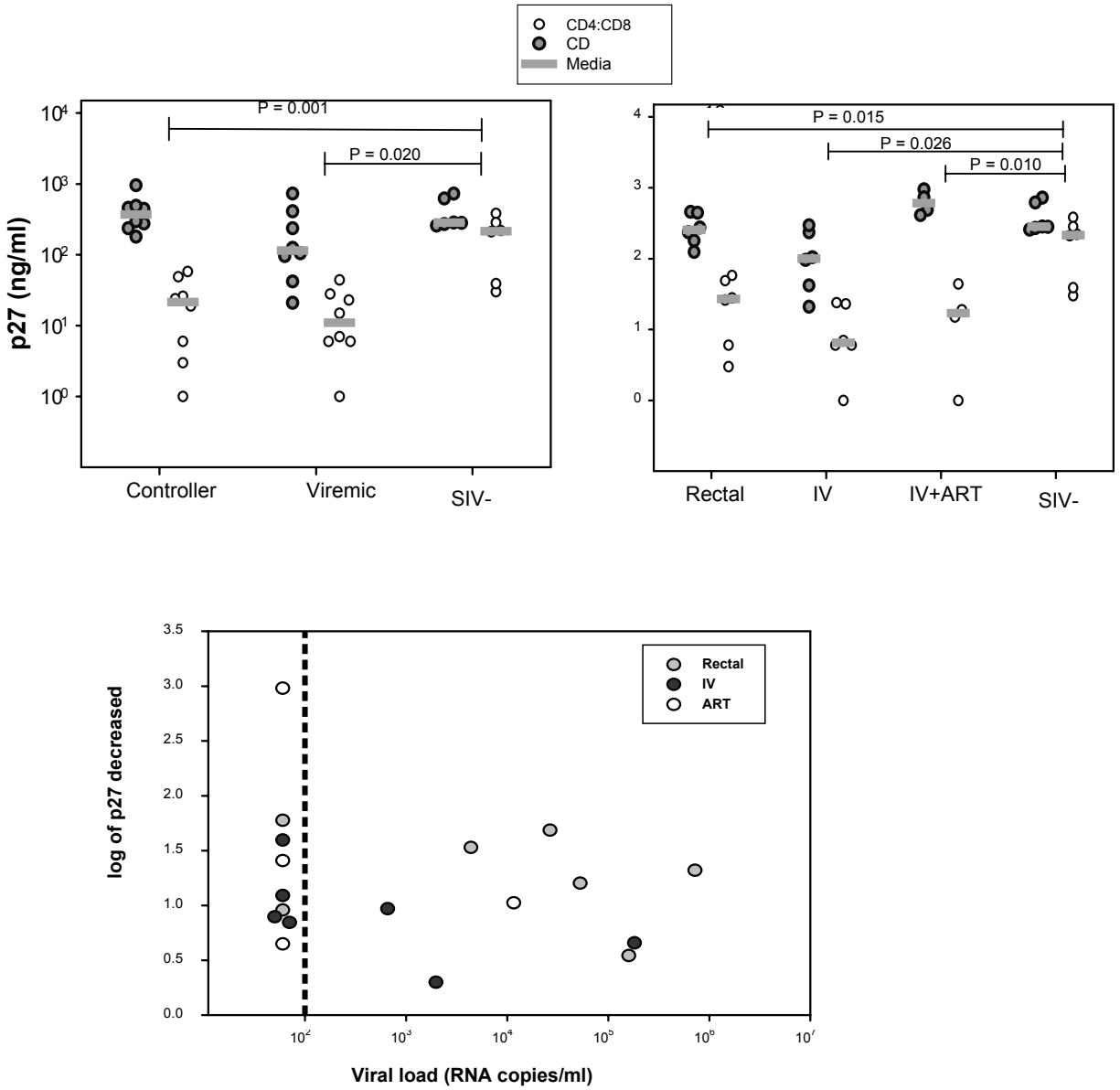


Figure18. Capacity of CD8+ T cells to inhibit SIV infection is unrelated to viral load or route of infection



A. Conclusion

Our results suggest that potent antiviral capacity of circulating CD8+ T cells is not determinant in the spontaneous control of viremia observed in the early phase of chronic infection in this group of SIV controllers. Mechanisms of control may differ from those in human HICs, who exhibit higher constitutive CD8+ T cell anti-HIV capacity than viremic patients. Other mechanisms such as an efficient restraint of SIV infection in the lymphoid tissues might account for the control of SIV infection.

V. Discussion

I have shown here that the HIV-suppressive capacity of CD8+ T cells from most of the HIV controllers is stable over time and is associated with the magnitude of HIV-specific CD8+ T cell responses, and in particular to those directed against Gag. We also have identified a group of HICs who carry infectious viruses and are able to durably control HIV infection despite a weak HIV-suppressive capacity of their CD8+ T cells.

Most of the HIC subjects in our study (14/19) had CD8+ T cells with marked and stable HIV suppressive capacities (strong responder HICs, p24 log decrease >2), that we have never observed in viremic [13] or HAART-treated individuals. The protective HLA alleles B27 and/or B57 were present in all strong responder HICs. However, CD8+ T cells from a subgroup of HICs, here called weak responder HIC, had only weak HIV-suppressive capacity. In agreement with recent reports [45, 47], the HICs I have studied had heterogeneous levels of HIV-specific CD8+ T cells, as estimated by the frequency of IFNγ-producing CD8+ T cells. The magnitude of the HIV-specific CD8+ T cell response correlated strongly with the capacity of CD8+ T cells from HICs to control HIV infection of autologous CD4+ T cells in vitro. Accordingly, the lowest frequencies of IFNγ-producing CD8+ T cells were found in weak responder HICs.

Some underestimation of the CD8+ T cell response in HICs may come for the use of peptides derived from consensus sequences for elispot determinations, or of a lab-adapted HIV strain for HIV-suppression analyses. However CD8+ T cells from weak responder HICs had limited suppressive capacity even when autologous viruses were used, which further evinced a truly weak CD8+ T cell response in these individuals. We cannot exclude that control of viremia in weak responders may be due to robust HIV-specific CD8+ T cell responses in lymphoid tissue, and actually Ferre and collegues have recently shown that HICs have polyfunctional HIV-specific T cell

responses in rectal mucosa that were frequently stronger than in blood.[61] However, the presence in this study of a few HICs with very weak responses both in the blood and in the rectal mucosa is interesting. Although a weak high quality CD8+ T cell resonse might be sufficient to control viremia in vivo, it seems unlikely to be the case in weak responder HICs. The absence of viral blips during the follow-up of weak responders and our finding that at least some of these HICs carry viruses that are highly infectious in vitro and readily detectable upon in vitro activation endorse the idea of a very tight and active host-restraint of HIV-1 infection. Our phenotypical analyses of the HIV-specific CD8+ T cells in weak responders showed an increased proportion of a CD27+CD45RA+ subset of cells, previously observed in patients treated during primary HIV infection, and that might represent a quiescent and stable memory pool able to proliferate and acquire effector capacities upon Ag stimulation[50]. Although these cells may provide an effective response in the eventuality of viral replication, their increased proportion in weak responder HICs together with the low expression of HLA-DR suggest a long period without antigenic stimulation of the CD8+ T cell response.

Therefore, an alternative mechanism is probably responsible for controlling HIV-1 in these HICs. The lower antiviral activity of CD8+ T cells in weak responder HIcs did not seem to be compensated for by other cell populations within PBMC (e.g., NK cells or γδ T cells), as illustrated by HIV-suppressive experiments where nonstimulated PBMC (depleted of CD4+ T cells), used instead of CD8+ T cells, were also unable to control HIV superinfection of autologous CD4+ T cells (not shown). Interestingly, persistent lack of low-levels of HIV Abs and remarkably low levels of T cell activation[62]. Further virologic studies (such as viral sequencing or determination of tissue viral replication) and the analysis of innate responses and regulatory T cells[63] might help to identify new mechanisms of control in HICs.

Unlike the cells from weak responder HICs, CD8+ T cells from strong responder HICs had a broad capacity to suppress superinfection of their own CD4+ T cells by a

wide range of HIV-1 strains[13], and at least partially, by other lentiviruses. This could be related to the presence of HIV-specific CD8+ T cells targeting epitopes located within highly conserved regions of the virus. Responses against Gag and Nef epitopes together accounted for the bulk of total CD8+ T cell responses in strong responder HICs, and no phenotypic differences were observed between Gag-specific and Nef-specific CD8+ T cell responses in these individuals[13]. Interestingly, I have observed a strong correlation between the HIV-suppressive capacity of CD8+ T cells in strong responder HICs and the number of Gag-specific CD8+ T cell reponses. Moreover, the analysis of the relative HIV-suppressive capacity of the Gag response in three strong responder HICs show that, for all three HICs, Gag-specific CD8+ T cells possess the strongest antiviral capacities. Thus, Gag responses appear to be strongly involved in the antiviral potency of CD8+ T cells. This is in agreement with a report showing evidence of CD8+ T cell selective pressure on gag in HICs[64]. Increasing evidence suggests that Gag-specific CD8+ and CD4+ T cell responses are associated with better control of HIV viremia[37-39, 45, 65]. Gag epitopes are presented on the surface of infected CD4+ T cells early after viral entry, before DNA integration and viral protein synthesis[66], and this might allow Gag-specific CD8+ T cells to recognize and eliminate infected cells before the infection is properly established and before Nef-mediated down-regulation of MHC class I molecule occurs[67]. Other factors such as functional avidity[65, 66] or lytic granule loading might contribute to an enhanced HIV-supressive capacity of Gag-specific CD8+ T cells.

No correlation was found between HIV-suppressive capacity of CD8+ T cells in strong responder HICs with Nef-specific CD8+ T cell responses. However, this observation does not preclude a contribution of responses targeting Nef (or other viral proteins) to the HIV-suppressive capacity of CD8+ T cells. Actually, our experiments of selective depletion of HIV-specific cell fractions showed variable capacities (from strong to none) of Nef-specific CD8+ T cells from HICs to suppress HIV infection, perhaps depending on the frequency of the Nef reponses that were targeting epitopes restricted by HLA-B57. Along these lines, escaping mutations are also found in Nef epitopes in HICs, although less frequently than in Gag epitopes[68].

Escaping mutations in epitopes located within structurally important regions of the virus could limit the capacity of the virus to mutate in order to escape immune pressure, as variations in these regions have a fitness cost [43, 69]. Although I did not directly address this issue, the difficulties to detect HIV-1 replication in the culture supernatants of activated CD4+ T cells from strong responder HICs might reflect the impact of the pressure exerted by CD8+ T cell responses on viral fitness. I cannot exclude, nevertheless, that, given the extraordinary antiviral potency of CD8+ T cells from strong responder HICs, the few remaining CD8+ T cells in the >97% pure CD4+ T cell fractions used in these experiments were enough to efficiently suppress autologous virus replication.

Several important questions await answers; that is, mainly whether the potent CD8+ T cell reponse observed in most HICs precedes or follows initial viremic control, and how such a potent CD8+ T cell response is maintained. The association presented here between blips in plasma viral RNA and stronger CD8+ T cell responses in HICs must be considered with care because of the limited number of weak responder HICs, but it is tempting to speculate that CD8+ T cell control of HIV might involve a feed back mechanism whereby occasional blips (or ongoing low-level viral replication) are needed to boost the antiviral response. The increased telomerase activity in these cells would further ensure their persistence[70]. Two scenarios can be envisaged: 1) if viremia is controlled by a common mechanism in weak and strong responder HICs, the presence of the protective HLA B27 and B57 alleles may help to sustain control over time, in the eventuality of viral escape, through the establishment of a robust CD8+ T cell response; 2) different mechanisms are responsible for initial control of HIV infection in weak and strong responder HICs. Detailed longitudinal studies of HICs will be necessary to answer these questions.

My results suggest that the remarkable HIV-1 suppressive activity of HIC $CD8^+$ T cells is due to a cytotoxic mechanism restricted by MHC class I molecules[13].

Two main mechanisms of cytotoxicity have been described in the literature [71]: cytotoxicity through (i) degranulation of perforin and granzyme; and (ii) Fas/FasL apoptotic pathway. I have further explored these mechanisms by:

1) Inhibiting degranulation or perforin production by $CD8^+$ T cells with colchicine or other suitable inhibitor.

These experiments were previously unfeasible using p24 ELISA for quantifying HIV replication because of the duration of the monitoring (14 days) and the cell toxicity of all available inhibitors. The intracellular p24 determination that I have set up requires much less time (24-48 hours) and provides therefore favorable conditions to develop these kinds of experiments. This experiment is currently under way.

2) Inhibiting the Fas/FasL apoptosis pathway with anti-Fas neutralizing antibodies

I have tried to block the CD95 on the infected CD4+ T cells using monoclonal antiCD95(Fas) antibody in order to determine if the blocking of the Fas/FasL apoptosis pathway abrogates the control of HIV replication mediated by the CD8+ T cells from HICs. Unfortunately, this experiment failed to yield the desired results.

I also have tried to investigate the exhaustion of CD8+ T cells in HICs by:

1) Anlaysis of antigenic driven exhaustion of CD8+ T cells

Functional capacity of HIV-specific CD8+ T cells is directly affected by HIV infection. Generally, during chronic infection these cells have an impaired capacity to produce cytokines and to proliferate and are more prone to apoptosis [72]. The functional impairment of HIV-specific CD8+ T cells in HIV viremic patients is thought to be due to T cell exhaustion by chronic antigen stimulation what may be reflected by their high expression of PD-1[73]. In contrast, PD-1 expression by HIV-specific CD8+ T cells is low in HICs [74] and functionality is preserved in these cells.

In order to evaluate whether HIV-specific CD8+ T cells from HIC are less susceptible to exhaustion driven by antigenic stimulation I have tried assesse the HIV-1 suppressive capacity of CD8+ T cells at different time-points after priming the cells with optimal HIV-1 peptides inducing CD8+ T cell response. However, this experiment was currently under set up. Furthermore, the restoration of CTL response by blocking PD-1/PD-L pathway in viremic patients didn't quite work due to the consistent low level of superinfection in the antibody treated group.

2) Determination of phosphorylation of FoxO3a in HIC CD8+ T cells

If a reduced susceptibility of HICs cells to antigenic exhaustion is found, this could be caused by an enhanced resistance of these cells to undergo apoptosis. In this regard, it has been recently shown that elevated levels of phosphorylated forkhead box03a FoxO3a might account for resistance of central memory CD4+ T cells to apoptosis and their enhanced survival [75]. FoxO3a is a phosphoprotein whose activity is regulated through phosphorylation. When dephosphorylated it is translocated into the nucleus and leads to the expression of proapoptotic targeted genes. Phosphorylation of FoxO3a is in part driven by TRC signaling [75], and I hypothesized that HIV specific CD8+ T cells in HIV controllers might possess favorable TCR that provide relevant survival signals. Interestingly the levels of pFoxO3a have been reported to be stronger in the CD4+ T cells from HIV controllers than in viremic patients independently of their maturation phenotype (Sekaly, R.P., oral communication, 2007, Institut Pasteur, Paris). Therefore I have tried to analyze by western blot the levels of unphosphorylated and phosphorylated pFOXO3a in the CD8+ T cells from HICs and controls before and after the antigenic stimulation with optimal peptides. However, this trial had been abandoned due to the very low amount of (mostly undetectable) pFOXO3a in the CD8+ T cells.

On the other hand, suppressive capacity of the CD8+ T cells in controlling HIV-1 infection could be used as a surrogate marker in evaluating efficacy of CD8+ T cells since i) this directly reflects the CTL response and ii) the viral suppression assay could differentiate the effective CD8+ T cell function of HIV controllers from uneffective CTL function of viremics patients. In this regard, I have also developed a rapid, and convenient virus “suppression” assay, which measures the capacity of CD8+ T cell mediated HIV suppression quantitatively, with small number of cells with relatively low cost. By this method, the antiviral capacity of circulating CD8+ T cells can be measured in 72 hours using less than 1~2 million PBMCs. This assay could be used as a surrogate marker in evaluating vaccine efficacy of HIV specific responses in vaccine trials.

Finally I have investigated the possibility to set up an animal model for studying HIV controllers by using cynomolgous macaques infected with SIVmac251. Unlike the human counter-parts, the capacity of *ex vivo* CD8+ T cells to inhibit SIVmac251 infection of autologous CD4+ T cells was no different in controllers than in viremic macaques. The inhibitory capacity of CD8+ T cells was not related to the infection route either. This suggests that in SIV controllers, potent virus suppression capacity by *ex vivo* CD8+ T cells is not necessarily needed in the control of virus. Although I have not observed significant differences between the SIV suppressive capacity of circulating CD8+ T cells from SICs and viremic or ART macaques, what suggest that these cells are not responsible alone of the spontaneous control of SIV infection in these SICs, we don't have any information on how it is on lymphoid tissues. A preliminary observation from Bruno Vaslin's group suggests an inverse relationship between the presence of SIV specific lymphocytes in lymph nodes and lower plasma viral loads [60]. This might imply that control in SICs may be efficiently achieved in lymphoid tissues. Therefore whether CD8+ T cells from lymph nodes from SICs possess an enhanced capacity *ex vivo* to control SIV infection should be investigated.

VI. Perspectives

This study will reveal important parameters of the function of the CD8+ T cell response in HIV Controllers. One of the most direct applications of our finding could be the quantification of the spontaneous capacity of CD8+ T cells to suppress HIV-1 infection as readout of induction of effective responses in subjects from vaccination programs. Intracellular p24 assay, which I have set up, could be used as a quantitative assay to measure the efficiency of novel vaccines in less than 72 hours. Although our current results on the SIV controllers reveal some differences with the HIV controllers, a deeper study of the characteristics of their CD8+ T cell response in lymphoid tissues will certainly provide new insight on the role of the $CD8^+$ T cell response in the control of SIV/HIV infection. Furthermore, since a longitudinal study of HIV controllers is hard to be done due to reasons such as extreme rarity of HIC patients and the lag time between the diagnosis and their identification, cross-sectional studies at different time points of other cohorts of patients will be done. In particular, patients from the ANRS "PRIMO" cohort (patients followed-up since the early times post infection, n~750) will be included in this study. If we find out that patients from PRIMO cohort who have low or undetectable plasma viral loads share peculiar characteristics of their CD8+ T cell response with HIV Controllers, this could give us valuable information on the early stages of HIV-1 infection in these patients and the evolution of an effective anti-viral immune response.

There has not been a through investigation on HIV controllers in East Asian region, including Korea. In fact, most of the reports are from several selected countries, such as the United States, and some European countries. It has been suggested that there is an HLA-dependent mechanism suggested by over-representation of 'protective' class I alleles, such as B*27 and B*57. However, in East Asia, particularly in South Korea and Japan, the frequency of B*57 in normal population is very low (0.2% in South Korea, 0.8% in Japan) compared to ~3% in Europe or U.S. Moreover, HLA B*2101, which is related to the rapid disease progression is relatively high 5.9% in South Korea, 8.8% in Japan) compared to very low frequencies in other ethnicity. In fact, in

the study of HLA-B polymorphism in Japanese HIV-1 infected long-tern surviving hemophiliacs, there was no increased frequency in the protective alleles such as HLA-B*5701; B*5801, and B27 but in HLA B*1507 and decreased frequency in B*5401. [76]The different genetic background in East Asian region could result in group of HIV controllers with different virus control mechanisms. To understand the actual mechanism of virus control in our region could give us valuable information to further applied to the vaccine development.

VII. Conclusion

1. My results suggest that *ex-vivo* capacity of CD8+ T cells to suppress HIV-1 infection is peculiar to HIV controllers (HIC). Some HICs are able to tightly control the virus with relatively poor HIV-suppressive CD8+ T cell capacities suggesting other mechanism of virus control. CD8+ T cell mediated suppressive capacity in SR HICs correlated strongly with the frequency of Gag-specific CD8+ T cells.

2. I have developed a rapid, and convenient form of viral suppression assay which could measure the effective anti-viral CD8+ T cell response. This novel method could be used as a new surrogate marker for vaccine studies. This was directly applied in the collaborative study to evaluate the role of avidity in HIV-1 CD8+ T cell clones in the viral suppression.

3. Unlike in HIV controllers, antiviral capacity of circulating CD8+ T cells is not determinant in the spontaneous control of viremia observed in the early phase of chronic infection in SIV controllers. Understanding the mechanisms of virus control in these individuals might explain the mechanisms of virus control underlying HICs.

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초록 (국문)

«HIV controller»의 CD8+ T cell 매개 바이러스 억제 기전

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HIV controller 란 HIV 에 감염된 후 항레트로바이러스제 치료없이 장기간 (>10 년) 동안 바이러스를 표준 측정방법으로는 측정 불가능한 수준으로 (<50 copies RNA/ml) 조절하는 매우 희귀한 인구집단을 의미한다. 최근 HIV 백신개발에 대한 접근방법은 HIV 바이러스의 감염 자체를 예방하는 것에서 감염된 바이러스를 조절하여 AIDS 의 발병을 늦추고 바이러스의 이차 감염을 막는 방향으로 전환되고 있으며, 이러한 접근방법은 백신접종자에게서 HIV controller 의 임상 경과와 유사한 경과를 유도하는 것이다. 따라서, 이러한 HIV controller 의 바이러스 조절 기전을 규명하는 것은 HIV 백신 및 면역치료의 개발에 있어서 매우 중요한 기초 연구가 될 것이다. 이에 저자는 HIV controller 에서 CD8+ T 세포 매개 HIV 조절기전에 대해 연구하기로 하였다.

HIV controller 에서의 바이러스 억제기전: *ex vivo* CD8+ T 세포에 의한 강력하고 자발적인 anti-HIV activity

본 연구는 ANRS HIV controller observatoires 를 통해 모집된 HIV controller 중 19 명의 HIV controller 를 대상으로 CD8+ T cell 매개 HIV 억제기전에 대해 알아보기로 하였다. 그 결과 대부분의 HIV controller 의 경우 *ex vivo* CD8+ T 세포에 의한 강력하고

자발적인 anti-HIV activity 가 존재함을 알 수 있었으며 (Strong responder HIV controllers), 이는 지속적이고 다른 렌티바이러스에도 작용하였다.

특징적으로, 이 anti-viral activity 는 Gag-특이 CD8+ T cell 의 frequency 와 강한 연관성을 보였다. 한편, 일부 HIV controller 의 경우 highly replicative virus 에 감염되었고, 비교적 낮은 CD8+ T cell 매개 HIV 억제능을 가지고도 강력하게 바이러스를 조절하는 양상을 보여 또 다른 기전이 존재함을 시사하였다. (Weak responder HIV controller).

Intracellular p24 assay 의 개발

현재까지 HIV 특이 CD8+ T 세포 반응을 측정하기 위한 측정방법으로는 정량적 ELISPOT assay 나 Intracellular cytokine staining 등이 사용되고 있다. 그러나 이러한 방법들로 CD8+ T 세포 자체의 기능을 정확히 측정하는 데에는 한계가 있다. 이에 저자는 빠르고 편리한 바이러스 억제 assay 인 Intracellular p24 assay 를 개발하기로 하였다. Intracellular p24 assay 는 superinfection 된 CD4+ T 세포의 HIV 감염을 *ex vivo* 자가 CD8+ T 세포가 억제하는 능력을 정량적으로 측정할 수 있으며, HIV controller 의 효율적인 CTL 반응과 Viremic 감염자의 비효율적인 면역반응을 감별할 수 있다는 점에서 IFN-ɣ 생성을 측정하는 ELISPOT assay 에 비해 더 정확한 방법이라 할 수 있다. 추후 Intracellular p24 assay 는 HIV 백신연구에서 효율적인 HIV 특이면역반응을 측정하는 surrogate marker 로 사용될 수 있을 것이다.

HIV controller 를 연구하기 위한 동물 모델 구축 - SIV 감염 짧은꼬리 원숭이 모델

HIV controller 의 연구에는 인구집단의 희소성, 초기 감염시기의 임상정보의 부재, 조직 샘플을 얻기가 어려운 점등의 난제가 있다. 이러한 점을 극복하기 위해 동물모델의 필요성이 대두되었으며 이에 저자는 CEA 의 Bruno Vaslin 연구팀과의 collaboration 을 통해 SIV controller (SIV 에 감염된 짧은 꼬리 원숭이 중 일정기간 이상 동안 바이러스를 <100 copies/ml 로 조절하는 개체)에서의 virus 조절 기전에 대해 알아보고 이 모델이 HIV controller 연구를 위한 동물모델로써의 가능성을 알아보기로 하였다. 그 결과 SIV controller 의 경우 HIV controller 의 경우와 달리 혈중 CD8+ T 세포에 의한 anti-HIV activity 는 *in vivo* SIV 조절능과 크게 관련이 없음을 알 수 있었다.

결론적으로 저자는 대부분의 HIV controlle 에서는 강력한 CD8+ T cell 매개 바이러스 억제능이 존재하며 (strong responder) 이는 gag-특이 CD8+ T cell 의 frequency 와 강한 연관성을 보여 HIV controller 에서 gag response 가 CD8+ T cell 의 바이러스 억제능에 매우 중요함을 밝혔다. 또한 이러한 결과를 바탕으로 빠르고 편리한 바이러스 억제 assay 인 Intracellular p24 assay 를 개발하였다. SIV 감염 짧은꼬리 원숭이 모델 연구에서는 HIV controller 와 달리 혈중 CD8+ T 세포에 의한 anti-HIV activity 는 *in vivo* SIV 조절능과 크게 관련이 없음을 알 수 있었으며 이는 비교적 낮은 CD8+ T cell 매개 HIV 억제능을 갖고도 강력하게 바이러스를 조절하는 일부 HIV controller (weak responder)에서의 바이러스 억제기전을 밝히는데 도움이 될 것으로 생각된다.

핵심되는 말: 인간면역결핍바이러스, CD8+ T 세포, 바이러스 억제기전, 원숭이 면역결핍바이러스

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