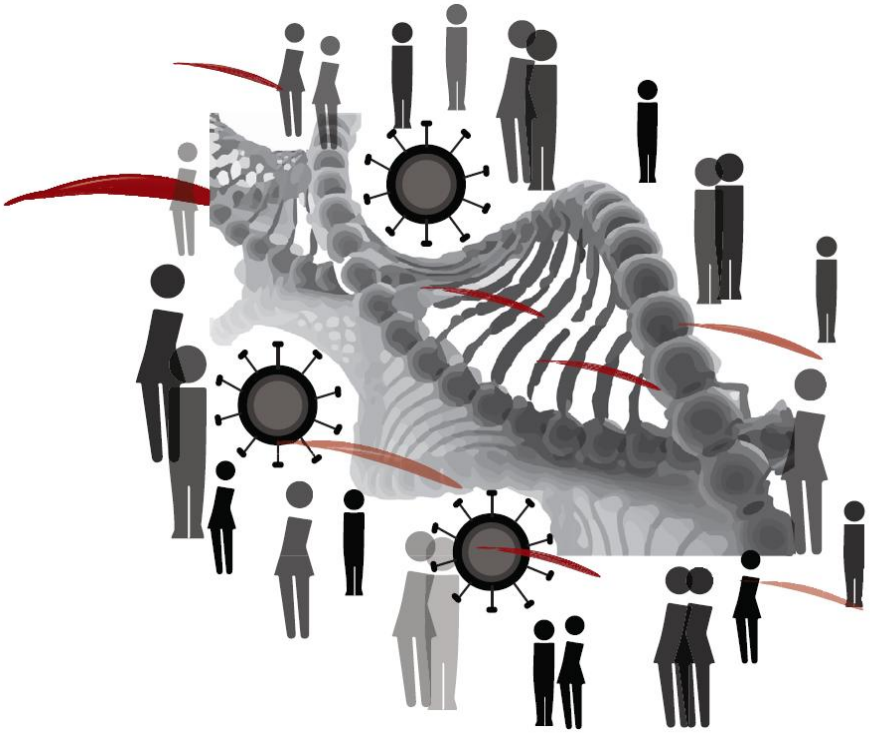
DOCTORAL THESIS 2014

Viral and host factors involved in rapid HIV-1 disease progression

Judith Dalmau Moreno

Institut de Recerca de la Sida – IrsiCaixa



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Departament de Bioquímica i Biologia Molecular

Programa de Doctorat en Bioquímica, Biologia Molecular i Biomedicina

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Viral and host factors involved in rapid HIV-1 disease progression

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Doctoral Thesis UAB 2014

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Certifica:

Que el treball experimental realitzat i la redacció de la memòria de la Tesi Doctoral titulada *"Viral and host factors involved in rapid HIV-1 disease progression"* han estat realitzats per na Judith Dalmau Moreno sota la seva direcció i considera que és apte per a ser presentat per a optar al grau de Doctor en Bioquímica, Biologia Molecular i Biomedicina per la Universitat Autònoma de Barcelona.

I per tal que en quedi constància, signa aquest document a Badalona, 30 de Gener del 2014.

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I per tal que en quedi constància, signa aquest document a Bellaterra, 30 de Gener del 2014.

Dr. Josep Vendrell Roca

Als meus pares, el meu

germà i tota la gent que estimo

"Viu com si anessis a morir demà.

Aprèn com si fossis a viure per sempre".

- Mahatma Gandhi

SUMMARY

Remarkable variation in clinical outcome can be observed following HIV-1 infection. While some HIV-1–infected individuals are able to suppress viral replication to very low levels (<2000 copies/mL) and/or maintain high $CD4^+$ T-cell counts over many years without antiretroviral therapy (HIV controllers), others quickly progress to AIDS or meet the current criteria for antiretroviral treatment within the first 3 years after primary infection (rapid progressors, RP). Furthermore, a minority of highly viremic individuals remain asymptomatic with high $CD4^+$ T-cell counts (viremic non-progressors, VNP), similar to those observed in the non-progressive disease model of SIV infection in natural hosts.

The study of extreme phenotypes can provide important information onthe interactions established between the viral variant and the host during primary HIV infection and the subsequent clinical evolution of the infection. Indeed, the study of HIV controllers (including elite controllers, who maintain plasma viral RNA under detectable levels [<50 copies/mL]) is providing relevant data on HIV immunopathogenesis. The other extreme comprises RPs, who account for a relatively small percentage of the HIV-1-infected population. Nevertheless, the implications of their immunogenetic and immunopathogenic characteristics are remarkable.

This thesis originates from the comprehensive study of 2 cases of extremely severe rapid progression that was later extended to the recruitment and study of an unprecedentedly large and well-defined cohort of RPs. The specific objective was to investigate a wide range of viral and host factors involved in rapid progression of HIV-1 infection, in contrast to other phenotypes, namely,individuals with an average progression profile (standard progressors, SP) and VNPs.

The results of this study demonstrate convergence of the viral and host factors contributing to the clinical severity of rapid progression. Individuals infected with highly replicative, dual-tropic, HLA-adapted viruses were shown to be more prone to develop AIDS-defining symptoms during primary HIV-1 infection, since in many cases they are also unable to mount humoral and cellular HIV-1-specific immune responses. Concordant HLA supertypes between the source and the recipient, the presence of common and risk HLA class I alleles, and the low frequency of protective HLA alleles were also shown to further accelerate disease progression. In addition, transcriptome analysis revealed that RPs have a specific $CD4^+$ and $CD8^+$T-cell transcriptome profile similar to that observed in pathogenic SIV-infected rhesus macaques and characterized by higher expression of interferon-stimulated genes. VNPs, on the other hand,were characterized by a gene regulation profile similarto that of non-pathogenic SIV-infected sooty mangabeys.

The present study provides important insights into the host and viral traits driving progression of HIV-1 infection, which have relevant implications for our knowledge of HIV pathogenesis and the importance of early monitoring of disease course.

RESUM

Els éssers humans mostren una notable variació en el desenvolupament clínic rere la infecció per VIH-1. Si bé algunes persones amb VIH-1 són capaces de suprimir la replicació viral del VIH a nivells molt baixos (<2000 còpies/ml) i/o mantenir els recomptes de CD4 alts durant molts anys en absència de teràpia antiretroviral (Controladors), altres progressen ràpidament a sida o compleixen els criteris actuals per a iniciar tractament antiretroviral en els 3 primers anys rere la infecció primària (Progressors ràpids, RP). D'altra banda, una minoria d'individus amb alts nivells de virèmia roman asimptomàtica i manté els recomptes de cèl·lules T $CD4^+$ elevats (Virèmics no progressors, VNP), similars als observats en el model no progressiu d'infecció per SIV en l'hoste natural.

L'estudi de fenotips extrems pot donar informació rellevant en termes de les interaccions que s'estableixen entre el virus i l'hoste durant la infecció primària pel VIH, així com de l'evolució clínica posterior a la infecció. De fet, l'estudi dels controladors (incloent els controladors d'elit, que mantenen l'ARN viral a nivells indetectables) està proporcionant dades rellevants de la immunopatogènesi de la infecció. L'extrem oposat són els RPs, els quals representen un percentatge relativament petit de la població infectada per VIH-1.No obstant això, les implicacions de les seves característiques immunogenètiques i immunopatogèniques són notables .

Aquesta tesi té el seu origen en l'estudi exhaustiu de 2 casos de progressió extremadament severa i ràpida, que posteriorment es va estendre a la creació i l'estudi d'una cohort gran i ben definida de RPs sense precedents. L'objectiu específic va ser investigar una àmplia gamma de factors virals i de l'hoste implicats en la progressió ràpida de la infecció per VIH-1, en comparació amb altres fenotips, incloent individus amb un perfil estàndard de progressió (progressors estàndard, SP) i VNPs.

Els resultats d'aquest estudi demostren la convergència de factors virals i de l'hoste que contribueixen a la gravetat clínica de la progressió ràpida. Les persones infectades amb virus altament replicatius, dual-tròpics i HLA-adaptats van mostrar ser més propenses a desenvolupar símptomes definitoris de SIDA durant la infecció primària per VIH-1, donat que en molts casos tampoc no són capaces de produir respostes immunitàrieshumorals i cel·lulars específiques contra VIH-1. La concordança de supertipus d'HLA, la presència d'al·lels d'HLA comuns i d'al·lels de risc, i la baixa freqüència d'al·lels protectius, també van mostrar associació amb l'acceleració de la malaltia. A més, l'anàlisi del transcriptoma va revelar que els RPs tenen un perfil transcriptòmic específic a les cèl·lules T $CD4^+$ i $CD8^+$, similar a l'observat en la infecció patogènica per SIV en rhesus macacs, i caracteritzat per una major expressió de gens estimulats per l'interferó. Els VNPs, en canvi,es caracteritzen per un perfil de regulació de gens similar a la infecció no patogènica per SIV dels sooty mangabeys.

El present estudi proporciona informació rellevant sobre les característiques virals i de l'hoste implicades en la progressió ràpida de la infecció per VIH-1, la qual té implicacions destacables en el nostre coneixement de la patogènesi del VIH-1 i en la importància de la monitorització primerenca de l'evolució de la malaltia.

RESUMEN

Los seres humanos muestran una notable variación en el desarrollo clínico tras la infección por VIH-1. Si bien algunas personas son capaces de suprimir la replicación viral del VIH a niveles muy bajos (<2.000 copias/ml) y/o mantener los recuentos de CD4 altos durante varios años en ausencia de terapia antirretroviral (Controladores), otras progresan rápidamente a SIDA o cumplen los criterios de inicio de tratamiento antirretroviral en los 3 primeros años tras la infección primaria (Progresores rápidos, RP). Por otra parte, una minoría de individuos con altos niveles de viremia permanece asintomática y mantiene los recuentos de células T $CD4^+$ elevados (Virémicos no progresores, VNP), similares a los observados en el modelo no progresivo de infección por SIV en el huésped natural.

El estudio de fenotipos extremos puede dar información relevante en términos de las interacciones que se establecen entre el virus y el huésped durante la infección primaria por el VIH, así como de la evolución clínica posterior a la infección. De hecho, el estudio de los controladores (incluyendo los controladores de élite, que mantienen el ARN viral a niveles indetectables) está proporcionando datos relevantes de la inmunopatogénesis de la infección. El extremo opuesto son los RPs, los cuales representan un porcentaje relativamente pequeño de la población infectada por VIH-1. Sin embargo, las implicaciones de sus características inmunogenéticas y inmunopatogénicas son notables.

Esta tesis origina del estudio exhaustivo de 2 casos de progresión extremadamente severa y rápida, que posteriormente se extendió a la creación y el estudio de una cohorte grande y bien definida de RPs sin precedentes. El objetivo específico fue investigar una amplia gama de factores virales y del huésped implicados en la progresión rápida de la infección por VIH-1, en comparación con otros fenotipos, incluyendo individuos con un perfil estándar de progresión (progresores estándar, SP) y VNPs.

Los resultados de este estudio demuestran la convergencia de factores virales y del huésped que contribuyen a la gravedad clínica de la progresión rápida. Las personas infectadas con virus altamente replicativos, dual-trópicos y HLA-adaptados mostraron ser más propensas a desarrollar síntomas definitorios de SIDA durante la infección primaria por VIH-1, dado que en muchos casos tampoco son capaces de generar respuestas inmunitarias humorales y celulares específicas contra el VIH-1. La concordancia de supertipos de HLA, la presencia de alelos de HLA comunes y de alelos de riesgo, y la baja frecuencia de alelos protectivos, también mostraron asociación con la aceleración de la enfermedad. Además, el análisis del transcriptoma reveló que los RPs tienen un perfil transcriptómico específico en células T $CD4^+$ y $CD8^+$, similar al observado en la infección patogénica por SIV en rhesus macacos, y caracterizado por una mayor expresión de genes estimulados por interferón. Los VNPs, en cambio, se caracterizan por un perfil de regulación de genes similar alde la infección no patogénica por SIV del sooty mangabey.

El presente estudio proporciona información relevante sobre las características virales y del huéspedimplicadas en la progresión rápida de la infección por VIH-1, la cual tiene implicaciones destacables en nuestro conocimiento de la patogénesis del VIH-1 y en la importancia de la monitorización temprana de la evolución de la enfermedad.

ABBREVIATIONS

Ab	Antibody
AIDS	Acquired immunodeficiency syndrome
APC	Antigen presenting cell
ART	Antiretroviral therapy
BL	Baseline
bNAb	Broadly neutralizing antibody
CCR5	Chemokine receptor 5, also known as CD195
CD4	Cluster of differentiation 4
CMV	Cytomegalovirus
CoRP	Cohort of Rapid Progressors
CRF	Circulating Recombinant Forms
CTL	Cytotoxic T lymphocyte
CXCR4	CXC chemokine receptor 4, also known as CD184
DC	Dendritic cell
DM	Dual/mixed
DNA	Deoxyribonucleic acid
DPS	Deep Pyrosequencing
EBV	Epstein-Barr virus
EC	Elite controller
ELISA	Enzyme-linked immunoSorbent assay
Env	HIV envelope glycoprotein
FDR	False discovery rate
GALT	Gut associated lymphoid tissue
GI	Gastrointestinal tract
GWAS	Genome-wide association studies
HAART	Highly active antiretroviral therapy
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen
HPC	Hematopoietic progenitor cells
IC50	Half maximal inhibitory concentration
IFN	Interferon
Ig	Immunoglobulin
IL-2	Interleukin-2
IQR	Interquartile range
IRB	Institutional Review Board
ISG	Interferon-stimulated gene
IVDU	Intravenous drug user

KIR	Killer-cell immunoglobulin-like receptor
Log	Logarithm
LPS	Lipopolysaccharide
LTNP	Long-term non-progressors
LTR	Long terminal repeat
MHC	Major histocompatibility complex
MIP-1α	Macrophage inflammatory protein-1-alpha
ml	Milliliter
mRNA	Messenger RNA
MSM	Men who have sex with men
NAb	Neutralizing antibody
NK	Natural killer
OR	Odd ratio
P	Post-baseline
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PHA	Phytohaemmagglutinin
Pol	HIV polymerase enzyme
RANTES	Regulated on Activation Normal T-cell Expressed and Secreted
RC	Replicative capacity
RNA	Ribonucleic acid
RP	Rapid progressor
RT	HIV reverse transcriptase enzyme
sCD14	Soluble CD14
SFC	Spot forming cells
SHCS	Swiss HIV cohort study
SIV	Simian immunodeficiency virus
SNP	Single nucleotide polymorphism
SP	Standard progressor
STD	Standard deviation
TCR	T cell receptor
TLR	Toll-like receptor
Treg	Regulatory T cell
VC	Cell virus isolates
VL	Viral load
VNP	Viremic non progressor
WT	Wild type

PRESENTACIÓ DE LA TESI

La present tesi doctoral ha estat redactada en un format que inclou una introducció completa amb els coneixements necessaris per a poder interpretar els resultats presentats, quatre capítols de resultats i una discussió global. Els capítols de resultats corresponen a quatre estudis del projecte en què s'engloba aquesta tesi, i a cadascun d'ells s'ha inclòs una petita presentació, una introducció, un apartat amb els materials i mètodes específics de l'estudi i una discussió. El motiu de no haver seguit el format més estàndard és perquè hem considerat que, pel seu estil, aquest treball seria més senzill de seguir i que la seva presentació seria molt més adequada si es seguia aquesta pauta.

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Chapter 1

INTRODUCTION

1. Human Immunodeficiency Virus type 1 and the infection

1.1. History and classification

The first cases of Acquired Immunodeficiency Syndrome (AIDS) were reported in Los Angeles (USA) in 1981 [1], but it was not until 1983 when a new human retrovirus, at the time known as lymphadenopathy-associated virus (LAV) or HTLVIII, was isolated from a lymph node biopsies of AIDS patients and identified as the etiological agent causing the disease [2, 3]. After further confirmation, this virus was finally designated as Human Immunodeficiency Virus (HIV) by the International Committee on the Taxonomy of Viruses [4]. In 1986, a related but less pathogenic virus was discovered in West African patients with AIDS and was named HIV type 2 (HIV-2) while the original virus isolates were termed HIV-1 [5].

HIV belongs to the group VI of reverse transcribing viruses, Retroviridae family, Orthoretrovirinae subfamily, Lentivirus genus, and includes both HIV-1 and HIV-2 species [6]. Despite clear similarities between both viruses, replicative and pathogenic capacity, virus evolution and targets of infection are different between them [7, 8]. The most prevalent virus is HIV-1, which is spread all over the world, while HIV-2 is confined to West Africa [9-11]. HIV-1 is classified into four major groups: M (major), O (outlier), N (new), and P (pending) [12, 13]. M group accounts for the majority of infections and can be divided into clades, which vary by 15-20%: A, B, C, D, F, G, H, J, K. Furthermore, a high number of Circulating Recombinant Forms (CRF) has been identified [14, 15]. Current evidence derived from phylogenetic and statistical analyses indicates that HIV viruses entered the human population around 1910 to 1930 [13, 16, 17] through multiple zoonotic infections from simian immunodeficiency virus (SIV)-infected non-human primates [16, 18]. Specifically, HIV-1 is closely related to SIVcpz isolated from the chimpanzee subspecies *Pan troglodytes troglodytes* [19], whereas HIV-2 is highly associated with SIVsm from sooty mangabeys *Cercocebus atys* [9].

Nowadays, HIV/AIDS is still one of the major causes of death worldwide. World Health Organization estimates that over 25 million people have died of AIDS since

the start of the epidemic and, in 2011, there were approximately 2.5 million new infections and a total of 34 million people living with HIV, the majority of them living in low and middle income countries. In addition, it was estimated that 1.7 million people died of AIDS-related causes (UNAIDS/WHO epidemic update 2011, www.unaids.org, **Fig. 1**).

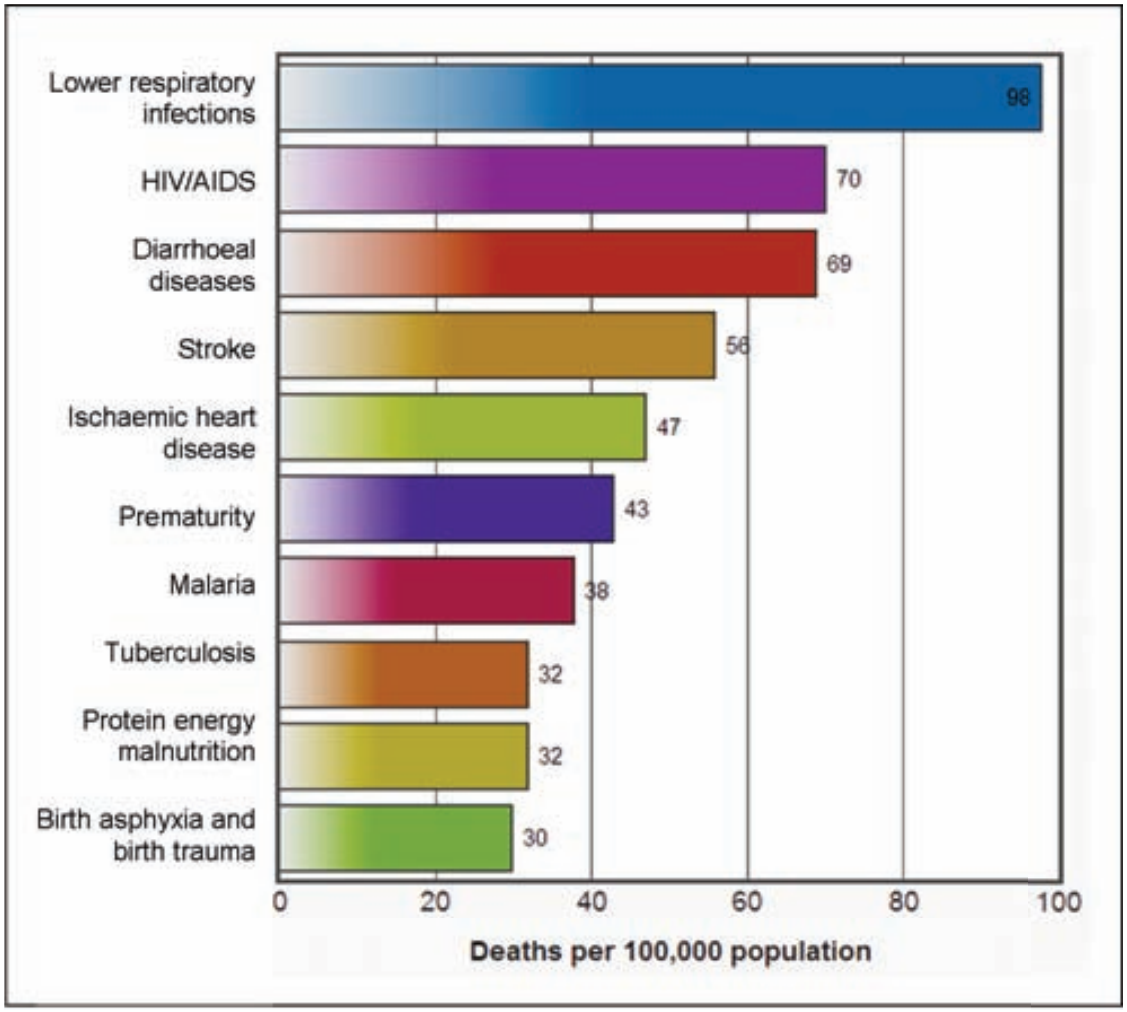

Figure 1. Top 10 causes of death in low-income countries 2011. *From World Health Organization (WHO) update 2011, www.who.int.*

1.2. Transmission, phases and immunopathogenesis

The three main transmission routes of HIV-1 infection are exposure to contaminated blood, sexual contact, and vertical transmission, being the two latter the major causes of viral spread [20]. Of note, more than 80% of HIV-1 infections occur via sexual transmission by exposure of mucosal surfaces to the virus [21]. The earliest events in acute HIV-1 infection determine the future health of each individual patient as well as the extent of transmission in the general population. Interestingly, the appearance of host and viral markers of infection is uniform and ordered, independently of the route of transmission, and replication rapidly converges in the lymphoreticular system of the gastrointestinal tract [22, 23]. During sexual transmission, both cell-free and cell-associated virions cross the mucosal barriers through microabrasions and/or migrating through the intact tissue, reaching quickly DCs and resting $CD4^+$ T cells in the epithelium or lamina propria [24, 25]. $CD4^+$ T cells are then activated by direct contact with DC, which fuel the initial infection [26]. There is a local viral expansion that is necessary to disseminate the infection to the draining lymph node, and subsequently through the bloodstream to establish an infection to secondary lymphoid organs with particular preference for gut associated lymphoid tissue (GALT). Once infection reaches the lymphoid tissues, local conditions favor the access of the virus to a large number of susceptible cells that are in close proximity, thus an exponential increase of viral RNA and infected cells are detected. The rapid expansion of HIV-1, first in the gut and then to the systemic level, are clinically important for the irreversible destruction of T-helper cells and the establishment of viral latency. After exposure, only a small number of viruses contained in the inoculum will be able to cross the mucosal barriers and establish infection. Later on, immune pressure will trigger the appearance of escape mutations, and the founder-virus sequence will be rapidly replaced by a pool of viral variants or quasispecies, which will continue evolving throughout the disease course [27].

After transmission, both humoral and cellular adaptive immune responses are progressively developed and directed against viral antigens during the course of HIV infection. However, the host is not capable of clearing the infection and,

subsequently, there is a progressive loss of the immune function, which in turn allows for the appearance of opportunistic infection and lastly death.

1.2.1. Phases of HIV infection

HIV-1 infection is characterized by a gradual decline in $CD4^+$ T cells, chronic immune activation and subsequent loss of immunological competence, eventually culminating in AIDS. In the 80% of HIV-1-infected individuals, the natural course of HIV-1 infection is characterized by a period of 7.7 to 11 years before clinical manifestations of AIDS occur [28]. Three main phases are distinguished in the HIV-1 infection course, in absence of antiretroviral treatment (**Fig. 2**):

a) **Acute infection**:

Acute infection lasts from 2 to 6 weeks after infection, until anti-HIV-1 antibodies are detectable (seroconversion). The first 7-21 days after transmission are called the 'eclipse phase', due to HIV-1 is still not detected in plasma [26]. From this point, plasma viremia will start an exponential increase and will reach a peak after 21-28 days (10^5-10^7 HIV-1 RNA copies/ml), while a massive depletion of $CD4^+$ T lymphocytes occurs. These high circulating levels of virus will be later partially controlled by immune responses, reducing and stabilizing viremia at a particular setpoint, several weeks after primary exposure (**Fig. 2**, Acute phase). The level of the viral setpoint and the number of blood $CD4^+$ T cells are clinically useful predictors of the progression of the disease.

The majority of recently HIV-1-infected people experience some mononucleosis-like symptoms, such as fever, lymphadenopathy, myalgia, rash and pharyngitis. However, about 30% remain asymptomatic during initial period of infection [29]. Therefore, in the absence of a high degree of clinical suspicion, the symptoms associated with acute HIV-1 infection are often too unspecific to perform a diagnostic. In the absence of seroconversion (Ab), confirmation of acute HIV-1 infection requires detection of RNA or p24 antigen, but so far these tests have not

been routinely available. The use of combo ELISAs (combined antibody and antigen detection) has increased the detection of acute infections.

It is relevant to note that, whereas the amount of circulating T-cells subsequently return close to normal, $CD4^+$ T-cell numbers in the GALT, which contain the majority of body T-cells [30], remain severely and irreversibly reduced [22, 31].

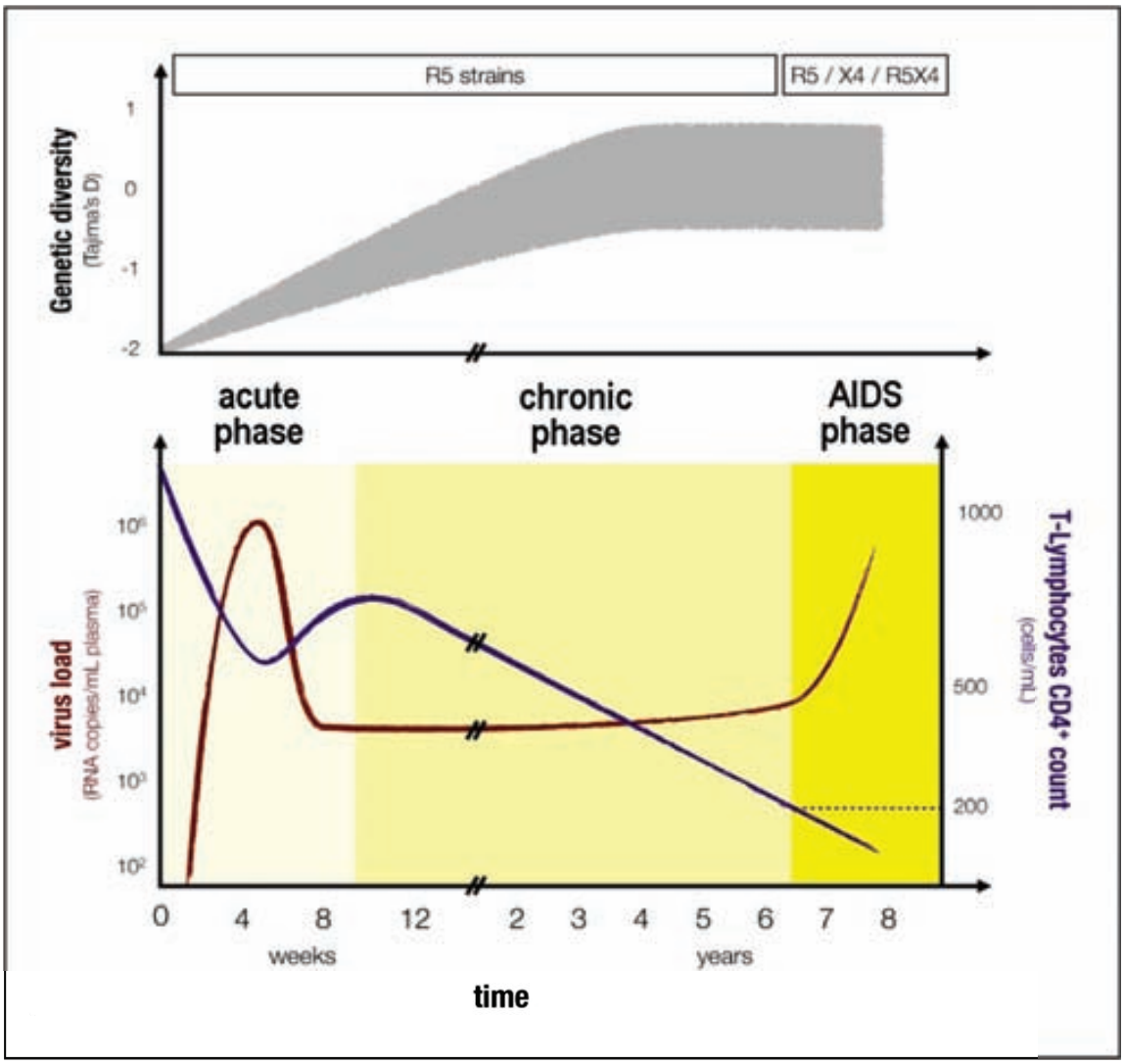

Figure 2. Typical course of HIV-1 infection. The top panel shows the diversity along with the type of HIV variant that dominates during the course of infection. The bottom panel shows the dynamics of the viral load (red), and the $CD4^+$ T-cell counts (blue). *Adapted from Alizon S and Magnus C, Viruses 2012*.

b) **Chronic phase:**

After the initial acute infection, HIV-1 viral load in blood decreases around 100 times, reaching a setpoint that is strongly predictive of the time of clinical disease onset. This second phase is characterized by continuous stimulation of the immune system by repeated exposure to viral antigens and altered cytokine environment. Despite the induction of innate and adaptive immune responses, the virus continues replicating and disseminates into the lymphoid organs, leading to general immune activation and cell death. Additionally, $CD4^+$ T cells are destructed at faster rate that can be produced, thereby shifting the dynamic equilibrium between production and destruction of T cells [32]. Consequently, the number of peripheral $CD4^+$ T cells progressively declines during the latent period (**Fig. 2**, Chronic phase).

In general, after a median of 10 years of infection in absence of antiretroviral treatment, the 50% of HIV-1-infected individuals develop signs of disease, including a marked decrease in $CD4^+$ T cells, lymphoid hyperplasia and impairment of immune functions [32]. The rate of immune progression will depend on multiple viral and host factors.

c) **AIDS:**

The progressive immune deficiency culminates in the development of AIDS. In this stage, the $CD4^+$ T-cell count drops below a critical level (about 200 cells/µl), viral load rapidly rises and immune responses sharply fail. Hence, this scenario enables the onset of opportunistic infections and other clinical components, such as HIV-1-associated cancers, kidney failure and central nervous system degeneration, which eventually results in death (**Fig. 2**, AIDS phase).

1.2.2. Immunopathogenesis of HIV-1 infection

1.2.2.1. CD4$^+$ T-cells homeostasis and depletion

HIV-1 targets different cells of the immune system, seriously compromising immune responses (**Fig. 3**). Its main cellular targets are CD4$^+$ T cells (**Fig. 4**), which are involved in many important pathways of immunity. HIV-1 has important detrimental effects on CD4$^+$ T-cell levels and the proportions of the different subpopulations in blood, lymphoid tissues and other compartments; these effects are caused by different mechanisms, most of them not directly related to HIV-1 infection of the cell *per se*. The most remarkable processes involved in CD4$^+$ T-cell loss are the dysregulation of cellular homeostasis and proliferation, HIV-1 direct cell-killing and bystander associated effects [33]. A brief summary of the mentioned processes is presented below.

CD4$^+$ T-cells homeostasis, the balance between cell production and cell death, is profoundly affected during HIV-1 infection. Thus, HIV-1 infection severely alters this process, reducing the *de novo* production and increasing the destruction of cells. Both mechanisms contribute to the continuous decline of CD4$^+$ T cells observed in untreated HIV-infected subjects. The alterations in cellular generation include an important dysfunction within the hematopoiesis and thymic cellular production. HIV-1 infects hematopoietic progenitor cells [34] and also induces cell-death through viral proteins such as gp120 in the bone marrow. The impairment of hematopoiesis may have devastating effects, since depletion of the earliest progenitor cells could affect all differentiated progeny cells, including granulocytes, myeloid cells, erythrocytes, and T- and B- cells [35]. Furthermore, HIV-1 can inhibit proliferation of immature thymocytes [36] and/or directly infect CD4$^+$ thymocytes, leading to impaired production of CD4$^+$ T cells.

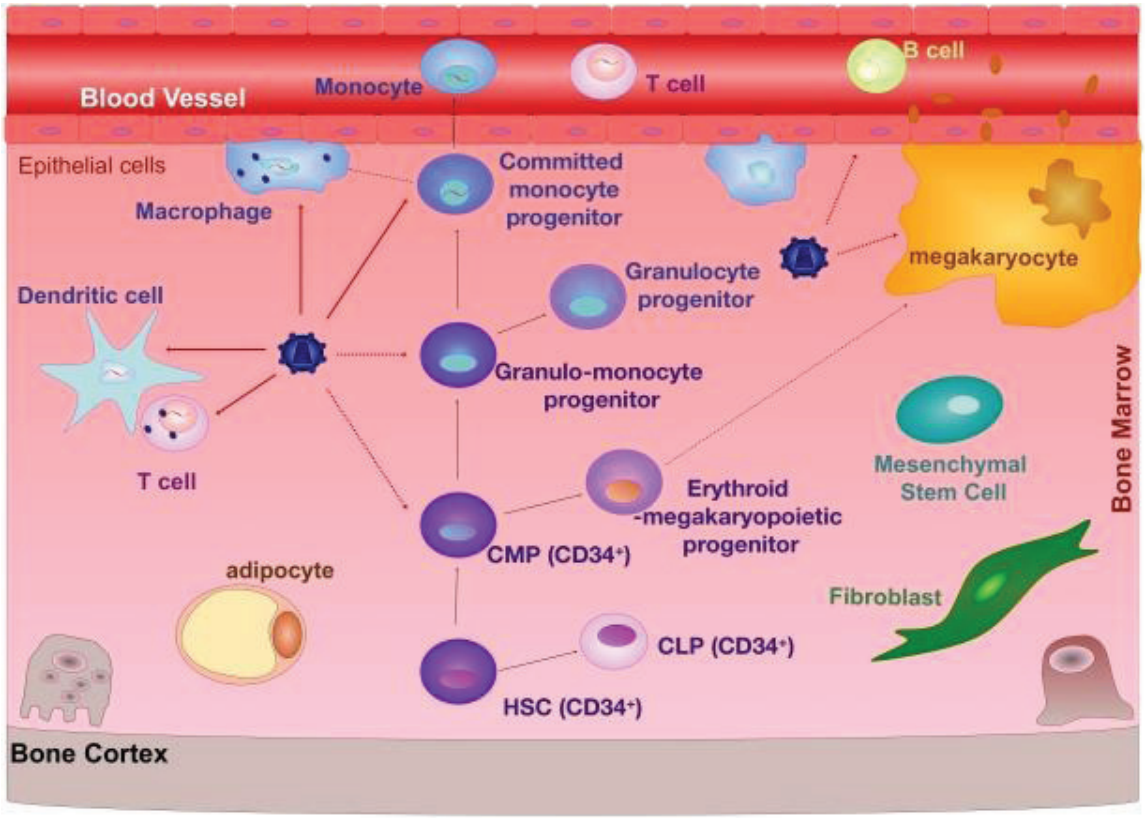

Figure 3. Cells of the bone marrow susceptible to HIV-1 Infection. The cellular components of the bone marrow include hematopoietic progenitor cells at all stages of differentiation, megakaryocytes, fibroblasts, endothelial cells, adipocytes, macrophages, osteoblasts, osteoclasts, and mesenchymal stem cells, while DCs, T cells, and B cells may also migrate from the blood into the bone marrow. Black arrows indicate the differentiation process from one cell type to another. Red lines points to cells that are known to be infected by HIV-1. *Adapted from Alexaki A, Wigdahl B, PLoS pathog 2008*.

In addition to the $CD4^+$ T-cell homeostatic dysfunction, HIV-1 provokes massive $CD4^+$ T-cell depletion and a loss of the integrity of the mucosal surface of the gastrointestinal (GI) tract during acute phases of the infection. Consequently, a local bacterial invasion across the damaged epithelial barrier may allow microbial products (especially LPS) to stimulate immune activation, leading to elevated levels of pro-inflammatory cytokines and T-cell proliferation. The induction of local inflammation causes $CD4^+$ T-cell activation and may serve to provide additional targets for the virus, thus augmenting its replication at this site [37]. Indeed, $CD4^+$ T cells from the GI tract are 10-fold more frequently HIV-infected than those in the

peripheral blood [38, 39]. Another source of T-cell destruction is the increased level of cell death; cell death is caused by different mechanisms, including: HIV direct killing, syncytium formation and single-cell killing through contact of uninfected cells with infected cells, and CTL responses against infected cells [40]. Finally, HIV-1 disrupts the lymphoid tissue architecture; the structure of secondary lymphatic tissue plays a critical role in immune system homeostasis, providing a structure that supports immune functions [41, 42]. Specifically, this disruption causes an important loss of naïve T cells, as well as the retention of effector T-cells in the lymph nodes [42].

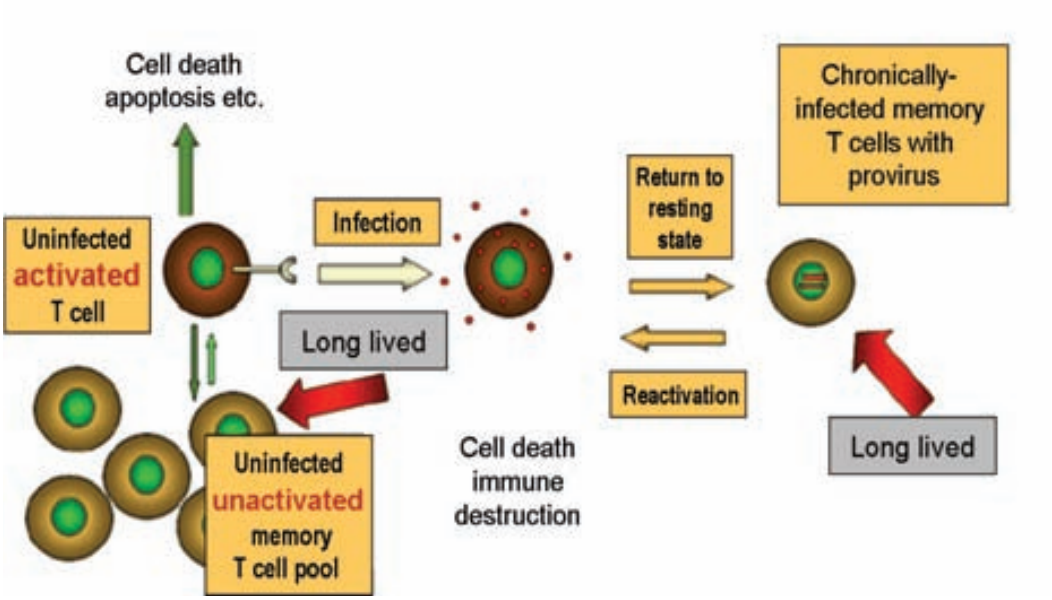

Figure 4. Dynamics of CD4 T cells in HIV-1 infection. *Adapted from Hunt R, Microbiology and Virology On-line 2010, www.microbiologybook.org.*

1.2.2.2. Immune activation

Immune activation abnormally persists during the chronic phase of HIV infection, with significant manifestations, including: (1) increased frequencies of T cells expressing activation markers and exhibiting a memory phenotype, (2) increased production of pro-inflammatory cytokines and (3) increased turnover and cell-death of T and B lymphocytes. Few years after HIV discovery, the idea that immune activation was involved in HIV pathogenesis emerged [43, 44] by the observation that soluble [45, 46] and $CD8^+$ T-cell activation markers [47] were independent predictors of disease progression.

Moreover, studies in SIV support the intimate association between immune activation and pathogenesis. Indeed, the pathogenic non-human primate model (Rhesus macaques) is characterized by strong T-cell activation; while minimal $CD4^+$ T-cell depletion and T-cell activation occurs in the non-pathogenic model of the natural host (Sooty mangabey and African green monkey), despite high levels of virus replication [48].

- Causes of immune activation:

Immune activation in HIV-1 infection includes two main components: (1) an inflammatory response mediated by HIV-specific immune responses and "bystander" immune activation; and (2) the homeostatic response to $CD4^+$ T-cell depletion. The main causes of immune activation are:

a) Persistent antigenic stimulation by HIV viral load which triggers activation of innate immunity and, consequently, of adaptive responses that attempt to clear the infection [49].

b) Direct stimulation by HIV gene products (i.e. gp120, Nef) that are able to activate lymphocytes and macrophages, inducing the production of pro-inflammatory cytokines and chemokines [50, 51].

c) Microbial translocation through increased permeability of the gut mucosa, increasing the levels of bacterial products such as LPS that may lead to enhanced immune activation of $CD4^+$ and $CD8^+$ T cells; additionally, microbial products can increase cytokine production by APC, such as IFN-α and IL-15. Indeed, chronic HIV disease is associated with increased levels of LPS compared to seronegative people, and its plasma levels correlate with other markers of systemic immune activation, such as HLA-DR and CD38 expression in CD8 T cells, as well as plasma levels of IFN-α and soluble CD14 (sCD14, surrogate marker of microbial translocation) [52].

d) Co-infections by other viruses arising because of the suboptimal control of other chronic viral infections, such as herpes and hepatitis virus, caused by the depletion of $CD4^+$ T cells and the release of pro-inflammatory cytokines during HIV-1 infection. For instance, there is an activation of Epstein-Barr virus (EBV) and Cytomegalovirus (CMV) specific $CD8^+$ T cells during HIV-1 acute infection [53]; hence, these viruses (and possibly other persistent viruses) induce a sustained antigen-mediated immune activation in the same way as HIV-1 does [54]. Furthermore, higher levels of immune activation have been found in HIV/HCV co-infected individuals [55, 56].

e) Imbalance between pro- and anti-inflammatory cells which is reflected in the effect of the loss of the Th17/Treg ratio in HIV-1-infected subjects with progressive disease but not in HIV controllers [57]. Tregs have a beneficial role as down-regulators of immune activation, but they might have a harmful effect as down-regulators of HIV-specific responses [57-59]. On the other hand, Th17 cells are selectively depleted in pathogenic SIV and HIV infection but maintained in non-pathogenic infections, and it is accompanied by a concomitant rise in the frequency of induced Treg cells. The unbalance between Th17 and Treg cells in mucosa is associated with high levels of immune activation and disease progression in SIV infection, and similar results have been also found in HIV infection [60].

f) The pro-inflammatory cytokines released during HIV-infection participate also in refueling the cascade of immune activation. For example, IL-1β, IL-6 and TNF-α can act synergistically to induce T-cell activation, growth and differentiation. IFN-α also plays an important role on HIV immunopathogenesis, and has been suggested to be involved in promoting chronic activation and immune dysfunction during HIV-1 disease, as well as in several cell-death pathways of both infected and uninfected cells [61-64], and in the characteristic destruction of lymph node architecture in advanced stages of HIV infection [62]. The induction of IFN-α by pDC has beneficial effects in the acute phase, but its continuous stimulation over chronic infection may drive the hyperactivation of the immune system contributing to the HIV immunopathogenesis [65].

- Consequences of immune activation:

The long-term status of immune activation and inflammation, due to the persistence of the virus in absence of antiretroviral treatment, has extensive and detrimental effects on the immune system and health:

a) Increased $CD4^+$ T-cell destruction. HIV infection promotes the establishment of a vicious cycle, in which virus infection promotes immune activation and death, and immune activation promotes HIV replication. The net result is an increased activation and activation-induced cell death in $CD4^+$ T-cell compartment [66].

b) Premature T-cell immunosenescence, exhaustion and ageing. HIV infected individuals show similar immunological profiles to HIV-uninfected elderly subjects. During ageing, a reduction in T-cell renewal and a progressive enrichment of terminally differentiated T-cells is observed and could be consequence of immune activation over a lifetime [67]. This process known as to immunosenescence can be associated to clinical immunodeficiency (increased risk and/or rapid progression of many infectious diseases) and increased risk of cancers, leading to increased morbidity and mortality [68, 69]

T-cell exhaustion was described as a dysfunction and subsequent physical depletion of antigen-specific T-cells during chronic viral, bacterial and parasite infections as well as during cancer [70]. During this process, loss of T-cell functions occurs in a hierarchical manner. If the severity and/or duration of the infection is high and/or prolonged, virus-specific T-cells can be completely eliminated, leading to loss of virus-specific T-cell responses. Exhaustion has been observed in HIV infection, as has been proposed as one mechanism for the incapability of the host to clear the persistent infection [71, 72].

2. Cohorts generation and groups of HIV-1 progression

Although the appearance of markers of infection is uniform and ordered in most infected individuals, humans show remarkable variation in the clinical outcome and the rate of disease progression following HIV-1 infection. Over 80% of cases of HIV-1 infection follow a pattern of standard progression, developing AIDS from 7.7 to 11.0 years post-infection, in absence of ART, depending on age at seroconversion [28]. However, there are departures from this estimate and the extremes of the spectrum are of particular interest for HIV research. The study of these extreme phenotypes is difficult due to recruitment and cohorts generation issues, but it is crucial for understanding HIV pathogenesis and may give important information for HIV vaccine development. Therefore, cohort generation is an important matter due to the complexity of creation of well-defined and well-characterized study groups in the case of some specific phenotypes.

2.1. Cohorts definition and challenges to cohort generation

The study of specific HIV-disease outcomes requires the generation of well-defined cohorts, with accurate definition of inclusion criteria. Many different research groups work with cohorts in the HIV-immunopathogenesis field but there is a tremendous variation on the parameters used to define the different clinical phenotypes, as well as in the accuracy in the patients and samples selection and definition.

One big challenge in cohorts' generation remains in those groups requiring a proper estimation of the infection or the seroconversion date to estimate the disease progression status. In the absence of clinical symptoms of infection or diagnostic tests that allow detecting very recent infections (i.e. p24 antigen, detuned ELISA, undetermined western blot…), the seroconversion date can be estimated as the midpoint between the date of the last HIV negative test and the first HIV positive test. However, this information and the documents that confirm it are generally not available for the majority of patients at the clinical care sites. Therefore, efficient identification and recruitment of patients requiring the determination of the estimated seroconversion date poses a significant challenge in clinical practice and research purposes.

Furthermore, the generation of the databases and the recompilation of biological material in the context of cohort studies are expensive and very time-consuming. Depending on the studied phenotype, automatic selection by clinical criteria in general databases may not be sufficiently accurate, being important an individual revision of the cases by experienced investigators and/or clinicians. It is important to note that, nowadays, clinicians tend to treat patients at acute infection, which makes even more challenging the generation of these special cohorts.

Finally, all candidates included in cohorts must sign the informed consent approved by the Institutional Review Boards (IRB) of the centers involved in the recruitment. These inform of consent might need to include signed genetic consent, or permission for genetic work as defined by national guidelines. In addition, a well-defined calendar of samples extraction is established for the prospectively recruited patients starting shortly after inclusion, and for those included retrospectively it is necessary to have available stored biological material in the timepoints of interest.

2.2. Groups of progression

2.2.1. Standard phenotypes

The natural course of HIV-1 infection is characterized by an acute phase where infection is established systemically and that typically lasts 2-6 weeks after infection [27], and a subsequent stabilization of VL and $CD4^+$ T-cell counts, so-called setpoint, that is quite constant over the chronic phase of the infection. In absence of antiretroviral therapy, this stable chronic phase lasts a median of 10 years in 80% of the cases, before patients enter in the AIDS phase. The nomenclature used to name the HIV-infected individuals following this average-progression pattern varies throughout the literature; an accepted title is 'Standard Progressors' (SP), which is the one used in our studies. Inclusion criteria for SP cohorts and control groups are very heterogeneous and our research group has done great efforts to find a good definition, consisting of: HIV seroconversion window of less than 1 year with documented negative and positive serology and maintenance of $CD4^+$ T-cell counts above 350 cells/µl within 3 years of seroconversion, with a VL setpoint above 2,000 copies/ml.

Standard progressors represent a valuable control group for several studies, where special phenotypes are compared to the average profile of progression in the general population of HIV-1-infected individuals.

2.2.2. Extreme phenotypes

A high percentage of HIV-1-infected individuals follow an average profile of disease progression, but there are remarkable differences of this standard clinical evolution. While some individuals with HIV-1 are able to suppress viral replication to undetectable levels and/or maintain high $CD4^+$ T-cell counts over many years in the absence of antiretroviral therapy (HIV controllers), others progress quickly to AIDS or meet the current criteria for antiretroviral treatment within the first 3 years after primary infection (rapid progressors). Interestingly, a minority of HIV-infected

individuals remains asymptomatic and show persistently high $CD4^+$ T-cell counts despite high plasma viremia for many years (viremic non progressors).

The study of extreme cases of HIV-1 infection course may give important information in terms of the interactions established between the viral variants and the host during primary HIV infection and the subsequent clinical evolution of the infection. Moreover, HIV controllers (including the long-term non-progressors and the elite controllers) are providing relevant data in immunopathogenesis of the infection. The opposite scenario is the rapid progressors and, especially those cases presenting severe disease evolution during primary infection. Similarly, contrasting individuals that show spontaneous control of HIV with those subjects that present with a rapidly progressing HIV disease development may further help to identify central mechanisms in HIV control that could guide immune-based prophylactic as well as preventative strategies. The study of such HIV disease extreme phenotypes (**Fig. 5**) has important implications in understanding HIV/AIDS pathogenesis and more importantly in the definition of good correlates of protection and the development of new strategies of vaccination and treatment.

In the next sections, some relevant information as well as inclusion criteria is provided for 3 different extreme phenotypes of interest.

2.2.2.1. Elite controllers

Elite controllers (EC) are the group that has been more profoundly studied during the HIV-research history as a model to define the correlates of protective immunity and virus control [73, 74]. This phenotype has been estimated to represent approximately 0.3% of the HIV infected population and it is of particular interest due to their ability to naturally control HIV infection and replication for many years, in absence of antiretroviral therapy. In the literature, different criteria have been used to define EC; a well-accepted definition includes: 1) asymptomatic HIV Infection, with a reduced rate of $CD4^+$ T-cell loss (18 cells/µl/year), over 10 year after seroconversion; 2) plasma HIV RNA levels without ART consistently below the level of detection for the respective assay (i.e. 50 copies/mL); 3) isolated

episodes of viremia (blips) up to 1000 copies/mL as long as they are not consecutive and represent the minority of all available determinations [75, 76]. Among the myriad factors associated to EC phenotype, host factors such as HLA and CTL immune responses seem to play a major role in their ability to control HIV infection [73, 77-85].

2.2.2.2. Rapid progressors

Despite rapid progressors represent a small group of the HIV-1-infected subjects, the implications of their immune-genetic and immune-pathogenic characteristics are remarkable. A complex interplay between multiple viral and host factors are most likely to be involved in accelerating disease progression. The phenotypic frequency of RP is low. Interestingly, epidemiological studies have shown that, in the absence of treatment, less than 0.5% of HIV-1-infected individuals progresses to AIDS within a year after primary infection, some of them with clinically severe presentations during acute HIV-1 infection, including AIDS-defining symptoms [86]. In contrast to EC, the study of RP has been subject of less research, being generally limited to particular case reports or small series [87-92]. The heterogeneity of the reported data has hampered an accurate interpretation of the rapid disease phenotype. The main reason is that the study of HIV-1 rapid progressors it has proved particularly challenging, due to the difficulty of efficient identification and recruitment of patients, especially if biological samples for multiple analyses need to be taken.

Rapid progressors (RPs) can be defined by a number of criteria — generally including progressive immunosuppression soon after seroconversion and, in many cases, high levels of viremia [75, 93]. Great efforts have been done in our project to define inclusion criteria for RP, which have finally included a HIV seroconversion window of less than 1 year with documented negative and positive serology and either of the following possibilities: (a) more than two $CD4^+$ T-cell counts below 350 cells/μl within 3 years of seroconversion and no subsequent rise of $CD4^+$ T-cells above 350/μl in the absence of combination antiretroviral therapy or (b) beginning antiretroviral therapy within 3 years of seroconversion and a $CD4^+$ T-cell count

within 1 month of starting antiretroviral therapy of less than 350/µl. Of note, $CD4^+$ T-cell values in the first 6 months after seroconversion are generally excluded to avoid the $CD4^+$ T-cell nadir during acute HIV infection.

2.2.2.3. Viremic non progressors

High levels of HIV-1 replication during the chronic phase of infection are usually associated with rapid disease progression. However, a minority of HIV-infected individuals can tolerate very high plasma viral loads, while remain asymptomatic and show persistently high $CD4^+$ T-cell counts for many years, in absence of antiretroviral treatment (viremic non progressors, VNP). The latter profile is reminiscent of the non-pathogenic model of SIV infection in natural hosts such as the sooty mangabey. Sooty mangabeys have non-progressive disease despite chronic virus replication that is characterized by low levels of immune activation, while pathogenic SIV infection of rhesus macaques is associated with chronic immune activation [94]. The importance of this special model for the understanding of HIV/AIDS pathogenesis has been underscored by studies in sooty mangabeys and in African green monkeys [95-99].

However, the low prevalence of this uncommon pattern of disease progression, estimated in the SHCS cohort to be only 0.1% of HIV-infected individuals for the strictest definition of VNPs, makes really challenging the generation of cohorts to generate reliable data on VNP immunopathogenesis. After deep analysis of clinical parameters and available information, selection criteria for VNP in our studies includes more than 3 years of follow-up, median HIV plasma viremia from more than 3 measurements of more than 100,000 copies/ml each, HIV plasma viremia consistently above 10,000 copies/ml, a median CD4+ T-cell count of more than 500 cells/µl, CD4+ T-cell counts consistently above 350/µl, and no HIV treatment during follow-up.

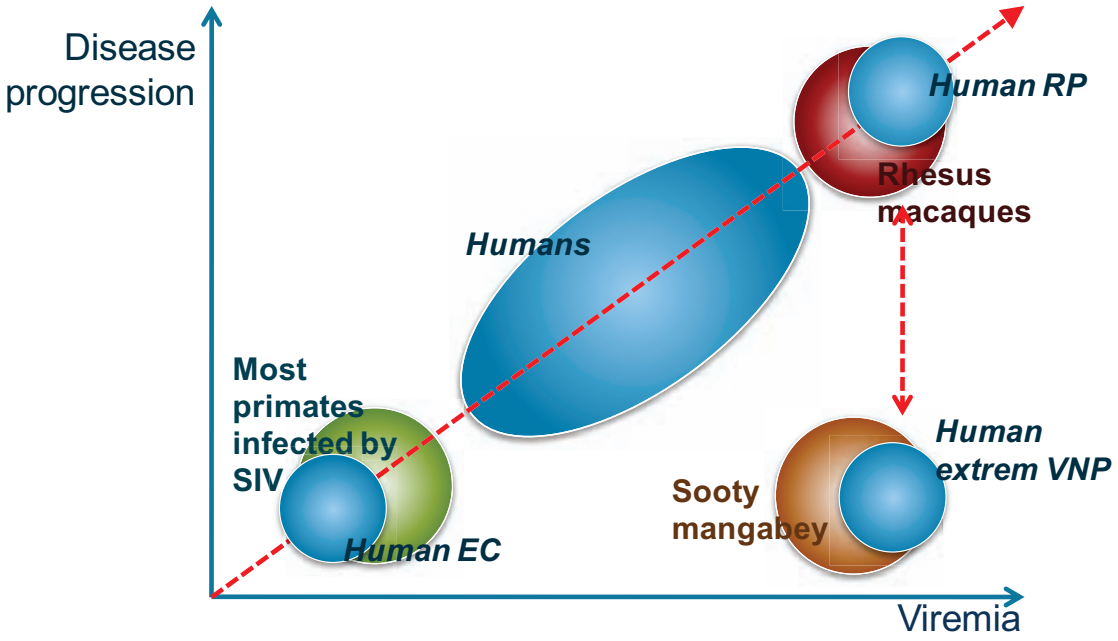

Figure 5. Variation in HIV-1 disease progression. *Adapted from Telenti A and Johnson WE, Cold Spring Harbor Lab Press 2011.*

3. Viral factors in HIV pathogenesis

Several studies have demonstrated the implication of viral factors in HIV pathogenesis, acting in a complex interplay with host factors and disease outcome. The specific weight of viral factors will depend on the studied phenotype, as the different progression profiles are not necessarily driven by the same factors.

Within the myriad viral factors studied in the literature, HIV tropism, viral fitness and mutational profiles of the different strains, appear to have a central role on the disease outcome following HIV-1 infection. In the next sections, a summary of the main traits of these features is presented.

3.1. Tropism in HIV-1 infection

HIV-1-entry in target cells requires binding to the CD4 receptor in the cell membrane [100], as well as to a coreceptor [101]. Although 14 chemokine

receptors or structurally related molecules have been identified that can function as coreceptors for entry of HIV-1 *in vitro* [102-105], the chemokine receptors CXCR4 and CCR5 are the major coreceptors for HIV-1 infection. The natural ligands for CCR5 are chemokines RANTES (CCL5), MIP-1α (CCL3) and MIP-1β (CCL4), while the natural ligand of CXCR4 is SDF-1 (CXCL4). For both coreceptors, these natural ligands have been shown to be potent inhibitors of viruses using the corresponding coreceptor *in vitro* [106-108]. Most HIV-1 variants manifest preferential binding to one or the other coreceptor, a phenotype that is referred to as **HIV coreceptor usage** or **tropism** [109, 110].

The HIV-1 envelope protein (gp160) mediates viral entry through CCR5 or CXCR4 attachment into the target cell; specifically, the V3 loop of its gp120 subunit is the primary genetic determinant for HIV coreceptor usage [111-114]. The preferential binding of gp120 to a specific coreceptor depends on different characteristics of the V3 loop, including single amino acid changes [115-118], increased positive charge [116, 119, 120] or the number of N-linked glycosylation sites. Increased positive charge and the loss of N-linked glycosylation sites have been often associated with CXCR4 usage. In addition but to a lesser extent, glycosylation sites near the V1/V2 loop have also been implicated in the efficiency of coreceptor usage, probably by maintenance of the conformation of the variable loops for interaction with the receptors [121-123].

The nomenclature used to describe viral variants depending on the tropism has varied over time. Early after the discovery of HIV-1, strains that efficiently infected macrophages and primary lymphocytes, but not immortalized T-cell lines, found in primary infection, were called M-tropic viruses, while those more cytopathic and found later in infection, which had the ability to replicate in these cell lines, but did not have the ability to infect macrophages were named T-tropic viruses. Because viral replication in these T-cell lines causes cytopathic changes and syncytium formation, such isolates are also named syncytium-inducing viruses. In contrast, M-tropic viruses do not replicate in T-cell lines (i.e. MT2), and hence are named non-syncytium-inducing viruses [105]. In the mid 90's, when the role of CCR5 and

CXCR4 in HIV-1 entry was discovered, it became apparent that viruses with the syncytium-inducing phenotype preferentially use CXCR4 coreceptors and were named X4 viruses, whereas non-syncytium-inducing strains use CCR5 coreceptors and were designated R5 viruses. Because is difficult to distinguish mixtures of R5 and X4 viruses from dual-tropic strains with the standard assays, viruses that are capable of using both CCR5 and CXCR4 coreceptors are often referred to as dual/mixed viruses (DM viruses) [124]. Different *in vitro* and *in silico* approaches are used for determination of HIV-1 tropism. Initially, SI phenotype in the MT2 cell line, which is a very reliable, yet laborious, method to determine CXCR4 usage, was most extensively used [125]. Later, other *in vitro* assays such as those using U87 cells were developed, which also allowed discriminating between X4-using viral populations and DM-populations. Furthermore, genotypic coupled with *in silico* analysis has been improved every year and reliable algorithms are now available. Specifically, geno2pheno is one of the most trustful algorithms available nowadays [126].

The vast majority of HIV-1 variants depends on either CCR5 or CXCR4 for replication in primary cells [127], and a differential coreceptor usage of HIV variants has been demonstrated for all different clades [128, 129], although an underrepresentation of CXCR4 using HIV variants in clade C has frequently been reported [130, 131]. Convincing explanations for this phenomenon are not available but the involvement of viral genotypic differences rather than environmental differences seems likely [132].

Co-expression of CD4 and an appropriate coreceptor have identified monocytes, macrophages, microglia, dendritic cells (DC), Langerhans cells and lymphoid cells such as thymocytes and $CD4^+$ T helper cells as potential target cells for HIV-1 [132]. However, the mere co-expression of CD4 and an appropriate coreceptor does not warrant the capacity of a cell to support productive infection.

3.1.1. HIV-1 tropism and disease outcome

At primary HIV-1 infection, R5 variants predominate in infected individuals and only a small proportion of patients are infected with X4-using viruses. The presence of R5 variants during early stages of HIV-1 infection suggests that macrophages are a principal target for the establishment of infection in a new individual [105, 133], and DCs and Langerhans cells have also been implicated in transmission of HIV-1 [132]. Moreover, the differential tropism of R5 and X4 variants for $CCR5^+$ and $CXCR4^+$ T-cell subsets [134] may offer an explanation for the predominance of R5 variants early in infection [102]. X4 variants predominantly infect $CXCR4^+$ cells with a resting phenotype, including naïve and resting memory $CD4^+$ T cells, which may not provide the intracellular requirements for productive infection, resulting in viral entry but not productive infection [135-137]. In contrast, R5 variants mainly target activated memory $CD4^+$ T cells [138-140], which are more susceptible to productive infection. Other studies have demonstrated the establishment of infection by X4 SHIV in macaques. However, when macaques were inoculated with a mixture of R5 and X4 SHIV, the X4 SHIV rapidly disappeared, suggesting a higher fitness of the R5 variant early in infection [141]. Clearance of X4 variants but not R5 variants has previously been reported in a parentally infected human as well [142].

Later in infection, when $CD4^+$ T-cell numbers drop below 400 cells/µl of blood, about half of HIV-1-infected patients experience a tropism-switch and X4 variants develop [143]. This proportion varies among HIV subtypes with the highest percentage reported in subtype D [144]. It is unclear why this change is only observed in about 50% of cases, when only a limited number of mutations is required for this phenotypic switch *in vitro* [115-118]. This suggests restraints on the ability to establish a productive infection or on the availability of susceptible target cells for X4 variants, rather than a difficulty to induce the switch *per se*. If so, the factors that limit the emergence of X4 variants in the course of infection may be similar to the factors that determine the predominance of R5 variants early in infection. Indeed, it seems likely that X4 variants evolve on multiple occasions

throughout infection, but may not be able to establish productive infection due to the resting phenotype of their $CXCR4^+$ target cells. The generalized immune activation in later stages of infection [145, 146] may result in proliferation of naive cells and/or a cytokine milieu that may relieve the post-entry block in infection. This may allow productive infection of $CXCR4^+$ cells or rescue of labile intermediates of HIV-1 replication [147]. At this stage newly emerging X4 HIV-1 variants may have a better chance of encountering appropriate target cells that efficiently support replication. However, it remains unclear why this only occurs in 50% of progressing HIV-1 infected individuals. The presence of a 'fitness valley' separating CCR5- and CXCR4-using genotypes is postulated to be a biological determinant of whether the HIV coreceptor switch occurs ([148] and reviewed in [102]).

Regarding the relationship between tropism and HIV disease progression homozygosis of CCR5-Δ32 genotype has been associated to a high degree of resistance to HIV infection (reviewed in [149, 150]), while effects of heterozygous expression of CCR5-Δ32 are modest or inexistent [131]. In addition, other mutations in CCR5 promoter or in CCR2 gene have been identified and are also associated with altered transmission or delayed disease progression, although to a lesser extent than the CCR5-Δ32 mutation [151, 152]. Those include the V64I substitution in the CCR2A protein sequence (CCR2-V64I), found to delay HIV disease progression, but without preventing HIV transmission. Intriguingly, CCR2 is rarely used as a coreceptor in HIV infection and its impact in global epidemic is unclear. Together with CCR5 mutations, approximately 29% of long-term non-progressors (LTNP) phenotypes in large cohorts have been estimated to be due to a mutant genotype for CCR2 or CCR5 [153].

In terms of CXCR4 usage, X4 variants are associated with an accelerated $CD4^+$ T-cell decline and progressive clinical course of infection [154-157]. Before the identification of chemokine receptors as the coreceptors for HIV-1, the differences in pathogenicity of R5 and X4 HIV-1 variants were thought to be due to differences in cytopathicity and replication rate. X4 variants in general replicate more rapidly and to higher levels than R5 variants and X4 infection results in a more massive

depletion of cells *in vitro* [102, 158-160]. Now it is apparent that CCR5 and CXCR4 are not evenly distributed on the cells that have been used in these *in vitro* assays. Indeed, R5 and X4 HIV-1 variants were equally cytopathic for the target cells expressing the appropriate coreceptors, resulting in depletion of the cognate target cells [161, 162].

The enhanced $CD4^+$ T-cell decline associated with the presence of X4 variants may therefore not merely be due to a broader target cell range and more extensive replication of X4 HIV-1 variants, but rather to the infection and killing of naïve T cells and deplete thymocytes more extensively than R5 variants [163]. The finding that naïve $CD4^+$ T cells are somewhat reduced in patients with X4 variants as compared to patients with only R5 variants, may indeed suggest that thymocytes are infected and depleted by X4 variants [164].

3.2. Viral fitness

Fitness is a complex evolutionary term that refers to the replicative adaptability of a particular organism in a particular environment [165], and it will be affected by several factors, especially derived from host immune pressure. Thus, in the case of HIV-1, the infecting virus will be exposed to several obstacles upon infection that it must have to deal with in order to adapt. These obstacles are variable and are mostly defined by the host, including: neutralizing antibody activity, cytotoxic T lymphocytes, target-cells availability, concentration of CCR5 and CXCR4 coreceptors, host coreceptor affinity for viral envelope, natural killer (NK)-cell activity, or administration of antiretroviral treatments [166]. All these features will limit viral spread in the infected individual and viral fitness will depend on the extent to which it is able to evolve and adapt to the host, while minimizing fitness loss and/or overcoming the decline on its replicative capacity (RC) [167-175].

HIV-1 rapidly adapts to host obstacles through viral replication by three main viral diversification mechanisms: 1) the low fidelity (high-error rate) of the HIV-1 polymerase, reverse transcriptase, 2) recombination of portions of genetically

distinct HIV-1 genomes, and 1) partial inhibition of a host cytidine deaminase APOBEC3G [166]. This high-level sequence diversity, and capacity for rapid evolution, is central to HIV pathogenesis and has created diversity in the pathogenic characteristics across infecting strains of HIV-1. Viral fitness is also an important factor in HIV-1 pathogenesis itself, but reliable determination of the *in vivo* fitness is not straightforward, due to the great number of factors in the actual environment of the virus. Different approaches are used for viral fitness determination and most of them focus on surrogate measures of this *in vivo* fitness: mainly *ex vivo* fitness or *in vitro* replicative capacity. Although the most reliable approach for determination of fitness would be the observation of *in vivo* competition of two or more variants to estimate relative fitness [176, 177], this is technically difficult. Therefore, many studies choose to perform direct RC measurements based on infection by single variants or viral populations *in vitro* in primary cells or immortalized cell lines [178].

Despite *in vitro* RC is not a direct measure of *in vivo* fitness and has clear limitations, correlation has been found between *in vitro* RC and *in vivo* VL and disease progression [179-183], although some studies have not corroborated these associations. For that reason, measurement of *in vitro* RC is considered a good marker of viral fitness, being important to study its contribution to HIV pathogenesis and disease progression.

Several studies have demonstrated the importance of RC in HIV-1 disease progression. Some of these important results are detailed below:

- HIV-1 isolates from long-term survivors were shown to be significantly less fit than HIV-1 isolates from patients with accelerated disease progression [184]. Moreover, sequence variation in *gag-pro* of EC [180, 181, 185] or deletions in *nef* in LTNP [186-188] have also been demonstrated to confer reduced replication capacity, when compared to wild-type HIV-1 isolates demonstrating the role of intrinsic viral characteristics in defining this marker of pathogenesis [171, 174, 182, 189].

- CTL-epitope escape mutations in the virus can allow higher virus replication in the host due to reduced immune pressure, despite negatively affect virus replicative capacity [168, 190-193], or they can affect virus fitness through this RC reduction [171, 183, 189]. However, the ongoing selection of additional mutations may allow the virus to compensate for these defects [168, 169, 171, 173, 174, 194] (**Fig. 6**). Moreover, transmitted CTL-escape mutations to a HLA-I-matched recipient imply elevated viremia and a replicative advantage to the transmitted variant, due to reduced immune pressure [190, 195]. In addition, CTL escape mutations associated with protective alleles (i.e. HLA*B57, B*58:01, B*27, and B*81 [170, 194, 196, 197]) often target conserved regions of the viral genome, which poses a difficulty for escape due to fitness costs. This may partially explain the enhanced protection from disease progression in individuals with these alleles (reviewed in [198]).

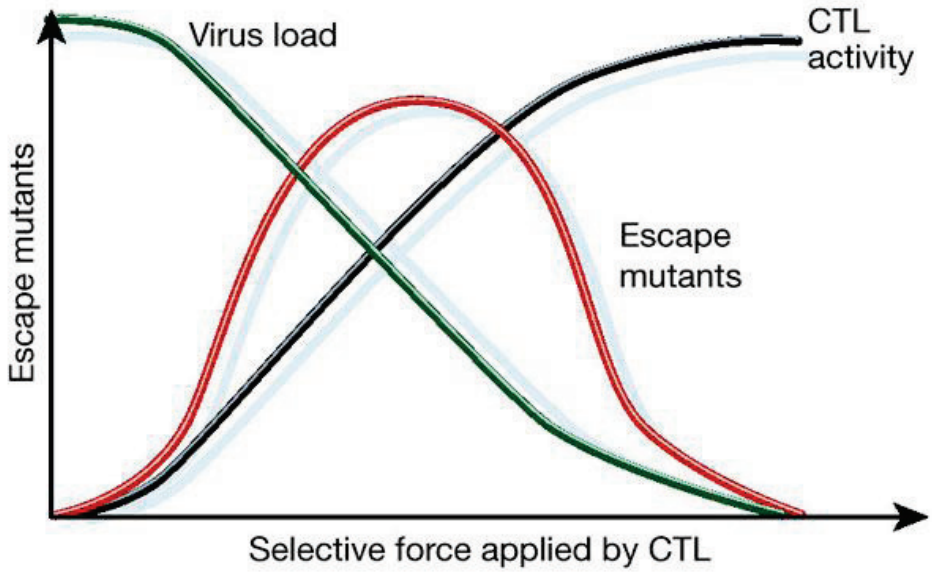

Figure 6. Schematic representation of the relationship between virus replication rate (viral load as representative), the level of CTL response and the selective pressure on epitope escape mutants.. When viral load is high, as in acute infection, there is no selective force until the CTLs appear. When the CTLs are maximally effective, selection occurs and viral load decreases to low level. In chronic HIV infection, the CTLs may be suboptimal and rapid HIV turnover allows many escape mutants to appear. *From McMichael and Rowland-Jones, Nature 2001.*

- RC has been correlated with the rate of $CD4^+$ T-cell decline over the first 3 years of infection in a manner that is partially independent of VL, suggesting that the replication capacity of HIV-1 during the earliest stages of infection is a determinant of pathogenesis beyond what might be expected based on setpoint of VL by itself [183].
- The HIV-1 pol replication capacity has been also associated to $CD4^+$ T-cell counts, yet is not strongly associated with HIV-1 RNA levels. The biological basis for elevated $CD4^+$ T-cell counts among those carrying a virus of low pol RC may be because of lowered virus infectivity, or restricted tissue replication [166].

Overall, available data suggest that fitness of the infecting HIV-1 isolate may be paramount to the subsequent disease and status of HIV-specific immune response [184, 199]. However, the actual contribution of RC in rapid disease progression has not been explored in large well-defined cohorts of RP, being limited to particular case reports and indirect studies in other HIV-disease phenotypes. Thus, the role of this viral factor needs to be further addressed for the RP phenotype.

3.3. Other factors

As detailed in sections above, viral tropism and fitness are central viral factors in HIV-1 pathogenesis and disease outcome. However, there are other viral features that may have important influences, often through indirectly disturbing replicative capacity, including resistance mutations to antiretroviral drugs, CTL escape mutations, viral subtypes and mutations in different HIV-1 genes.

As previously mentioned, the extreme heterogeneity of HIV is a result of rapid viral turnover (~2.6 days/replication cycle), a high virus burden (10^{10} viral particles/day) and the error-prone nature of the reverse transcriptase (RT) enzyme which lacks proofreading activity ($3x10^{-5}$ mutation per base-pair per cycle) [200-203]. HIV can also recombine, giving rise to viruses leading to major antigenic shifts and, if stable, alterations in fitness or virulence [204-207].

One example of viral features affecting HIV pathogenesis is that several drug resistance mutations have been shown to induce a certain degree of attenuation in terms of impaired replicative capacity, especially in absence of compensatory mutations, which may have a role in both transmission and virulence (i.e. mutation M184V is associated with lower plasma VL) [208, 209].

Aside from treatment-induced viral variants, several genetic defects and polymorphisms that impair replicative capacity have been implicated in mediating relative viral control, such as: deletions in the nef gene or in the overlap of nef and the U3 region of the long terminal repeat (LTR) [186], unusual, difficult-to-revert polymorphisms and 1-2 amino-acids deletion in gp41 and Gag, a four amino acids insertion in Vpu [210]. Moreover, the greatest genetic differences are observed in the *env* gene (up to 30% nucleotide diversity) followed by *gag* (20%) and *pol* (15%) [211]. *Ex vivo* fitness of primary HIV isolates typically maps to the *env* gene and is highly controlled by the efficiency of viral entry into the host cells [212-214]. On average, sequence changes occur at a rate of 1% per year in *env*. Even though the relationship between disease progression and HIV *env* genetic diversity from founding or infecting viruses has been shown to increase continuously and peaks at the onset of AIDS [186, 187, 215].

Furthermore, others have also been able to isolate viruses with impaired replicative capacity from PBMC cultures generated from LTNP, supporting the hypothesis that primary infection by ‘attenuated virus strains’ with slow replication kinetics may facilitate control of viremia [216].

Regarding the viral subtypes, nine clades have been recognized (A-D, F-H, J-K), and they differ by 15-20% [217]. HIV-1 clade C is the most prevalent transmitted virus and its infections are characterized by a high viremia set point and higher virus levels in genital fluids [218]. Moreover, viruses from different lineages within group M have recombined to form circulating recombinant forms (CRF). CRFs account for about 10-20% of all new infections [14, 219, 220], and the most dominant forms in the epidemic are the CRF01_AE, CRF02_AG, CRF07,08_BC, and the CRF_BF-like viruses [221-224]. Despite the fact that the characterized viral

subtype CRF02- AG in case report 2 is rather unusual in our area (1.1% of pol sequences tested for antiretroviral resistance during 1999–2007), it is still the second most common non-B subtype. This CRF is the predominant and most rapidly spreading HIV strain in West Africa and western Central Africa [225, 226], which raises concerns about its superior replication fitness and/or transmission efficiency. In fact, primary HIV-1 CRF02-AG isolates from Cameroon exhibited higher *ex vivo* replicative fitness than did subtype A and G viruses from the same geographic region [227, 228].

Finally, effect of HIV-1 proteins on different cellular antiviral factors, such as Vif or Vpu on APOBEC3G [229-231] or tetherin [232], respectively, has been demonstrated to influence viral replication or viral particles budding from the cell membrane. Moreover, transcriptional regulation of HIV-1 is only one step in the retroviral lifecycle, but it can affect replicative fitness [233]. Mutations or specific polymorphisms on HIV-1 genes might then have also an effect on viral virulence through these mechanisms.

4. Host factors in HIV infection

As mentioned in previous sections, HIV pathogenesis is driven by a complex interplay between viral and host factors. Host factors in particular encompass a large variety of factors, from human genetics to immune system and epidemiological or social characteristics. In the following sections, an overview of some of these important features is presented.

4.1. Immunogenetics of HIV disease: HLA class-I, -II and KIR

Several genetic polymorphisms, gene variants and the copy-number of certain genes have been studied and persistently related to HIV pathogenesis and disease progression. The vast majority of genetic factors having remarkable effects on HIV-1 infection are directly related to the immune system.

Among these myriad factors, polymorphisms in viral coreceptors, such as CCR5 or CCR2 have been described to have an effect on HIV transmission and disease

progression. The most relevant is the CCR5Δ32 genotype associated to resistance to HIV infection (reviewed in [149, 150]), but other mutations in CCR5 and CCR2 genes have been also identified and associated with altered transmission or delayed disease progression . Interestingly, approximately 29% of long-term non-progressors (LTNP) phenotypes in large cohorts have been estimated to be due to a mutant genotype for CCR2 or CCR5 [153].

Moreover, numerous polymorphisms in chemokine receptors and/or some chemokine ligands have been also identified to influence HIV disease outcome and pathogenesis. One important example are 2 single nucleotide polymorphisms (SNPs) found in the RANTES gene promoter associated to reduced RC and delayed HIV disease progression [234-236]. In addition, copy number variation of certain genes has been also related to progression, as is the case for the CCL3L1 (MIP-1α) gene; individuals with higher CCL3L1 copy numbers show a lower VL setpoint, suggesting an increased rate of HIV disease progression [237]. Furthermore, some polymorphisms in the DC-SIGN gene promoter have been related to an increase or decrease in susceptibility to HIV infection, particularly also in parenterally acquired HIV infection [238]. However, results need to be interpreted carefully, as they have not been validated in GWAS analysis performed in different cohort studies.

The *human leukocyte antigen* (HLA) class I genes exhibit the strongest and most consistent association, underscoring a central role for $CD8^+$ T cells in resistance to the virus [239]. HLA proteins play important roles in T-cell-mediated adaptive immunity by presenting immunodominant HIV epitopes to cytotoxic T lymphocytes (CTLs) and $CD4^+$ T cells [239]. Thus, HLA has been intensively studied and implicated in disease course, prognosis and even mortality; an overview of these observations is summarized in the next section.

4.1.1. HLA and HIV infection

Evidence for favorable or unfavorable relationships of certain HLA-I markers to virologic and immunologic outcomes of HIV infection is conclusive [240-247].

Combinations of HLA-I markers associated with more favorable outcomes would be predicted to enhance control of viremia [78, 242].

The HLA class I and class II molecules (**Fig. 7**) are encoded by genes located within the human major histocompatibility complex (MHC) on chromosome 6p21.3. The classical HLA class I (HLA-A, -B and -C) and class II (HLA-DR, -DQ and -DP) genes within the MHC are the most diverse loci in the human genome with the number of alleles varying from 31 for DQA1 to >2000 for HLA-B (http://www.ebi.ac.uk/imgt/hla) [239].

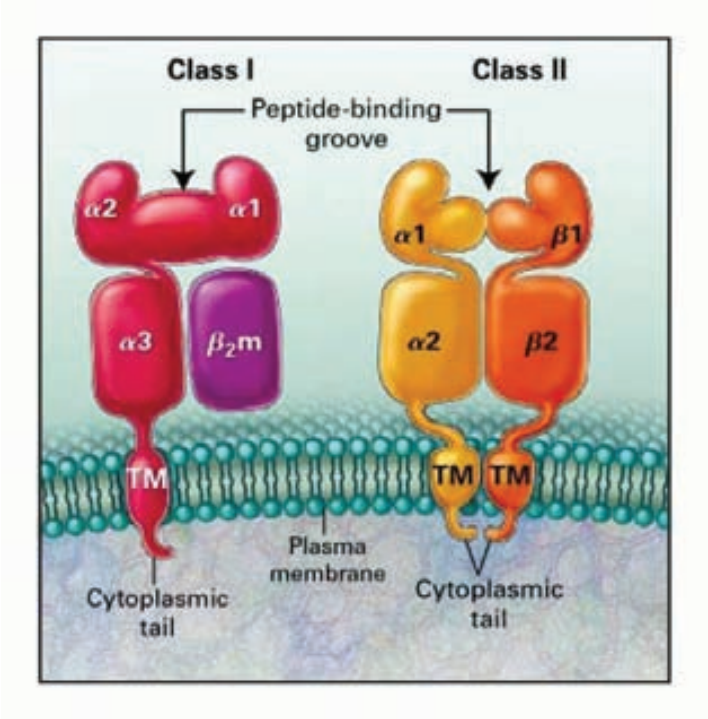

Figure 7. Structure of HLA class I and HLA class II molecules. *From Klein J and Sato A, NEJM 2000.*

Variation is mainly concentrated in the regions encoding the peptide binding groove and they have been the main focus of research in terms of the effect of HLA on HIV infection. During HIV-1 infection, the HLA class-I genes encode molecules that are expressed on the cell surface and bind antigenic peptide epitopes derived from the pathogen or self-stress proteins, and present them to $CD8^+$ T cells, thereby initiating a cytotoxic T-cell (CTL) response. In addition, HLA

class-I molecules also regulate NK cell activity via interactions with NK cell receptors. The class-II genes encode molecules expressed on the surface of antigen-presenting cells (APCs) and mainly bind extracellular peptides and present them to $CD4^+$ T cells, which generally induces cytokines production that will help other immune cells to respond (reviewed in [239]).

HIV infection is one of only a few infectious diseases showing a clear-cut and consistent HLA association, which has been confirmed by several genome-wide association studies (GWAS) [74, 82, 248-250]. Indeed, HLA represents the most significant locus in differential control of HIV across humans.

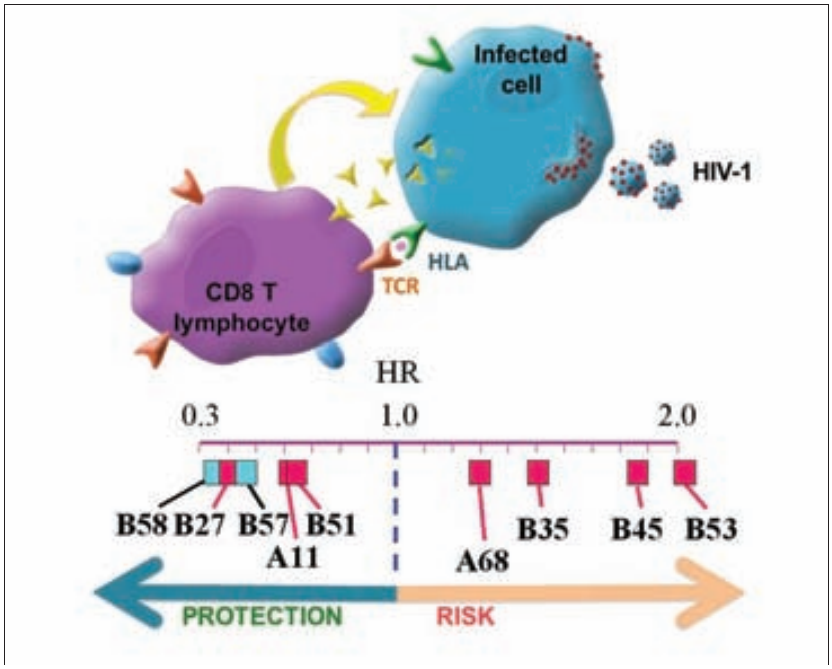

Figure 8. **HLA role in HIV-1 infection.** *Adapted from Sáez-Cirión et al. CD8 T Cell-mediated HIV-1 suppression in HIV controllers (2009), at https://www.pasteur.fr/.*

HLA heterozygosity/homozygosity. Most HLA allelic frequencies are quite evenly distributed [251, 252], so homozygosity is not very common (especially in class I alleles due to extensive variation). Individuals who are heterozygous at the HLA locus will be able to present a broader repertoire of antigenic peptides to T cells as compared to homozygotes, thereby exerting greater pressure on the

pathogen. Under this model, it could be expected that HLA homozygous individuals would progress more rapidly to AIDS than HLA heterozygous individuals after HIV infection, which have been demonstrated by several studies [190, 253, 254]. Other reasons for this phenomenon are that it may also take the virus a longer time to accumulate escape mutations in HLA heterozygous individuals relative to homozygous individuals [239], in agreement with the model of frequency-dependent selection (or rare allele advantage), which argues that HIV adapts to HLA alleles that are common in the population [255, 256].

HLA class I variation. Distinct HLA allotypes may mediate differential responses to HIV infection based on their capacity to contain HIV replication. As a general rule, HLA-B appears to have the most obvious differential effects on HIV outcome, perhaps as a consequence of its extensive diversity. A large study of HIV-infected patients showed that most $CD8^+$ T-cell responses to HIV were HLA-B restricted and that variation in viral load was predominantly associated with variation at the HLA-B locus [79]. Specific HLA alleles have also been identified to have protective or detrimental effects on HIV disease outcome (**Fig. 8**). Among the diverse publications, some alleles consistently associated with disease progression, include: 1) Protective alleles: B*57, B*27:05, B58:01, and 2) Risk alleles: B*35Px.

Among the protective HLA effects identified to date, one of the most consistent associations has been with B*57 [73, 79, 81, 257]. Nearly 40% of elite controllers carry B*57 compared to only 9% in non-controllers, which is about the same frequency as that observed in the general population [73, 81]. Multiple GWAS have also demonstrated that SNPs in LD with B*57 showed strong association with low HIV-1 viral load set point [82]. The B*57 molecule binds a number of immunodominant epitopes located in conserved regions of Gag [167, 190, 258, 259] with the most frequently targeted epitope being Gag TW10 [247], and CTL responses to these highly conserved HIV epitopes may sufficiently maintain a low viral load. The Caucasian B*57:01 and the closely related African B*57:03 molecules both bind the same Gag peptide even though they utilize mutually exclusive T-cell receptor (TCR) repertoires [260, 261]. The HLA-B*27 is also

associated with slower progression to AIDS and control of viral load levels [73, 78, 262]. Like B*57, B*27 recognizes a highly conserved immunodominant gag epitope (**Fig. 9**), but unlike B*57, its restriction is focused on a single gag epitope KK10 [77, 263]. Restriction of KK10 efficiently suppresses HIV replication and delays the onset of AIDS. The KK10 peptide is a conserved epitope and can tolerate little variation. Consequently, under B*27 pressure, the virus must undergo a complex pattern of mutations in order to escape recognition, but once the virus fully escapes, it is able to replicate at wild-type levels [66], resulting in rapid disease progression [77, 263, 264].

Unlike B*57 and B*27, HLA-B*35 alleles consistently associate with susceptibility to HIV outcomes (reviewed in [265]). Analysis of B*35 subtypes indicated that more rapid progression to AIDS among B*35 positive subjects is due to the less common B*35:02 and B*35:03, and not the most common B*35:01 [266]. B*35:01 is the predominant B*35 subtype in individuals of African descent, while B*35:02 and B*35:03 are only sporadically detected, which likely explains the lack of B*35 association with AIDS progression in African Americans. This dichotomy of B*35 subtypes effects may be based on their peptide-binding specificity. The common B*35:01 allele preferably binds epitopes with proline at position 2 and tyrosine at position 9 (referred to as B*35PY). B*35:02/03 and the related B*53:01 are more broadly reactive and bind epitopes with proline at position 2 but accept several amino acids at position 9 (referred to as B*35Px) [267-269]. These results further underscore that minor variations in the class I MHC can have a significant impact on AIDS pathogenesis. Functional assays demonstrated that the level of the gag-specific CTL is inversely correlated with HIV viral loads in B*35PY but not B*35Px individuals, suggesting a difference in the quality and/or the quantity, of HIV-specific CTL activity between B*35Px and B*35PY positive individuals [270].

Apart from the major associations described above, other class I associations with HIV outcomes have also been described. HLA-B alleles with the Bw4 public epitope (as defined by amino acids 77–83) have been correlated with AIDS protection in a number of studies [271, 272].

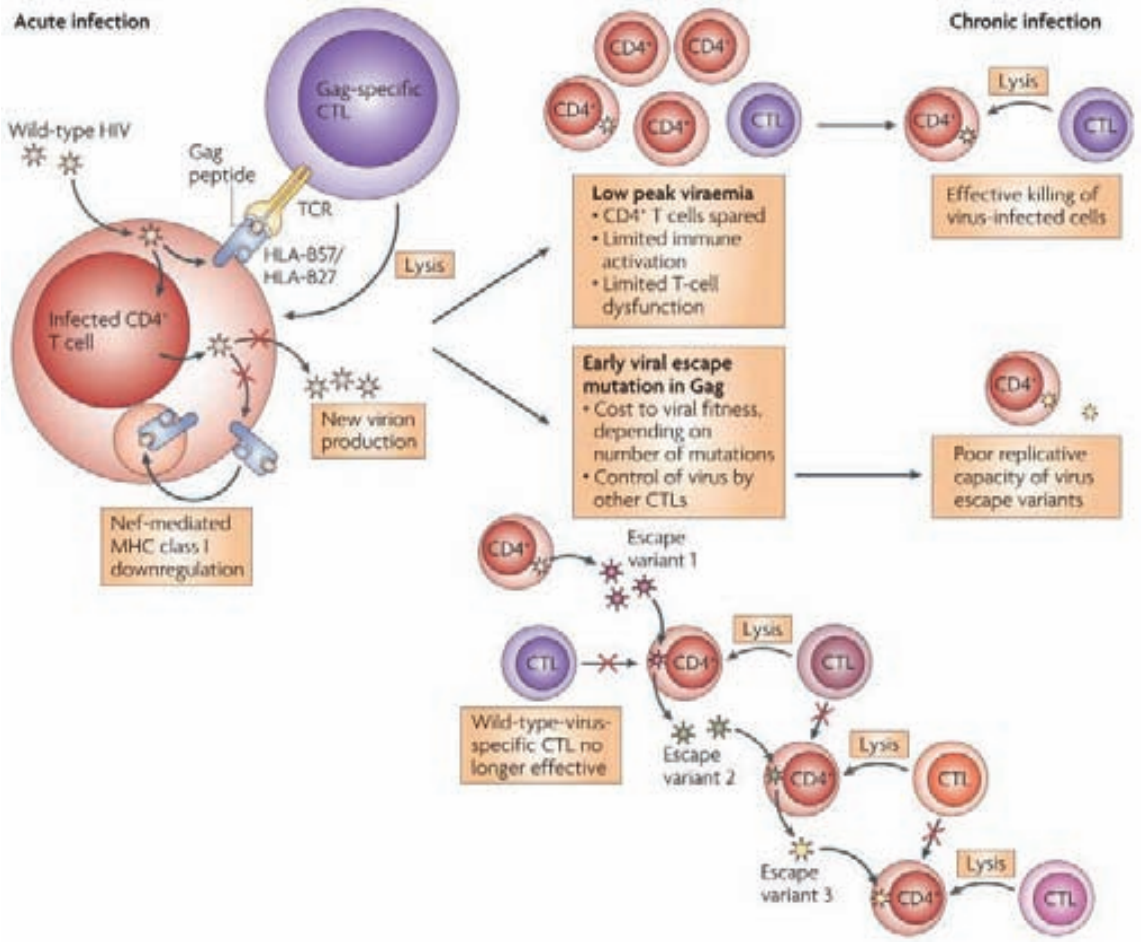

Figure 9. Schematic model of immune control of HIV mediated by HLA alleles presenting multiple gag epitopes. In HIV-infected individuals carrying protective HLA alleles, associated with effective control of the virus (such as HLA-B57 and HLA-B27), Gag-specific responses are generated in acute infection. Wild-type virus-specific-CTLs recognize infected cells early in the viral life cycle, before Nef-mediated MHC class I downregulation. Escape mutations that are typically selected in Gag reduce *in vitro* viral fitness. Remaining wild-type virus-specific CTL responses can contain the infection until new escape variants are selected. However, increasing numbers of costly escape mutations further limit the replicative capacity of the virus, thereby facilitating effective killing of virus-infected cells by remaining CTLs. TCR, T-cell receptor. *From Goulder PJ and Watkins DI, Nat Rev Immunol 2008.*

When treated as a group, the Bw4 protection is typically weaker and less consistent compared to B*27 or B*57 both of which carry the Bw4 epitope. However, when HLA alleles are ranked by relative hazard there is a clear trend for $Bw4^+$ alleles to cluster on the protective side (Fig. 3). Other alleles that associate with HIV outcomes include the protective B*13 [273, 274], B*51:01 [78], B*58:01

[79, 255], and B*81:01 alleles [189, 275], and the susceptible B*58:02 [79, 246]. Some of these alleles have frequency distributions that differ across populations, which may explain their somewhat inconsistent associations in different cohort studies.

HLA class II variation. $CD4^+$ T cells play a critical role in the immune response against HIV-1 despite being the principal targets of HIV infection. Although most HIV specific $CD4^+$ T cells are not infected [276] and are therefore capable of contributing to the antiviral response, there is significant impairment in their proliferative capacity in viremic patients [277, 278]. $CD4^+$ T cells are also required for the development and maintenance of memory and cytotoxic $CD8^+$ T cells [279-282]. Despite the role of $CD4^+$ T cells in the antiviral response, the data regarding the effect of HLA class II genetic variation on HIV disease outcomes are not as convincing as those for class I. Nevertheless, there is some evidence from several studies indicating an effect of specific class II alleles/haplotypes on disease outcome. A protective effect for HLA-DRB1*01 and a susceptible effect of DRB1*15:03 have been described among African populations [283-285]. DRB1*13:03 associates with reduced viral load levels in both B and C clade populations [286], and the DRB1*13-DQB1*06 haplotype has shown an association with viral control after ART interruption [287]. DRB1*13:01 carriers were also reported to be less likely to transmit virus to their seronegative partners, despite the lack of an effect on viral load [285].

Several studies have also indicated a function of HLA in natural killer cell-mediated innate immunity through interaction with killer cell-immunoglobulin-like receptors (KIR). KIR genes, a polymorphic set of molecules that modulate natural killer (NK) cell activity (and some T cells) [288], especially in the context of their relationship with HLA, have also been shown to have important roles in HIV pathogenesis. Particularly, HLA-B*57 has been implicated in regulating NK cell function via its interaction with KIR3DL1/S1 (see below), which may also contribute to the B*57 protection against AIDS progression. A brief summary of the effect of KIR genes in this specific frame is presented below.

4.1.2. KIR and HLA

The killer cell immunoglobulin-like receptor genes are arranged in a 'head-to-tail' cluster on human chromosome 19q13.4 within the leukocyte receptor complex and encode molecules that belong to the immunoglobulin (Ig) superfamily of receptors [289]. They consist of a group of regulatory molecules that are expressed on NK cells and a subset of T cells. KIR2DL and KIR3DL genes encode molecules with long cytoplasmic tails and are inhibitory. KIR2DS and KIR3DS genes encode molecules with short cytoplasmic tails that transmit activating signals [290]. There is extensive diversity of KIR haplotypes as a consequence of non-allelic homologous recombination (NAHR), but two basic groups of KIR haplotypes termed A and B have been described [291]. Haplotype A is uniform in terms of gene content and is composed of nine genes that predominantly encode inhibitory receptors. The B group of haplotypes, on the other hand, contains variable numbers of genes encoding activating and inhibitory receptors ranging from 4 to 17 [292-294]. Multiple alleles also exist for each KIR gene (http://www.ebi.ac.uk/ipd/kir/alleles.html), encoding products that can vary in expression level or functional capacity. Another notable feature of the KIR locus is that there is variegated expression of KIR on NK cell clones, such that a given KIR gene is expressed in some but not all NK cell clones in a given individual [295].

Thus far, only HLA class-I allotypes have been identified as ligands for KIR. KIR3DL1 recognizes HLA-B molecules and a subset of HLA-A molecules that have the serologically defined Bw4 motif (determined by amino acid positions 77-83). Some KIR3DL1 subtypes exhibit a stronger inhibitory effect in the presence of HLA-B Bw4 subtypes that have isoleucine at position 80 (Bw4-80I) as opposite to threonine at the same position (Bw4-80T) [296, 297]. HLA-B Bw6 allotypes, on the other hand, do not serve as ligands for KIR.

Due to their spontaneous killing of targets that lack self MHC (missing self-hypothesis), NK cells were initially thought to be non-MHC restricted [298], but it is now clear that inhibitory KIR are involved in restraining NK cell cytotoxicity. More recent data suggest that during the developmental process of NK cell education,

interactions between inhibitory receptors expressed on the NK cell surface and self MHC class I molecules are important in determining functional potency to respond to and eliminate abnormal cells expressing aberrant levels of MHC [299].

4.2. Immune responses against HIV-1

Human immune system is a key player in HIV infection; first, because it is seriously compromised due to many of their cellular components are targets for the virus, especially the $CD4^+$ T cells. On the other hand, both innate and adaptive immune responses have been demonstrated to have a decisive role in controlling the HIV-1 infection and viral replication, although being unable to the complete clearance of the virus, once the infection is established. Protection against viral pathogens is mediated by an intricate interaction of innate and adaptive immune responses that work together to prevent infection or to control viral replication [300]. Different components and mechanisms of the immune response have been studied in the frame of HIV research and some important highlights are presented within the next sections.

4.2.1. Innate immunity in HIV-1 infection

Innate responses to HIV are detected very early after HIV-1 infection and consist of non-specific responses that humans have developed to face invasion from foreign pathogens or antigens. The study of innate immunity in HIV-1 infection contemplates several elements of the innate immune system, including anatomical barriers (such as epithelial cells), broad antiviral mechanism (secretory molecules and cellular restriction factors) and cellular populations such as DCs, NKs, granulocytes and other phagocytes [301].

Innate responses are activated after detection of pathogens through pattern-recognition receptors (PRRs) that recognize particular pathogen hallmarks, known as pathogen-associated molecular pathogens (PAMPs). Toll-like receptors (TLRs) are a major class of PRRs that recognize both intracellular and extracellular PAMPs and lead to signal transduction. An important consequence is the activation

of DC precursors and immature DCs, leading to DC maturation and cytokines production. DCs act as a bridge between innate and adaptive responses by activating innate responses but also migrating to lymph nodes, where they develop a strong adaptive immune response.

Type I interferons (IFN), represented by IFN-α and IFN-β, are cytokines promptly secreted upon the interaction between microbial pathogens (viruses, bacteria, parasites or tumor cells) and the immune system. These cytokines are indispensable in inducing antiviral immunity, being plasmacytoid DCs (pDC) the major producers of both IFN-α and IFN-β in response to viral infection. Type I IFNs activate a variety of immune effector cells that belong to the innate as well as the adaptive immune system [302, 303].

Another immune-cells population with a remarkable role in innate immunity is natural killer (NK) cells. NKs act as a bridge between innate and adaptive responses, by production of cytokines and chemokines that mediate activation of effector cells involved in the adaptive immune response [304], They play a role in early responses against infected or transformed cells, through the production of cytokines and direct cytotoxicity [305, 306]. Different subsets of NK cells have been identified and have been observed to have different roles, capacities and characteristics.

Following HIV infection, NK cells rapidly expand in response to a variety of cytokines before the expansion of $CD8^+$ T cells. However, HIV has devised strategies to evade recognition by NK cells, including the downregulation of HLA class I expression on the cell surface of $CD4^+$ T cells, through its Nef protein, in an effort to escape $CD8^+$ T-cell lysis, rendering the cells vulnerable to NK cell lysis. The HIV-1 Nef protein selectively downregulates HLA-A and HLA-B but expression levels of the major contributors to NK cell inhibition (HLA-C and HLA-E) remain intact.

Several studies have also shown that NK cell phenotype and function are altered during the course of HIV-1 infection [307-310]. Acute HIV-1 infection is characterized by elevated NK cell numbers. Additional defects in NK cell function,

receptor expression, and effects on other immunomodulatory cells have also been described [311-313]. NK cell cytokine production was also increased in a group of exposed-uninfected intravenous-drug users, suggesting that enhanced NK cell function contributed to the protection against HIV-1 infection [314]. Taken together, the data suggest that NK cells confer some level of protection against HIV and that their anergy in chronic infection may contribute to disease progression.

4.2.2. Adaptive immunity in HIV-1 infection

Adaptive immunity, in contrast to the innate immune response, is antigen-specific and requires days/weeks to mature in order to recognize and eliminate the invading organism. The main components of the adaptive immune response are the cellular and the humoral immune responses (**Fig. 11 and 12**), which are mainly directed to recognition of non-self-antigens to eliminate pathogens and to develop immunological memory. Within these two large fields, $CD8^+$ T-cytotoxic responses, $CD4^+$ T-cell responses and neutralizing antibodies have given and continue providing clue information in HIV infection and pathogenesis, especially for their role in protection as well as for the immune pressure generated over the virus, forcing HIV to mutate and evolve to escape from them.

4.2.2.1. $CD4^+$ T-cells as helpers in immune responses against HIV

$CD4^+$ T cells act as helper cells in many immune processes. After complete activation, CD4 T-helper cells (Th0) release IL-2 and produce IL-2 receptor, activating T-cell proliferation. Five different kinds of helper T-cells have been identified: Th1, Th2, T follicular helper (Tfh), Th17 and regulatory T-cells.

Th1 cell differentiation requires IL-12, produced by innate immune cells, as well as IFN-γ, produced by both NK cells and T-cells. Th1 cells produce IFN-γ and are involved in cellular immunity against intracellular pathogens, by providing cytokine-mediated help to CTLs, and favor phagocyte-mediated immunity [315, 316].

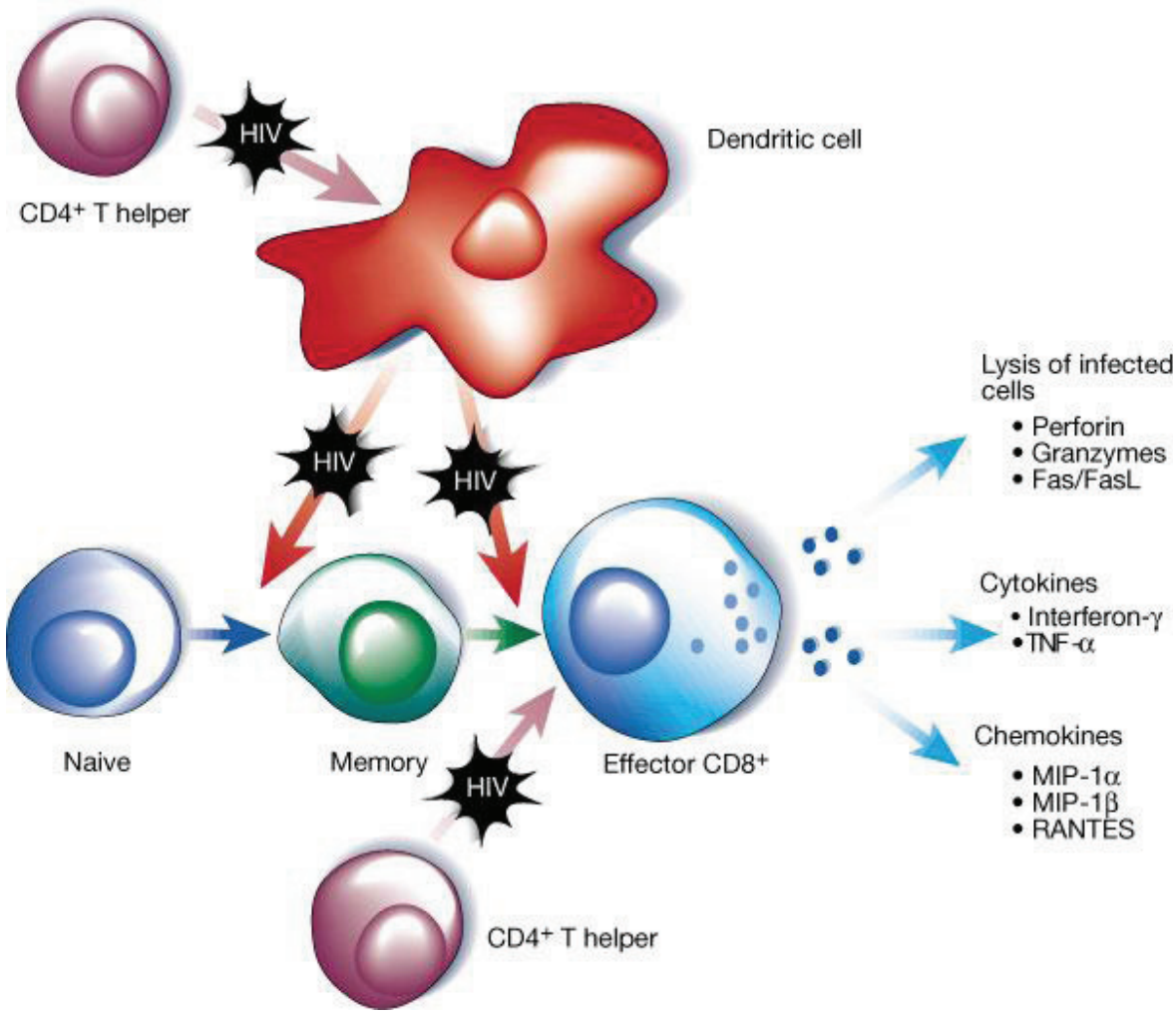

Figure 10. CD4⁺ T-cell dependence of CD8⁺ T cells. CD4⁺ T cells are important for priming dendritic cells to initiate CD8⁺ T-cell responses, to help maintaining memory T cells and in maturation of CD8⁺ T-cell function. All of these actions are impaired by HIV infection. In addition, HIV can directly infect and impair dendritic-cell function. *From McMichael and Rowland-Jones, Nature 2001*.

Th2 cell differentiation requires IL-4 and they produce different cytokines including IL-4, IL-5 and IL-13. Th2 regulate B-cell class switching to IgE through their production of IL-4 (humoral immunity), to control helminthes and other extracellular pathogens. Th2 cells also migrate to the lung or intestinal tissues where they recruit eosinophils (through IL-5) and mast cells (through IL-9) leading to tissue eosinophilia and mast cells hyperplasia [317].

Follicular helper T cells (Tfh) require the combined stimuli of antigen binding to the TCR and exposure to IL-6 and IL-21 activate them, and cause the Tfh cells to collect in the follicles in the lymph node, where they stimulate B-cells to undergo class switching with the synthesis of all antibody classes (except IgE), undergo affinity maturation and form memory B cells [318].

Th17 cells are closely related to Treg; while Treg inhibit autoimmunity and anti-inflammatory response, Th-17 show pro-inflammatory properties. Th17 and Treg share a reciprocal maturation pathway and function together in opposite ways to control the inflammatory response. Th17 cell differentiation requires transforming growth factor (TGF)-β in combination with the pro-inflammatory cytokines IL-6 or IL-21. Th17 cells produce IL-17 and IL-22 and play important roles in clearance of extracellular bacteria and fungi, especially at mucosal surfaces [319].

Regulatory T-cells (Treg) differentiation requires only TGF-β. Treg cells have essential roles in maintenance of immune homeostasis, regulating these effector T-cell responses and thus preventing their potentially pathogenic effects through a variety of mechanisms. They can suppress the system by secreting inhibitory cytokines (IL-10), inducing cytolysis of target cell, metabolic disruption or modulation of DC maturation or function [320].

Thus, $CD4^+$ T cells play a key role in many different immune processes and, consequently, HIV-induced depletion of CD4 T-lymphocytes results in deficiencies in general immunity (**Fig. 10**) to face the infection and in an increased susceptibility to infection by a number of opportunistic microorganisms.

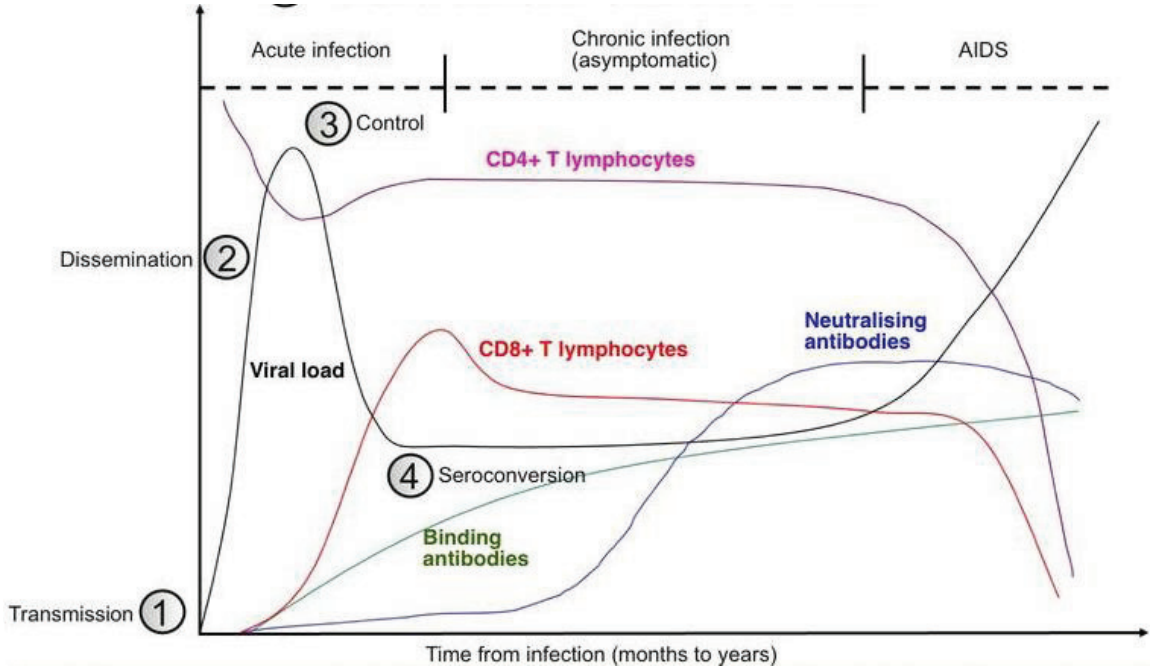

Figure 11. Typical immune response to HIV-1. A typical immune response to HIV infection shows a rapid increase in viremia in the early acute phase which declines to a setpoint and that coincides with a decline in $CD4^+$ T cells. HIV-specific $CD8^+$ CTL responses reduce the viral load and an increase in CD4+ T cells is observed. HIV-specific antibodies appear after the reduction of viremia, but antibodies are detectable by ELISA only later in acute infection. During chronic infection, $CD4^+$ T cells decline slowly and viral load remains stable (setpoint), and neutralizing antibodies begin to appear. Continued HIV replication and immune evasion exhausts the immune system leading to opportunistic infection and AIDS. *From immunopaedia.org.*

4.2.2.2. Cytotoxic $CD8^+$ T-cell responses

The cellular immune response plays a critical role in controlling HIV replication, especially through the $CD8^+$ T-cytotoxic potential, which is responsible for mediating the elimination of infected $CD4^+$ T-cells, contributing to immunological control of the infection [321]. Strong and early $CD8^+$ cytotoxic T-cell and $CD4^+$ T-helper cell responses directed toward HIV-infected cells appear to curb HIV pathogenesis [184].

Antigen-specific $CD8^+$ T cells are a heterogeneous population capable of performing multiple functions and responses, including production of cytokines and chemokines, cytolytic effector molecules, and antigen-specific lysis of major

histocompatibility complex (MHC) class I matched target cells. The majority of responding $CD8^+$ T cells exerts multiple functions following stimulation against a variety of viruses.

One of the most important functions of $CD8^+$ T cells is to recognize and kill infected cells. This function has been shown to be important for control of several infections, including EBV [54], CMV [322, 323], hepatitis B virus (HBV) [324], and hepatitis C virus (HCV) [325]. $CD8^+$ T cells predominantly mediate killing through the secretion of granzymes and perforin upon recognition of viral epitopes in the surface of infected cells [326, 327]. Both of these proteins are contained within lytic granules and are released early after $CD8^+$ T-cell activation into the immunological synapse formed between the $CD8^+$ T cell and the target cell. This process of degranulation is MHC class I-restricted and antigen-specific and likely plays an important role in control of viral infection *in vivo* [328]. $CD8^+$ T cells can also mediate killing by the Fas-Fas ligand (FasL) pathway.

Evidence that cytotoxic $CD8^+$ T lymphocytes are important for control of HIV replication comes from both HIV infection in humans and simian immunodeficiency virus (SIV) infection in non-human primates. First, the resolution of peak viremia during acute HIV infection is temporally associated with the expansion of HIV-specific $CD8^+$ T cells [329, 330]. Second, immunological pressure exerted HIV- and SIV-specific $CD8^+$ T cells is linked to the emergence of viral escape mutations during acute and chronic infection [331-335]. Third, there is a strong correlation between specific MHC class I alleles and non-progressive infection in both humans and rhesus macaques [336-338]. Finally, experimental depletion of $CD8^+$ T cells in SIV-infected rhesus macaques results in a concomitant loss of control of viral replication [339, 340].

Despite a clear role for $CD8^+$ T cells in the initial control of HIV replication, a correlate of protection has remained elusive. Much of the research in the field has focused on HIV seropositive individuals who maintain normal $CD4^+$ T-cell counts and are clinically healthy for 10 or more years (long-term non-progressors), and those who control viral replication to below the limit of detection (elite controllers or

ECs). Studies seeking to define the mechanism(s) of control have largely relied on the comparison of HIV-specific $CD8^+$ T-cell responses from ECs to those from chronic progressors (CPs). Characterization of these responses has evolved over the past two decades and has provided some of the first clues to cellular mechanism(s) of control.

While an inverse relationship between simple frequency of HIV-specific $CD8^+$ T cells and plasma viral load was initially established on the basis of tetramer staining [341], this finding was not supported by subsequent studies that found no relationship between the frequency of IFN-γ-producing HIV-specific $CD8^+$ T cells and HIV viral load [49, 85, 342]. It was proposed that these disparate findings were the result of a significant portion of the circulating tetramer-staining $CD8^+$ T-cell population being functionally impaired [70, 280]. While early studies in this area have shown that the majority of tetramer-positive HIV-specific $CD8^+$ T cells can produce IFN-γ [343, 344], high expression of inhibitory markers, including PD-1, CD160, 2B4, and Lag-3 on HIV-specific $CD8^+$ T cells may indicate some degree of functional insufficiency [72, 345, 346].

4.2.2.3. Neutralizing antibodies

Humoral responses against HIV comprise the production of both neutralizing and non-neutralizing antibodies, especially against the HIV-1 envelope glycoprotein (Env). The HIV-1 Env is highly immunogenic and induces antibodies against its subunits gp41 and gp120 shortly after infection. Soon after HIV discovery, these antibodies were shown to block infection of cells or inhibit cell-to-cell spread *in vitro* [347, 348].

The hierarchy of the humoral immune response during acute HIV-1 infection has recently been studied in detail [29, 349]. The first HIV-1 Env-specific antibodies are observed as early as 8 days after plasma viremia is detected [349]. The initial humoral immune response is non-neutralizing and does not select for viral escape. Over time, virus neutralizing antibodies (Nabs) develop in most HIV-1-infected

individuals, demonstrating that the humoral immune system can target epitopes on the native viral spike. The initial specific NAbs against the autologous virus and antibodies involved in cellular cytotoxicity are generally not detected until more than 3 months after infection [350-353]. Most NAbs are directed to the gp120 subunit of the HIV-1 Env and they are highly strain specific. Thus, NAbs exert a strong immune pressure on the autologous virus, but display little breath of activity against heterologous viruses. Initial cross-reactive NAb may arise after several months of infection, but the more broadly reactive NAb that targets highly conserved epitopes are generally not found until more than 2 years of infection [352, 353]. Therefore, it is important to note that HIV-infected individuals are able to develop potent antiviral NAb responses against HIV-1, normally already ancestral, once the NAb responses mature.

The structural and functional analysis of HIV-1 NAbs b12, 2F5, and 4E10 led to some pessimism about the potential of the immune system to generate broadly neutralizing antibodies (BNAbs) against HIV-1 [354]. This concern was highlighted by the observed lack of natural immunity to HIV-1 [355] and by studies demonstrating the ability of HIV-1 Env to continuously evolve to evade the autologous NAb response [350, 351]. Additionally, as the full genetic diversity of HIV-1 was appreciated, formidable immune evasion mechanisms that are intrinsic to the HIV-1 Env were also highlighted, including carbohydrates masking of Env [356-358], only transient exposure of receptor binding sites or gp41, or limited spatial accessibility to antibody molecules [356, 358].

This pessimistic view of the HIV-1 humoral immune response started to change after the observation that some HIV-1-infected individuals are able to mount NAb responses that are more potent and cross-reactive than those generated by current vaccine immunogens [359-361]. Several research groups reported that sera of HIV-1 infected subjects could potently neutralize genetically diverse HIV-1 strains (broad neutralization capacity) [352, 362-364]. Early work assessing serum-mediated HIV-1 neutralization was often not accurate enough and generated high variability among studies, due to several technical limitations [365-368]. Starting in

2005, a concerted effort to generate accurate high-throughput neutralizing assays was initiated, based on the construction of recombinant Env-pseudoviruses that mediated a single round of viral infection [369, 370]. The advantage of such assays was that each pseudovirus contained a well-defined clonal sequence and that large panels of diverse HIV-1 Envs could be rapidly constructed, thus better representing the global genetic diversity of HIV-1. These assays allowed the screening of large panels of HIV-1 sera and the identification of individual donors whose sera were able to neutralize the majority of HIV-1 strains. Overall, these data revealed that there was a heterogeneous spectrum of serum neutralization with some sera displaying weak neutralization and others able to neutralize most HIV-1 strains tested. Depending on the cohort tested and the definitions used, these studies revealed that between 10% and 25% of HIV-1-infected subjects generate relatively potent cross-reactive NAbs (reviewed in [371-373]).

The contribution of HIV-1 NAbs to disease progression is controversial [350, 374] and most cohort studies performed to date have not demonstrated a remarkable role of humoral responses in HIV disease outcome, although impairment of neutralizing responses have been observed in some case reports of highly progressive disease.

A necessary component of most effective viral vaccines is the induction of virus-specific antibodies that inactivate or neutralize the invading pathogen [375]. Since HIV-1 discovery, great efforts have been performed through preventive vaccination, and the specific viral proteins that would be the target of potentially protective antibodies have been studied in detail. It is now clear that HIV-1 is different from other viruses for which successful vaccines have been made. Furthermore, the ability of Env to shield vulnerable regions from NAbs provides yet another obstacle for vaccine design [357, 358, 371]. Thus, HIV-1 poses a unique set of impediments to the induction of fully protective immune responses and, therefore, also to effective vaccine development.

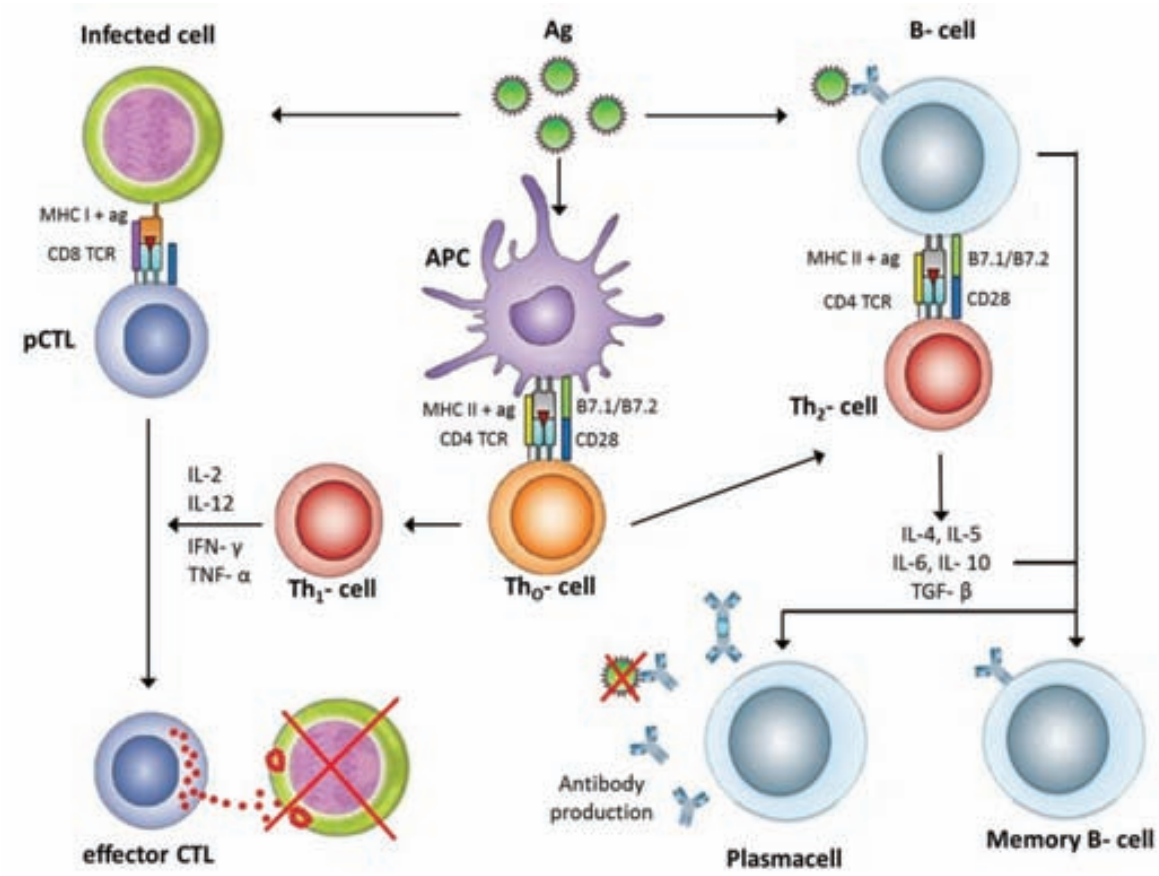

Figure 12. Overview of the adaptive immune responses after virus recognition by antigen presenting cells. Virus antigens are presented by dendritic cells and B cells to T cells. Infected cells present peptides together with MHC I molecules on the plasma membrane. The peptide-MHC I complex is recognized by precursor CTLs. Th1 cells, induced by antigen presenting cells, produce IL-2, IFN-γ, and TNF-α. This results in activation and differentiation of the precursor CTLs into memory or effector CTLs. Effector CTLs can directly kill infected cells by the production of perforines and granzymes. Activated Th2 cells, also induced by antigen presenting cells, produce B cell stimulating cytokines (including IL-4, IL-5, IL-6, IL-10, TGF−β) that activate naive B cells. This facilitates/induces B cell differentiation into memory B cells and plasma cells that produce large amounts of IgG, IgA, IgE antibodies that prevent further virus infection.
Ab: antibody, Ag: antigen, APC: Antigen Presenting Cell, DC: Dendritic Cell, IL: Interleukin, TCR: T Cell Receptor, Th: CD4+ T helper cell. *From De Haes et al., Immunodeficiency 2012; available from: http://www.intechopen.com/books/immunodeficiency/-wrapped-up-vaccines-in-the-context-of-hiv-1-immunotherapy.*

Chapter 2

HIPOTHESES & OBJECTIVES

Hypotheses

1. Extremely severe progression of HIV disease is a very rare phenotype that might be driven by a complex interplay between viral and host factors. This phenotype might be characterized by infection with highly pathogenic strains.
2. Plasma primary HIV-1 isolates might be different to those from peripheral blood mononuclear cells and thus generate different results, owing to compartmentalization and latency.
3. RPs generally have specific genetic, immunogenetic, and transcriptomic traits that distinguish them from other phenotypes of HIV-1 infection.
4. RPs could be more prone to infection by highly pathogenic variants with a particular adaptation/escape profile for host immunity, thus favoring the rapid CD4 loss observed.
5. The immune system of RPs could be seriously compromised, owing to different clinical and immunogenetic factors.

Objectives

1. To determine the main viral and host factors involved in severe progression of HIV-1.
2. To define the clinical selection criteria for rapid progressors, standard progressors, and viremic nonprogressors and generate well-defined cohorts that can provide data and biological samples.
3. To evaluate our laboratory techniques for obtaining whole-virus primary isolates in order to take greater advantage of available samples.
4. To identify genetic and transcriptomic markers of rapid progression of HIV-1 infection, in comparison to other disease phenotypes.
5. To define the role of viral factors (eg, coreceptor usage and replicative capacity) in rapid progression of HIV-1 infection.
6. To study the host immunogenetic factors and immune responses involved in rapid progression.

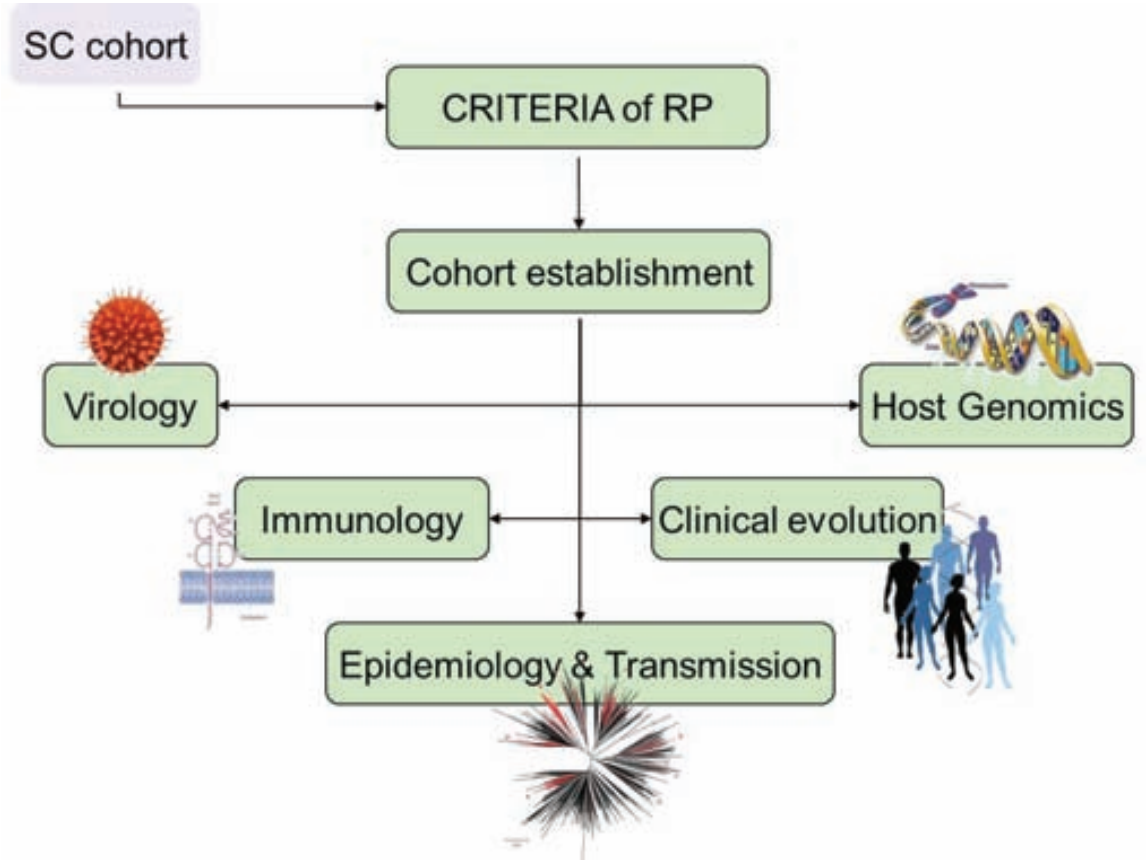

Figure 13. Project flow-chart. In order to elucidate the factors involved in rapid disease progression, a comprehensive set of studies were designed, after the Cohort of Rapid Progressors (CoRP) was established.

Chapter 3

RESULTS

Chapter 3a

STUDY I

Contribution of immunological and virological factors to extremely severe primary HIV type 1 infection

This chapter corresponds to the manuscript:

Dalmau J., Puertas M.C., Azuara M., Mariño A., Frahm N., Mothe B., Izquierdo-Useros N., Buzón M.J., Paredes R., Matas L., Allen T.M., Brander C., Rodrigo C., Clotet B., Martinez-Picado J. ***Contribution of immunological and virological factors to extremely severe primary HIV type 1 infection***. *Clinical Infectious Diseases*. 2009; 48: 223-232.

PRESENTATION

The first study included in this thesis is the origin of the project. In 2007, two special cases of extremely severe primary HIV-1 infection were detected in two different hospitals in Spain. These patients presented symptoms of advanced HIV-disease, despite the fact that laboratory tests indicated that they were at primary HIV-infection stage. Therefore, great efforts were carried out in order to elucidate the factors involved in such a severe phenotype, in order to give as much information as possible to the clinicians working in the case and to investigate the pathogenic factors behind these presentations for its great interest in the HIV pathogenesis field.

INTRODUCTION

Symptoms of acute retroviral syndrome typically coincide with high-level viremia and the induction of the host's initial adaptive immune response [329, 376]. However, clinically severe presentations during acute HIV-1 infection, including AIDS-defining symptoms, are considered to occur infrequently [377]. Furthermore, epidemiological studies have revealed that, in the absence of treatment, in <0.5% of HIV-1–infected individuals, the infection progresses to AIDS within 1 year after primary infection [86]. A complex interplay between multiple viral and host factors is most likely to be involved in accelerating disease progression. Among these myriad factors, CXCR4 tropism has been associated with high virulence [155]. Moreover, HLA class I concordance between individuals and the inability to elicit specific cytotoxic T lymphocyte (CTL) responses have been suggested to increase the rate of transmission of HIV-1 infection and disease progression [378, 379].

In our study, we investigated the immunological and virological factors that contributed to the development of AIDS-defining pathogenesis in 2 independent case reports of unusually severe, acute, sexually-transmitted HIV infection. The clinical and diagnostic outcomes are shown in figure 10.

PATIENTS, MATERIALS AND METHODS

The study participants provided written informed consent to participate in this study, which was approved by the institucional review boards of the hospitals where the participants received medical care. HIV-1 was isolated from the patients' PBMCs, and the viral stocks were titrated in TZM-bl cells [380].

Coreceptor usage of primary HIV-1 isolates was assessed by infection of U87.CD4 cells expressing either CCR5 or CXCR4 [381]. Syncytia induction was determined *in vitro* in MT-2 cells. CCR5 was genotyped in genomic DNA extracted from cryopreserved PBMCs to detect the Δ32 deletion. The growth rate of the viral isolates was determined by infecting phytohemagglutinin-stimulated donor PBMCs [382]. To assess the presence of drug resistance–associated mutations, we sequenced the HIV protease region (codons 1–99) and reverse-transcriptase region (codons 40–247) from a plasma sample obtained before the initiation of antiretroviral therapy.

To determine whether the index patient in case report 2 harbored the same virus as the suspected source patient, viral RNA was extracted from plasma. The *pol* (protease and first 235 codons of the reverse transcriptase) and *env* (C2 to V5 regions) genes were sequenced [382, 383]. In addition, a total of 46 molecular clones encompassing the *env* gene were used to estimate diversity in the plasma viral RNA for the source and index patients [383]. Sequence alignments were obtained using Sequencher, version 4.6 (Gene Codes), and ClustalW and were manually edited in the regions of variable length. Genetic distances and evolutionary rates were computed using a Kimura 2–parameter model. Neighbor-joining phylogenetic trees of each patient's *pol* and *env* sequences were constructed using MEGA3. The reliability of phylogram clustering was assessed by bootstrapping analyses. Coreceptor use was inferred from *env* clonal sequences with use of phenotype prediction Tools [126].

HLA class I and class II genotypes were identified by high resolution sequencing in an approved clinical laboratory. HLA class I supertype assignment was based on

functional classification for the many different 4-digit, high-resolution HLA alleles that overlap in their peptide-binding specificities [384].

Cellular immunity to HIV and Epstein-Barr virus (EBV) was assessed by IFN-g enzyme-linked immunospot assays. T cell responses to an overlapping peptide set spanning the entire HIV clade A and clade B protein sequence were detected [385]. In addition, optimal epitopes known to be presented by the patients' HLA class I alleles were either included in their clade-specific consensus version or based on sequence variants identified in the index or the source patient. To assess general immune reactivity, 3 peptide pools containing a previously described set of EBV-derived optimal CTL epitopes were also tested [386]. Positive responses were determined on the basis of specific cutoffs that are defined elsewhere [387].

CASE REPORTS

Case report 1

A 26-year-old Spanish man with a history of recurrent conjunctivitis and mild bronchial asthma who reported having had only 1 heterosexual partner during the previous 2 years was admitted to a hospital in Ferrol, Spain, for progressive dyspnea, tachypnea, tachycardia, fever, and nonproductive cough. Four months before hospital admission, the patient had received a diagnosis of leukopenia but had negative results of HIV serological examination. Four weeks before hospital admission, he developed maculopapular rash, conjunctivitis, balanitis, and oral thrush.

At hospital admission, laboratory testing revealed leukocytosis, bilateral alveolar-interstitial lung infiltrates, and severe hypoxia. Two days later, he fulfilled the criteria for acute respiratory distress syndrome and required orotracheal intubation, mechanical ventilation, and broadspectrum antibiotic therapy. An EIA was weakly positive for HIV-1 antibodies, and the Western blot results were undetermined. *Pneumocystis jiroveci* was detected in a bronchoalveolar lavage specimen, and *Candida albicans* was isolated from oropharyngeal exudates. The patient's plasma

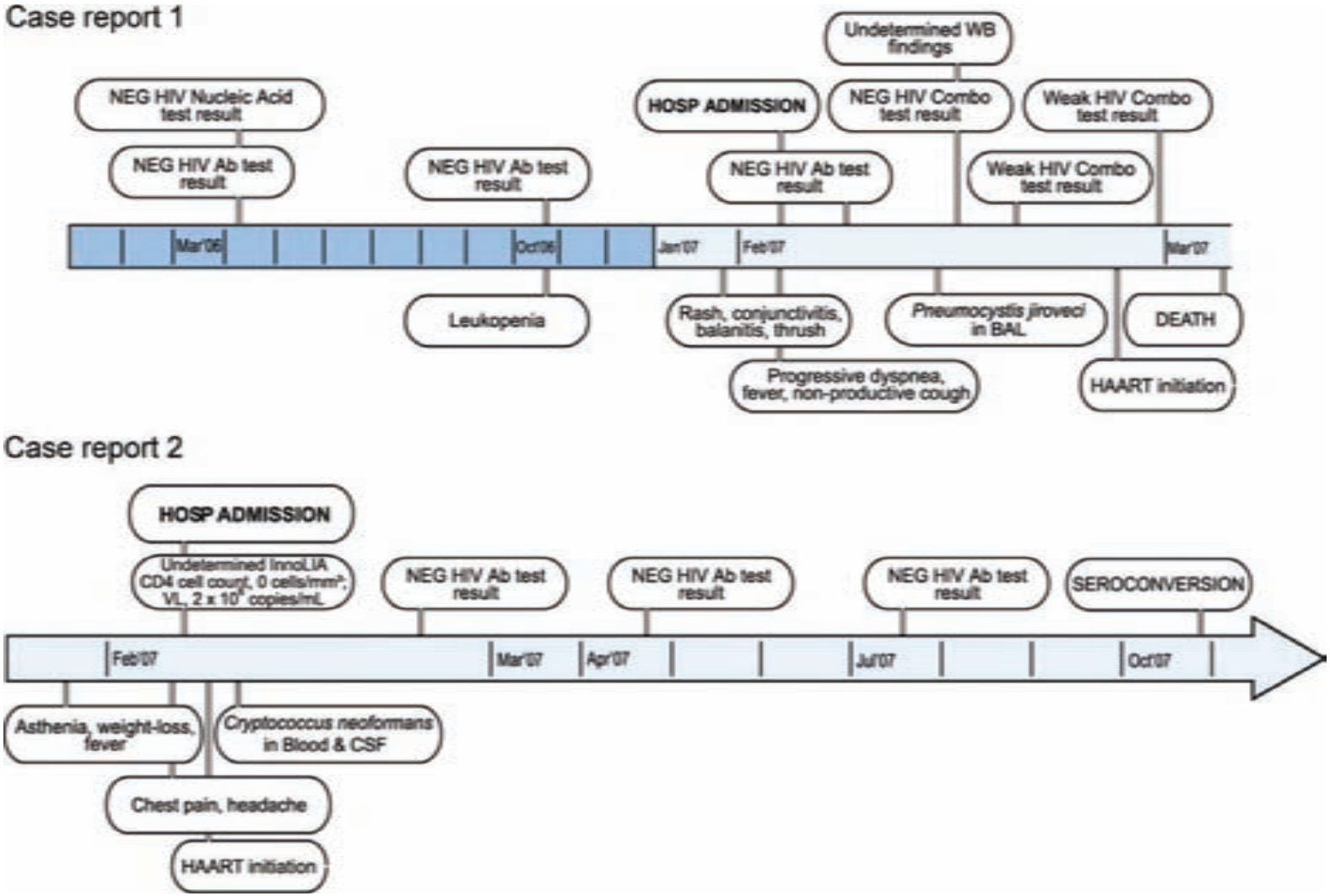

Figure 14. Description and outline of clinical and diagnostic outcomes in the 2 case reports of sexual transmission of severe primary HIV-1 infection in this study. Ab, antibody; HOSP, hospital; NEG, negative; WB, Western blot.

HIV-1 RNA level was 31,600 copies/mL, and his $CD4^+$ T-cell count was 108 cells/mm3. Antiretroviral therapy with tenofovir, emtricitabine, lopinavir-ritonavir, and enfuvirtide was initiated. The acute respiratory distress syndrome was refractory to treatment, and the patient died of multiorgan failure 3 weeks after hospital admission.

Case report 2

Case report 2 involved an index patient and the presumed source patient. The index patient was a 15-year old girl from Ecuador who was admitted to a hospital in Badalona, Spain, with fever, severe headache, fatigue, malaise, severe odynophagia, and a 4-day history of continuous retrosternal chest pain. She had a 1-month history of asthenia, a 2-kg weight loss, and fever. Her previous medical history was unremarkable. The index patient reported having had only 1 regular sexual partner during the previous 2 years.

At hospital admission, she was febrile and had enlarged bilateral cervical lymph nodes, oral thrush, and soft, non-swollen hepatomegaly (1.5 cm). *C. albicans* was subsequently isolated from oropharyngeal and vaginal exudates. *Haemophilus influenzae* (biotype 2) was isolated from a sputum sample. Serological testing was reactive for IgG against cytomegalovirus and *Toxoplasma gondii.* An EIA for HIV-1 antibodies was weakly reactive, and a line immunoassay for detection of IgG antibodies against HIV-1/2 disclosed only 1 band for gp41 in 2 independent samples obtained 5 days apart. The index patient's HIV-1 RNA level was $2x10^6$ copies/mL, and her $CD4^+$ T-cell count was 0 cells/mm^3. The patient initiated therapy with tenofovir, emtricitabine, and lopinavir-ritonavir. Three days later, she developed cognitive deficits. Cranial MRI revealed ischemic and hemorrhagic supratentorial and infratentorial lesions in the cortical-subcortical interface and basal ganglia. *Cryptococcus neoformans* was isolated from blood and CSF cultures.

The patient had a favorable clinical course with appropriate treatment. Two months later, her HIV RNA level was undetectable, and her $CD4^+$ T-cell count was 51

cells/mm^3 (CD4$^+$ T-cell percentage: 3%). Despite repetitive antibody testing, complete seroconversion did not occur until 9 months after initial presentation.

The index patient's partner, presumably the source patient, was a 23-year-old man who was also from Ecuador. He confirmed having had several sexual partners, but he had never been tested for HIV-1 infection. EIA and line immunoassay were reactive for HIV-1. His plasma HIV-1 RNA level was 40,000 copies/mL, and his CD4+ T cell count was 32 cells/mm^3 (4%). The source patient initiated therapy with tenofovir, emtricitabine, and lopinavir-ritonavir. Nine months later, his HIV RNA level was undetectable, and his CD4$^+$ T cell count was 130 cells/mm^3.

RESULTS

Case report 1

Laboratory assessment of the patient indicated a change in HIV-1 antibody reactivity near the time of presentation. The results of 3 previous assays (HIV-1 antibody–, nucleic acid–, and antigen-based assays) performed within 9 months before presentation were negative. Antibody and antigen tests and Western blots became partially reactive, and plasma HIV-1 RNA was present at the time of presentation, suggesting HIV-1 primary infection (figure 10 and table 1).

The replication-competent virus isolated from PBMCs was able to infect and replicate in both CCR5 and CXCR4-U87.CD4 cells, as concluded from the p24 antigen production and the formation of syncytia in the cell cultures (figures 15*A* and 15*B*).

The patient did not have a Δ32 genotype in the CCR5 chemokine receptor gene that might have explained an early selection of CXCR4-tropic viruses [388]. The production of p24 antigen in growth kinetics cultures of donor PBMCs was similar to the production seen with the laboratory-adapted viral strain HIV-1$_{NL4–3}$ (fig. 15*C*). Phylogenetic analyses with boot-scanning methods for the genetic subtyping of *pol* indicated the presence of a subtype B virus. The HIV-1 genotype had no drug resistance–associated mutations. The results of HLA typing are shown in table 1.

Table 1. Laboratory assessment of the patients involved in 2 case reports of sexual transmission of severe HIV-1 infection.

Variable	Case report 1: index patient	Case report 2	
		Index patient	Source patient
Plasma HIV-1 RNA level, copies/mL	32×10^4	2×10^6	4×10^4
$CD4^+$ T cell count, cells/mm^3	108	0	33
$CD4^+$ T cell percentage	27	0	4
$CD8^+$ T cell count, cells/mm^3	Not performed	203	481
$CD8^+$ T cell percentage	Not performed	59	59
Result of standard Ab HIV test	Negative	Weak[a]	Positive
HIV Western blot findings	Result undetermined	Result undetermined	Positive
Result of nucleic acid and/or viral load test	Positive	Positive	Positive
Plasma p24 level, pg/mL	6.3	72.6	12.5
Viral subtype	B	AG	AG
Drug resistance genotype			
Protease	K20M and M36I	L10V, I13V, G16E, M36I, H69K, and L89I	L10V, I13V, G16E, K20I, M36I, H69K, and L89I
Reverse transcriptase	None	None	None
Coreceptor use[b]	R5/X4	R5/X4	R5/X4
CCR5 Δ32 genotype	WT/WT	WT/WT	WT/WT
HLA alleles			
Class I[c]	A*0201 (A2), A*1101 (A3) B*3503 (B7), B*4001 (B44) Cw*0304, Cw*0401	A*0201 (A2), A*0301 (A3) B*0702 (B7), B*1801 (B44) Cw*0702, Cw*1203	A*6802 (A2), A*6801 (A3) B*5101 (B7), B*5301 (B7) Cw*0401, Cw*1502
Class II	DRB1*0701, DRB1*1302 DQB1*0202, DQB1*0604	DRB1*0405, DRB1*1201 DQB1*0301, DQB1*0302	DRB1*0101, DRB1*0701 DQB1*0202, DQB1*0501

NOTE. Ab, antibody; WT, wild type.

[a] Different HIV antibody tests provided nonreactive or weakly reactive results.

[b] Viruses from all of the patients were syncytia-inducing viruses.

[c] Supertypes are shown in parentheses (A*68 and B*53 [increased susceptibility] and B*51 [increased protection]).

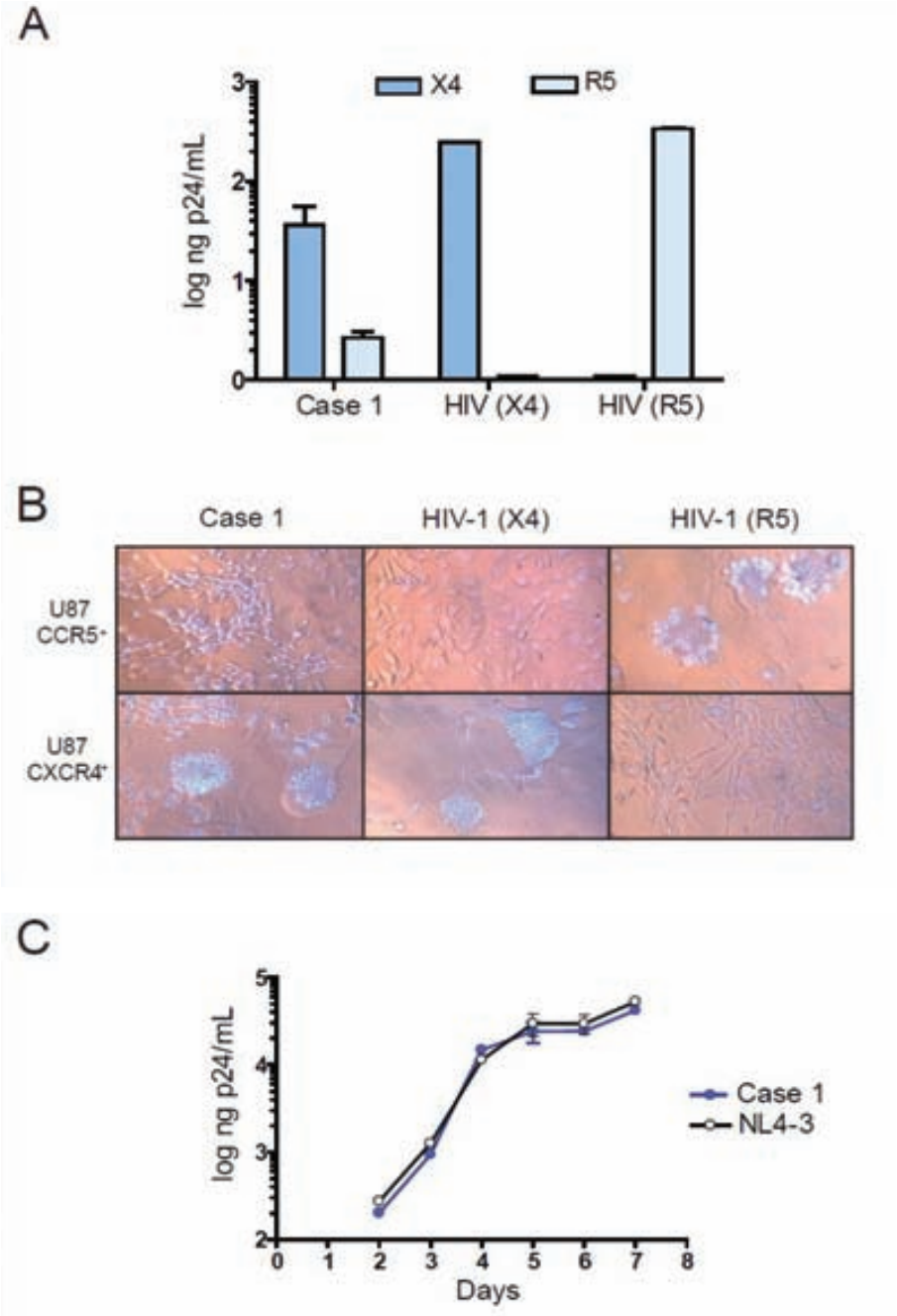

Figure 15. Virological data for case report 1. Viral coreceptor use based on p24 production *(A)* and syncitia formation *(B)* in U87.CD4 cells expressing either CXCR4 or CCR5. Control viral strains HIV-1_{NL4-3} (CXCR4-tropic, syncitia inducer) and HIV-1_{NFN-SX} (CCR5-tropic, nonsyncitia inducer) were included in both assays. *C.* Viral replication growth rates in phytohemagglutinin-stimulated primary donor PBMCs infected with the patient's viral isolate. The laboratory-adapted HIV-1_{NL4-3} reference strain was grown in parallel. One of 2 representative experiments with PBMCs from 2 different donors is shown.

Case report 2

Clinical symptoms and analytical results for the index patient were consistent with a diagnosis of advanced HIV-1 infection and AIDS. However, the patient denied HIV risks other than sexual contact with her partner during the previous 2 years. The patient's mother tested negative for HIV-1 infection, thus excluding potential vertical transmission.

Moreover, the viruses isolated from the source and index patients were similar both phenotypically and genotypically (figure 16 and table 1). Laboratory assessment of the index patient's original sample provided clear reactivity data on the presence of HIV-1 antigens, but despite high levels of immunoglobulins, antibody-based tests showed partial reactivity, indicating a lack of HIV-1–specific antibodies (figure 14 and table 1). Bootstrap analysis of *pol* and *env* sequences from the index and source patients revealed values of ≥99% in 1000 replicates (figure 16*A* and 16*B*), indicating that sequence clustering was unlikely to have occurred by chance. The genetic distance between the *pol* sequences from the index and source patients was <0.1%, whereas the mean genetic diversity between randomly selected sequences from local, epidemiologically unrelated HIV-infected individuals with the CRF02-AG subtype was 3.0%. The high degree of similarity between viral sequences indicates a likely viral transmission from one patient to the other. Clonal analysis of *env* sequences indicated that all sequences from the index patient were closely related (mean diversity, 1.8%), and the source patient's sequences had a mean diversity of 2.4% (figure 16*C* and 16*D*).

After 5 days of culture, the viruses isolated from the source and index patients were able to infect and replicate in both CCR5 and CXCR4-U87.CD4 cells, as indicated by the p24 antigen production and the formation of syncytia in the cell cultures (figure 16*E* and 16*F*). Neither of the individuals showed a Δ32 genotype in the CCR5 chemokine receptor gene. Phenotypic inference of the V3 amino acid sequence in multiple clones from each individual suggests that all clones from the index patient could use CXCR4 (R5X4 tropism) for viral entry, whereas the source patient had clones that could use only CCR5 and clones that could use CXCR4 (R5X4 tropism) (figure 16*C*).

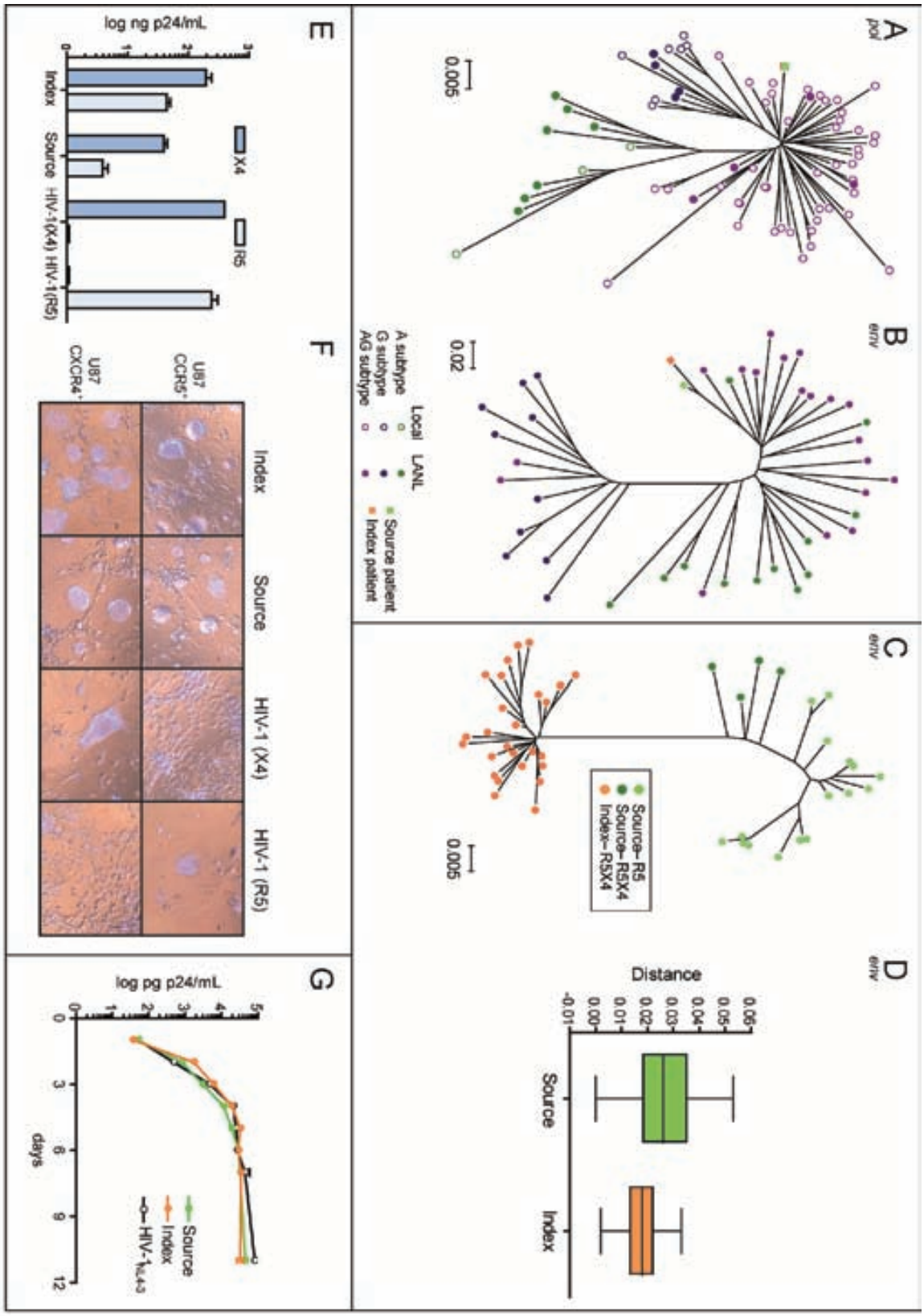
A
pol
0.005
B
env
0.02
A subtype
G subtype
A/G subtype
Local
LANL
Source patient
Index patient
C
env
Source- R5
Source- R5X4
Index- R5X4
0.005
D
env
Distance
Source
Index
E
log ng p24/mL
X4
R5
Index
Source
HIV-1(X4)
HIV-1(R5)
F
U87 CCR5+
U87 CXCR4+
Index
Source
HIV-1 (X4)
HIV-1 (R5)
G
log pg p24/mL
days
Source
Index
HIV-1NL4-3

Figure 16. Virological data for case report 2, including data on samples from the source patient and index patient. Neighbor-joining phylograms of pol *(A)* and env *(B)* sequences derived from viral RNA in plasma are shown. The scale for the genetic distance is based on the Kimura 2-parameter method. *C*, Phylogenetic analysis of the env C2V3 clonal sequences derived from the index and source patients' viral isolates, with different color patterns to represent the virtual tropism of each clone. *D*, Intrapatient diversity. Viral coreceptor use based on p24 production *(E)* and syncytia formation *(F)* in U87.CD4 cells expressing either CXCR4 or CCR5 are shown. Control viral strains HIV-1_{NL4-3} (CXCR4-tropic, syncytia inducer) and HIV-1_{NFN-SX} (CCR5-tropic, nonsyncytia inducer) were included in both assays. *G*, Viral replication growth rates in phytohemagglutinin-stimulated primary donor PBMCs infected with the patients' viral isolates. The laboratory-adapted HIV-1_{NL4-3} reference strain was grown in parallel. One of 3 representative experiments involving different PBMC donors is shown.

The production of p24 antigen in growth kinetics cultures in PBMCs was equal in the 2 viral isolates and comparable to that in the laboratory-adapted viral strain HIV-$1_{NL4–3}$ (figure 16*G*). Genetic subtyping of the *gag, pol,* and *env* genes in the patients' viruses indicated that both patients had the AG circulating recombinant form 02 (CRF02-AG). Drug-resistance genotyping revealed no drug resistance–associated mutations in the reverse transcriptase. Several polymorphisms were detected in the protease gene; this might have been associated with possible tipranavir resistance in non–subtype B viruses (table 1).

Cellular immune responses. The patient in case report 1 showed a single weak response against 1 overlapping peptide that was not subsequently observed in the reconfirmation test and only a borderline response to 1 EBV-peptide pool. This atypical lack of EBV-specific CTL responses suggests a widespread impairment of the ability to mount adequate CTL responses [387].

The index and source patients in case report 2 expressed HLA class I alleles that were highly related. In fact, 3 of the 4 HLA-A and -B alleles had matched HLA supertype for both patients (table 1). Because the transmission of escape mutants arising in the source patient may have prevented the induction of an effective T-cell response in the index patient, the cellular immune response to the entire viral proteome was assessed in both patients at 1 and 9 months after the transmission event. The analyses included 2 comprehensive sets of overlapping peptides

spanning HIV clade A and clade B consensus sequences, as well as autologous peptide variants (from the index and source patients) of optimally defined epitopes presented by the patients' HLA types (table 2).

Table 2. Optimal epitopes showing sequence diversity between viruses from the source and index patients on case report 2.

Epitope	Protein	HXB2 position	Reference	Sequence
A02 AL9	Vpr	59-67	con B	AIIRILQQL
			source/index	AIIRILQQL
			ConAG	AIIRILQQL
A02 FK10	Gag	70-79	con B	FLGKIWPS**Y**K
			source/index	FLGKIWPS**H**K
			ConAG	FLGKIWPS**Y**K
A02 GT9	Vpr	41-49	con B	GLGQHIY**E**T
			source	GLGQHIY**N**T
			index	GLGQHIY**D**T
			ConAG	GLGQHIY**E**T
A02 SL9	Gag	77-85	con B	SL**Y**NT**V**ATL
			source/index	SL**F**NT**I**ATL
			ConAG	SL**F**NT**I**ATL
A02 RI9	Vpr	62-70	con B	RILQQLLF**I**
			source/index	RILQQLLF**T**
			ConAG	RILQQLLF**V**
A02 RI10	Env	311-320	con B	**R**GPG**RA**F**VTI**
			source/index	**I**GPG**QT**F**YTI**
			ConAG	**I**GPG**QT**F**YAT**
A02 YV9	Pol	127-135	con B	YTAFTIPS**V**
			source/index	YTAFTIPS**L**
			ConAG	YTAFTIPS**V**
A03 AK9	Pol	158-166	con B	AIFQ**S**SMTK
			source/index	AIFQ**A**SMTK
			ConAG	AIFQ**A**SMTK
A03 RK9	Gag	20-28	con B	RLRPGGKK**K**
			source/index	RLRPGGKK**Q**
			ConAG	RLRPGGKK**K**
A03 RY10	Gag	20-29	con B	RLRPGGKK**K**Y
			source/index	RLRPGGKK**Q**Y
			ConAG	RLRPGGKK**K**Y
A03 TN10	Vpr	19-28	con B	TLELLEELK**N**
			source/index	TLELLEELK**H**
			ConAG	TLELLEELK**H**

Epitope	Protein	HXB2 position	Reference	Sequence
A68 DL9	Pol	30-38	con B	DTVLE**EWN**L
			source/index	DTVLE**EIN**L
			ConAG	DTVLE**IWL**L
A68 IV9	Pol	3-11	con B	ITLWQRP**L**V
			source/index	ITLWQRP**V**V
			ConAG	ITLWQRP**L**V
A*6801 DR11	Vpr	52-62	con B	DTW**A**GV**E**AIIR
			source	DTW**E**GV**M**AIIR
			index	DTW**E**GV**V**AIIR
			ConAG	DTW**E**GV**E**AIIR
A*6802 EV10	Vpr	48-57	con B	**E**TYGDTW**A**GV
			source	**N**TYGDTW**E**GV
			Index	**D**TYGDTW**E**GV
			ConAG	**E**TYGDTW**E**GV
B07 RI10	Env	298-307	con B	RP**N**NNTRK**SI**
			source	RP**S**NNTRK**NI**
			Index	RP**N**NNTRK**GV**
			ConAG	RP**N**NNTRK**SV**
B07 TL9	Gag	48-56	con B	TPQDLN**T**ML
			source/index	TPQDLN**M**ML
			ConAG	TPQDLN**T**ML
B18 FK10	Gag	161-170	con B	FRDYVDRF**Y**K
			source/index	FRDYVDRF**F**K
			ConAG	FRDYVDRF**F**K
B51 LI9	Env	416-424	con B	L**P**CRIKQII
			source/index	L**Q**CRIKQII
			ConAG	L**P**CRIKQII
B51 TI8	Pol	128-135	con B	TAFTIPS**I**
			source/index	TAFTIPS**L**
			ConAG	TAFTIPS**I**
B53 QW9	Gag	176-184	con B	QA**S**QEVK**N**W
			source	QA**T**QEVK**H**W
			index	QA**T**QEVK**N**W
			ConAG	QA**T**QEVK**N**W
B53 TL9	Gag	48-56	con B	TP**Y**D**I**N**Q**ML
			source/index	TP**Q**D**I**N**T**ML
			ConAG	TP**Y**D**I**N**Q**ML
X-GL12	Vpr	9-20	con B	GPQREP**H**NEWTL
			source/index	GPQREP**F**NEWTL
			ConAG	GPQREP**F**NEWTL

NOTE. Changes in the sequences are represented by boldface type.

The source patient showed weak responses to 3 different regions of the virus (table 3), which represented an overall weak response rate, compared with a median of 17 responses among 1300 previously tested patients with chronic HIV infection (authors' unpublished data) [385]. The index patient showed an even weaker HIV-specific T cell response to only a single peptide, which was detected before the patient received treatment but was subsequently lost 9 months after infection. Importantly, the index patient was able to mount a T-cell response to a peptide pool containing EBV-derived CTL epitopes, which indicated that the absence of HIV-specific T cells was not attributable to poor cell viability or a general immune incompetence in this individual.

Although some responses to autologous sequence variants that were not tested may exist, the data are in line with a remarkable absence of HIV-specific T cell immunity in both the patient in case report 1 and the index patient in case report 2. This absence of immunity may be related to the extraordinarily fast disease progression in these individuals.

Table 3. Cellular immune responses in patients involved in 2 case reports of sexual transmission of severe primary HIV-1 infection.

Patient, peptide	Protein	HXB2 position	Sequence	SFC per $1x10^6$ PBMCs
Case report 1: OLP 83 con B	Nef	118-135	**tQGYFPDWQNY**TPGPGIRY	47
Case report 2				
Source patient				
OLP 42 con A	Gag	172-189	LRAE**QATQEVKGW**MTETL	41
OLP 42 con B			LRAE**QASQEVKNW**MTETL	72
B53 QW9 con B	Gag	176-184	**QASQEVKNW**	97
B53 QW9 index/con A			**QATQEVKNW**	97
OLP 84 con A	Nef	126-143	NYTPGPGIRYPLCFGWCF	10
OLP 84 con B			NYTPGPGIRYPLTFGWCF	103
OLP 223 con A	Pol	583-600	QLEKDPIAGAETFYVDGA	52
OLP 223 con B			QLEKEPIVGAETFYVDGA	21
OLP 224 con A&B			GAETFYVDGAANRETKL	72
Index patient				
OLP 296 con A&B	Env	52-69	**LFCASDAKAY**DTEVHNVW	18
A24 LY10 con B&A/index	Env	52-61	**LFCASDAKAY**	24

DISCUSSION

The interplay between the viral and host factors influencing accelerated disease progression is complex and poorly understood. The 2 temporarily coincident case reports described here suggest immediate progression to AIDS from primary HIV-1 infection after sexual transmission. In both cases, the diagnosis of primary HIV-1 infection was supported by nucleic acid– and antigen-based screening tests, with an evolving antibody pattern. The patient in case report 1 tested negative for HIV several times before presentation and subsequent HIV-1 Western blot was only partially reactive. The index patient in case report 2 lacked previous negative test results but presented with very high plasma HIV RNA level, which is consistent with acute HIV infection [389]. In addition, HIV-1–specific antibody tests were only partially reactive and did not become positive until 9 months after the initiation of antiretroviral treatment. Although detection of HIV-specific antibodies after the onset of symptoms of primary infection can take 5–15 days [390], complete seroconversion may occasionally be delayed until 12 months after identification of infection by antigen testing, when virological control has been achieved with effective antiretroviral therapy [92, 390]. The fact that serum levels of IgG, IgM, and IgA (in the index patient in case report 2) were within the reference range or higher suggests polyclonal B cell activation [391]. Moreover, positive IgG responses to cytomegalovirus, *T. gondii,* and hepatitis A virus indicate the ability of antibodies to maintain an appropriate response against microorganisms that cause persistent latent infection. Although plasma viral load had greatly decreased with treatment, $CD4^+$ T-cell recovery increased slowly from total absence at presentation, which could have delayed HIV seroconversion.

The diversity of the viral population in HIV-1 *env* increases in parallel with divergence at a rate of 1% per year for a few years after seroconversion, before reaching a peak and then leveling off or decreasing [215]. Nevertheless, the rates of diversity are higher among patients who have a sharp decrease in their CD4 T-cell count [392]. In case report 2, the mean HIV-1 diversity was lower in the index patient than it was in the source patient, indicating that viral evolution took longer in the latter. Although viral diversity tends to decrease in the later stages of infection,

most of the genetic distances would remain 12% [215], thus supporting the direction of transmission in this pair and the theory of a very early presentation after viral transmission to the index patient.

The development of acute retroviral syndrome typically coincides with high-level viremia and the host's initial immune response. However, these 2 case reports reveal primary HIV-1 infection with unusually severe clinical symptoms. Other reports have described severe presentations during primary HIV-1 infection, including acute myopericarditis, renal failure, acute liver failure, and opportunistic infections [87-90], but viral and host factors have not been addressed in detail.

In both case reports, the virus isolated from the patients' PBMCs was able to use CCR5 and/or CXCR4 as entry coreceptors and to replicate very efficiently in phytohemagglutinin-stimulated donor PBMCs. These data indicate that both viral isolates were either dual-tropic viruses or a mixed population of CCR5-tropic and CXCR4-tropic viruses with high replication capacity. This observation would suggest that the transmitted virus had the ability to deplete $CCR5^+$ and $CXCR4^+/CD4^+$ T lymphocytes, which may help to explain the total loss of the $CD4^+$ T-cell population and rapid clinical progression observed in the index patient at the time of transmission. Infection with dual-tropic HIV-1 variants in injection drug users has been associated with an immediate and rapid decrease in total T-cell count and progression to AIDS within 4 years after the estimated time of infection [393]. Furthermore, patients who experienced seroconversion of $CCR5^{\Delta 32/\Delta 32}$ and showed the uncommon pattern of early syncytia-inducing virus and rapid decrease in $CD4^+$ T-cell count had a uniformly high viral load and dual-tropic coreceptor use [394]. A link between the detection of syncytia-inducing variants and a rapid decrease in $CD4^+$ T-cell count *in vivo* has already been established [395]. Despite the fact that the characterized viral subtype CRF02- AG in case report 2 is rather unusual in our area (1.1% of *pol* sequences tested for antiretroviral resistance during 1999– 2007), it is still the second most common non-B subtype. CRF02-AG is the predominant and most rapidly spreading HIV strain in West Africa and western Central Africa [225, 226], which raises concerns about its superior replication fitness and/or transmission efficiency. In fact, primary HIV-1 CRF02-AG

isolates from Cameroon exhibited higher *ex vivo* replicative fitness than did subtype A and G viruses from the same geographic region [227, 228]. These observations are consistent with the high replication rate that we observed in primary phytohemagglutinin-stimulated PBMCs, although we compared these rates with the rate of replication of the laboratory-adapted subtype-B HIV-$1_{NL4–3}$ strain. In our case reports, concurrent host factors may have also contributed to higher susceptibility to HIV-1 infection or disease progression. For example, specific HLA haplotypes have been proposed as an important risk factor in this context [396]. Among these, HLA-B*35, which is in high linkage disequilibrium with HLA-Cw*04, has been consistently associated with rapid progression to AIDS [253, 397, 398]. Specifically, the allele HLA B*3503, present in the patient in case report 1, has been reported to increase the risk of progression to AIDS by 2.7-fold (95% CI, 1.7–4.3; $P < 0.001$) [266]. In case report 2, the source patient expressed the HLA-A*68, HLA-B*53, and HLA-Cw04 alleles, which have been associated with rapid disease progression [396]. Although, none of the alleles in the index patient have been associated with accelerated disease progression, the donor and recipient expressed 3 of 4 HLA-A and HLA-B alleles that were in the same HLA supertypes (i.e., clusters of functionally related, 4-digit, high-resolution HLA class I alleles) [384]. This may have facilitated the transmission of viruses with CTL escape mutations, thus diminishing the number of epitopes recognized in the newly infected individual [255, 378, 379, 399].

Remarkably, optimal epitope variants representing autologous sequence diversity did not elicit a response, which suggests effective CTL escape (table 2). This hypothesis would fit with the fact that the index patient in case report 2 showed almost complete absence of HIV-1–specific CTL responses; this is rather unusual during primary HIV-1 infection. In a previous study, only 1 of 5 patients who presented with primary HIV-1 infection showed absence of precursor CTL specific for cells expressing viral proteins [329]. Another study involving acute and early HIV-infected patients reported a slightly higher breadth and magnitude of HIV-1–specific CTL responses [400]. However, that study used a less comprehensive pool of overlapping peptides, and contrary to our case reports, in which there was

a persistent absence of responses at month 9, CTL responses increased after 6–12 months of treatment. Although we could not identify the source patient in case report 1, the lack of HIV-1–specific CTL responses in the index patient might allow us to speculate a potential HLA class I concordance at transmission. Nonetheless, the inability to elicit HIV-1–specific CTL responses at the time of primary infection was paralleled in these 3 patients with AIDS-defining pathogenesis and severe clinical presentation. Although HIV-1–specific CTL responses have been considered to be a crucial factor in HIV disease progression, we had limited experimental and clinical evidence of the detrimental effect that the inability to elicit these responses might have in symptomatic primary HIV-1 infection [329, 330]. Moreover, the coincident inability in these 2 case reports for the patients to mount an effective adaptive immune response against HIV-1, albeit not to other pathogens, might be a consequence of a potential defect in the innate immunity.

Clearly, further studies involving these and other patients with accelerated disease progression will be needed to address these factors.

In conclusion, we describe 2 case reports of sexual transmission of highly replicative, dual-tropic HIV-1 of subtypes B and CRF02-AG that resulted in an aggressive clinical progression to severe symptomatic AIDS in young patients. Adaptive cellular and humoral immune responses in the host might have simultaneously failed to control the virus.

Chapter 3b

STUDY II

Comparative transcriptomics of extreme phenotypes of human HIV-1 infection and SIV infection in sooty mangabey and rhesus macaque

This chapter corresponds to the manuscript:

Rotger M.*, **Dalmau J.***, Rauch A.*, McLaren P.*, Bosinger S.*, Martinez R., Sandler N.G., Roque A., Liebner J., Battegay M., Bernasconi E., Descombes P., Erkizia I., Fellay J., Hirschel B., Miró J.M., Palou E., Hoffmann M., Massanella M., Blanco J., Woods M., Günthard H.F., de Bakker P., Douek D.C., Silvestri G., Martinez-Picado J.[#], Telenti A.[#]. ***Comparative transcriptomics of extreme phenotypes of human HIV-1 infection and SIV infection in sooty mangabey and rhesus macaque***. *The Journal of Clinical Investigation*. 2011; 121:2391-2400.

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PRESENTATION

The deep analyses of the 2 case reports of extremely severe HIV-1 infection lead us to interesting results, which required further confirmation and an extension in a larger cohort. Therefore, rapid progression criteria and a well-defined cohort were generated, and a full project to study different aspects of potential factors of this pathogenic profile was designed. The first part of the project consisted of a detailed genomic and transcriptomic analysis of RPs, in contrast to other phenotypes, especially those individuals having comparable viremia levels while maintaining normal CD4 counts (VNPs). In this section, the results for this first part of the project are presented.

INTRODUCTION

HIV infection leads to severe immunodeficiency in most infected subjects, in an average of 10 years; however, there are marked departures from this estimate. Attention has been directed at understanding the determinants of nonprogressive disease, as exemplified by the clinical course of long-term nonprogressors and of elite controllers [74, 401, 402]. The other extreme of the spectrum of disease — rapid progression — has been subject of much less research. Rapid progressors (RPs) can be defined by a number of criteria — generally including progressive immunosuppression soon after seroconversion and, in many cases, high levels of viremia [75, 93]. Limited data suggest that the concurrence of viral and host factors contributes to the severity of early disease [403]. There are, however, few such individuals in clinical cohorts — the main limitations for prospective recruitment are the need to identify patients with a known date of infection (seroconverters), and the short window of clinical observation before antiretroviral treatment is initiated. These constrain the availability of relevant biological material for study.

There are also very rare individuals that can tolerate very high viral loads, comparable to those of RPs, while maintaining stable $CD4^+$ T-cell counts for many years in the absence of treatment. Choudhary et al. [404] described 3 HIV-infected individuals with long-term asymptomatic disease who maintained stable CD4+ T

cell counts and low levels of immune activation, despite viral replication in the range of 104 to 105 HIV-1 RNA copies per ml of plasma. This profile of tolerance of viral replication is reminiscent of the pattern of SIV infection in the natural host. The importance of such model for the understanding of HIV/AIDS pathogenesis has been underscored by studies in sooty mangabeys and in African green monkeys [95-99]. Sooty mangabeys have nonprogressive disease despite chronic virus replication that is characterized by low levels of immune activation, while pathogenic SIV infection of rhesus macaques is associated with chronic immune activation. The consequences of immune activation include increased cell turnover, the skewing of lymphocytes toward more activated and differentiated subpopulations, and the induction of cellular exhaustion, senescence, and low renewal potential (reviewed in [94]).

The first goal of the present study was to explore a set of standard criteria to identify HIV-infected individuals presenting those 2 distinct clinical patterns: rapid progression and the contrasting setting of nonprogressive disease, despite prolonged and very high levels of viremia (extreme viremic nonprogressors [VNPs]). We then used immunogenetic, genomic, and transcriptomic tools and biomarkers to identify differences between those extreme groups as well as exploring genomic patterns previously defined in comparative studies of SIV infection in the pathogenic and the nonpathogenic models of rhesus macaques and sooty mangabeys, respectively [95-97]. The study revealed characteristic biomarkers and transcriptome patterns and highlighted several genes of relevance for the understanding of pathogenesis of HIV-1–induced immunosuppression.

MATERIALS AND METHODS

Ethics statement. All participating centers provided local institutional review board approval for genetic analysis, and each participant provided informed consent for genetic testing. The Institutional Review Boards are Comission d'Ethique de la Recherche Clinique, Faculté de Médecine, Université de Lausanne, Lausanne,

Switzerland, and Comitè Etic d'Investigació Clínica, Hospital Germans Trias i Pujol, Badalona, Spain.

Patients and definition of clinical profiles. Study participants were followed in the SHCS (www.shcs.ch) or at the HIVACAT. The selection criteria for RPs included a HIV seroconversion window of less than 1 year with documented negative and positive serology and either of the following possibilities: (a) more than 2 $CD4^+$ T-cell counts below 350 cells/µl within 3 years of seroconversion and no subsequent rise of $CD4^+$ T cells above 350/µl in the absence of combination antiretroviral therapy or (b) beginning antiretroviral therapy within 3 years of seroconversion and a $CD4^+$ T cell count within 1 month of starting antiretroviral therapy of less than 350/µl. $CD4^+$ T-cell values in the first 6 months after seroconversion were excluded to avoid the $CD4^+$ T-cell nadir during acute HIV infection. The selection criteria of VNPs included more than 3 years of follow-up, median HIV viremia from more than 3 measurements of more than 100,000 cp/ml, HIV viremia consistently above 10,000 cp/ml, a $CD4^+$ T cell count above 350/µl, and no HIV treatment during follow-up. Study candidates were identified by a standardized database search. Subsequently, the individual $CD4^+$ T cell profiles of all candidates were visually inspected before final inclusion. In addition to individuals fulfilling the definition of RP or of VNP, the study included 9 ECs as reference group.

Immunogenetics and genome-wide association analyses. High-resolution genotyping of HLA-A, HLA-B, HLA-Cw, and DRB1 alleles was performed by sequence-based typing methods. KIR gene typing was performed by a sequence-specific oligonucleotide probe using the Luminex microbead technology. For genome-wide association analysis, participants were genotyped using Illumina BeadChips Human660W-Quad. For quality control purposes, SNPs were removed based on their absence (locus absence >5%), minor allele frequency (>1%), and Hardy-Weinberg Equilibrium deviation ($P < 1\times10^{-6}$). Participants were filtered based on call rate, gender check (heterozygosity testing), cryptic relatedness, and population structure [405].

Cell isolation, RNA extraction, and transcriptome profiling. For transcriptome analysis, we included all RPs (n = 27) for whom viable cells were available from the time of seroconversion (>6 months to 3 years from acute infection) and before initiation of antiretroviral treatment. Samples from all VNPs were included. The CD4 and viral load values at the time of transcriptome analysis are presented in Table 6. $CD4^+$ and $CD8^+$ T cells were positively selected from frozen PBMCs (median time of cryopreservation was 1,485 [IQR, 821–2,558] days) using magnetically labeled $CD4^+$ or $CD8^+$ microbeads and subsequent column purification according to the manufacturer's protocol (Miltenyi Biotec). The median $CD4^+$ T cell purity, verified by flow cytometry, was 96.8% (range, 93.9%–98.9%), whereas the median $CD8^+$ T cell purity was 88.8% (range, 84.8%–92.1%). $CD4^+$ and $CD8^+$ T-cell viability was assessed by the trypan blue dye exclusion method using the Vi-CELL (Beckman Coulter). Total RNA was extracted from purified $CD4^+$ and $CD8^+$ T cells using the mirVana miRNA Isolation Kit (Ambion) according to the manufacturer's protocol for total RNA extraction. The amount of RNA was estimated by spectrophotometry using the Nanodrop 1000 (Thermo Fisher). RNA quality was determined by the Agilent RNA 6000 Pico Kit on an Agilent 2100 Bioanalyzer. Samples were collected between 1993 and 2008 and investigated in 2009. The median of $CD4^+$ T cell viability for samples that were successfully analyzed was 79% (IQR, 64%–87%). The median of $CD8^+$ T-cell viability for samples that were successfully analyzed was 82% (IQR, 74%–87%). Viability was minimally dependent on time of cryopreservation and more dependent on collection center. These covariates were assessed in the analyses. Target preparation was performed starting from 200 ng total RNA using the Illumina TotalPrep-96 RNA Amplification kit (Ambion). cDNA and cRNA were purified using the MagMAX Express Magnetic Particle Processor (Applied Biosystems). cRNA quality was assessed by capillary electrophoresis on the Agilent 2100 Bioanalyzer. Hybridization on HumanHT-12 v3 Expression BeadChips (Illumina) was carried out according to the manufacturer's instructions.

Transcriptome data analysis. Bead summary data were the output from Illumina's BeadStudio software without background correction, as this has

previously been shown to have detrimental effects [406]. Genes declared as nonexpressed (P > 0.01) were excluded from analysis. Data preprocessing, including quantile normalization and log2 transformation was completed in the Partek Genomics Suite package (Partek Inc.). Outliers were identified based on principal component analysis using 3 standard deviations as the cutoff for inclusion. For the differential expression analysis, we applied an empirical Bayes analysis approach, as implemented in the "limma" package of the R programming language, to model the variation profiles of all genes and used that information as prior knowledge to better estimate the variance of each gene expression [407].

The selected analysis included the following genes: APOBEC3H, BST2, EIF2AK2, IFI27, IFI35, IFI44, IFIH1, IFITM1, IFITM3, IRF1, IRF9, ISG15, JAK1, JAK2, MX1, MX2, OAS3, STAT2, TAP, TRIM22, TYK2, ZBP1, APOBEC3F, APOBEC3G, IFI6, IFIT1, IFIT3, OAS1, OAS2, OASL, PSMB8, PTPN2, RNASEL, STAT1, and TRIM5 as previously described [408].

Signature analysis and validation. Because of the rarity of individuals with a VNP profile, we used a heuristic approach to assess possible genetic markers associated with the clinical profile. This approach included the analysis of a preliminary signature, including genes identified as possibly associated with the VNP profile upon transcriptome analysis because of concordant signals in both $CD4^+$ and $CD8^+$ T cells as well as genes identified as potentially relevant in studies of SIV infection in the natural host: sooty mangabey and African green monkey. The signature was tested in an independent validation set of 153 individuals from a previous transcriptome analysis [408].

Pathway and network analyses. STRING (http://string.embl.de/) was used to identify known and predicted interactions (derived from 4 sources: genomic context, high-throughput experiments, coexpression, and previous knowledge). IPA (http://www.ingenuity.com/) and KEGG (http://www.genome.jp/kegg/pathway.html) were used for the analysis of pathway enrichment.

GSEA and gene set selection. The GSEA algorithm uses a Kolgorimov-Smirnov statistic to determine the significance of distribution of a set of genes within a

larger, ranked data set [409]. To evaluate the enrichment of SIV-inducible genes in the rhesus macaques and sooty mangabeys and in our human data set, we performed GSEA as follows: transcriptome data from VNPs and RPs were ranked according to their calculated Bayesian statistic; genes in which the mean was greater in VNPs were classified as positive, and genes with a greater mean in RPs were classified as negative. The data were ranked by the inverse Bayesian P value, resulting in a data set in which the most significant genes, overexpressed in VNPs, were listed at the top, and the most significant genes, overexpressed in RPs, were listing at the bottom. We next defined discrete query gene sets (Supplemental Table 4, Addendum) from a large microarray data set, detailing longitudinal SIV infection in rhesus macaques, which develop disease, and sooty mangabeys, a nonpathogenic, natural host species, described previously [96]. The ISG set comprised genes known to be regulated by type I interferon that were found to be differentially expressed in SIVmac239-infected rhesus macaques after 180 days of infection. The immune activation gene set was defined by multiple criteria: significant correlation of expression with lymphocyte activation assessed by circulating levels of $Ki67^{+}CD8^{+}$ T cells in SIVmac239-infected macaques (FDR = 0.0106), significant induction of expression assessed by ANOVA (FDR = 0.0075), a minimum of 2-fold upregulation in macaques at 1 or more time points, and expression in sooty mangabeys not exceeding 1.5x at any interval. To determine whether gene expression maintained chronically in VNPs shared similarity with that of sooty mangabeys, we defined the sooty mangabey chronic query gene set as follows: robust multiarray average log10 intensity values from baseline samples were subtracted from chronic timepoints for individual animals of both species, and 2-sample t test was performed on the subsequent fold-change data; genes with a higher average fold change in sooty mangabeys relative to that in rhesus macaques were ranked according to P value, with the top 50 most significantly overexpressed genes selected for gene set inclusion. GSEA was performed using the desktop module available from the Broad Institute (www.broadinstitute.org/gsea/). GSEA was performed on the pre-ranked human

data sets using 1,000 permutations, median collapse of duplicates, and random seeding.

Analysis of sCD14 levels. sCD14 levels were quantified in plasma samples using a commercially available ELISA assay (Diaclone). Plasma samples were diluted (1:50 or 1:100) and tested in duplicate. Plasma aliquots were collected either in EDTA (n = 55) or BD Vacutainer CPT Cell Preparation Tube with Sodium Citrate (CPT) tubes (n = 12). The CPT tubes contained a nonnegligible amount of molar sodium citrate solution (1 ml for the tubes, 8 ml draw capacity) and polysaccharide/sodium diatrizoate solution (FICOLL Hypaque solution; 2 ml for the tubes, 8 ml draw capacity), therefore samples collected with these tubes were considered to be diluted 1.44 times, and values were corrected accordingly.

Statistics. Comparisons of clinical and demographic characteristics used Fisher's exact tests for dichotomous variables and the Wilcoxon rank-sum test for continuous variables (STATA SE, release 11; StataCorp LP). In genome-wide association studies, association between genotype and phenotype (rapid progression) was tested using logistic regression, including top population principal components as covariates to correct for stratification. Genome-wide significance was assessed, using a cutoff of $P < 5\times10^{-8}$ to correct for multiple tests. In transcriptome analysis, we used a FDR method [410] to control for multiple testing. Probes selected for further analysis had an FDR-adjusted P value of less than 0.05. Statistical analyses dedicated to GSEA are detailed in the relevant section (see GSEA and gene set selection). Multiple regression analyses and graphical representations were performed by using the statistics package R (www.r-project.org).

Microarray data accession number. Microarray results have been deposited in the Gene Expression Omnibus database; the accession number is GSE28128.

RESULTS

Clinical and immunogenetic profiles

We identified 6 individuals that fulfilled strict clinical criteria of VNPs and had material available for analysis; plots of the infection course for each VNP individual are shown in Figure 17.

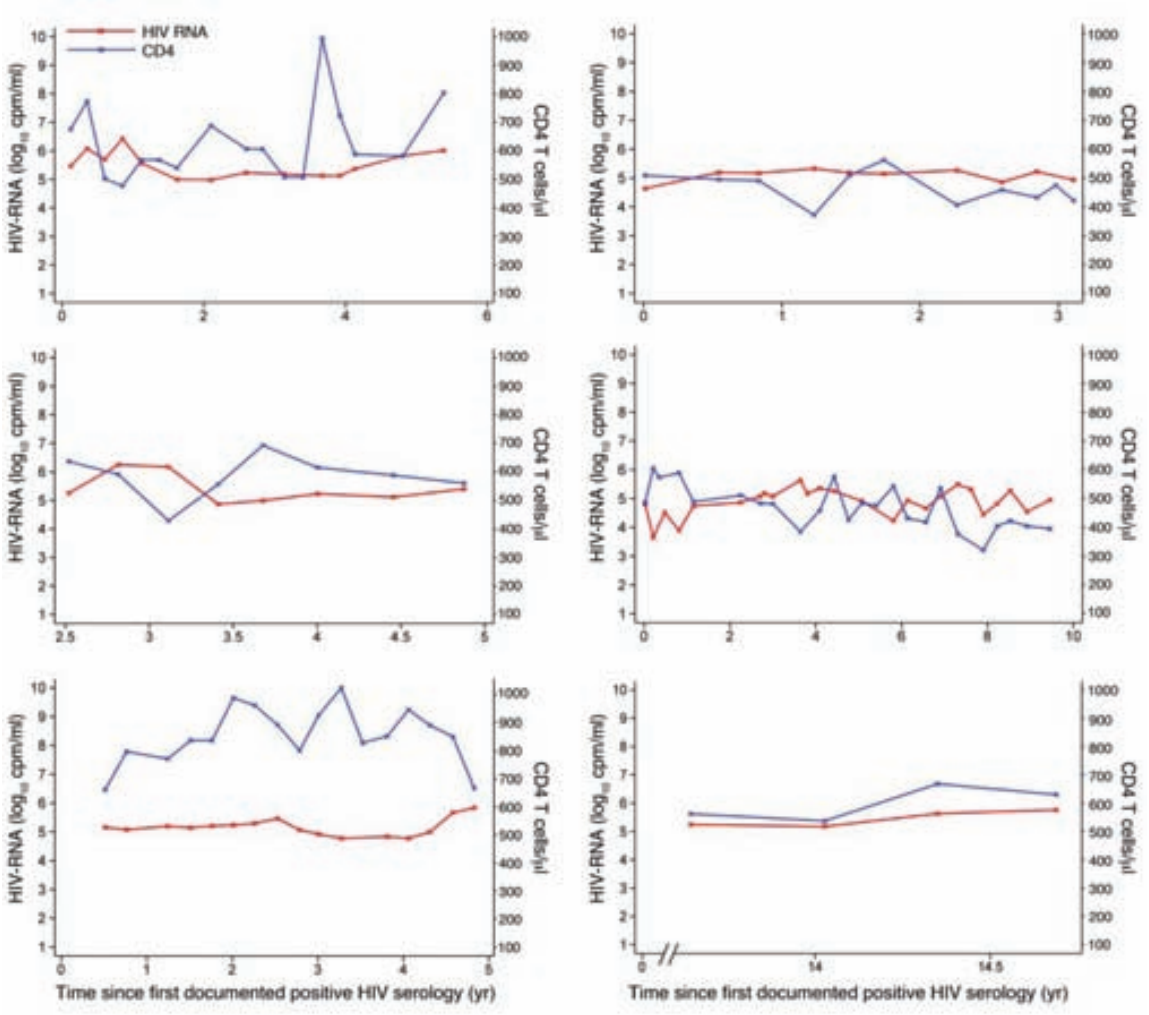


Figure 17. Individual viral loads and CD4⁺ T-cell profiles of VNPs. Viremia is shown in red, and CD4⁺ T-cell count is shown in blue.

We further identified 66 individuals who fulfilled the criteria of rapid progression and had materials available for study; the collective plot is shown in Figure 18.

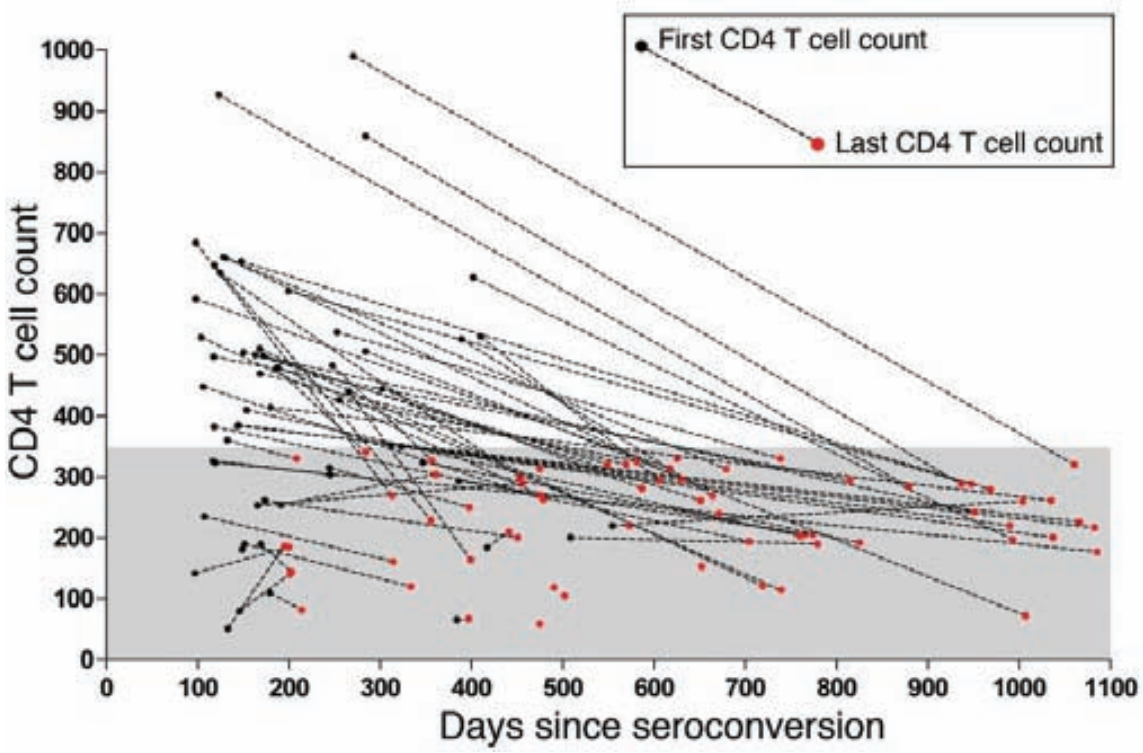


Figure 18. Evolution of T cell count in individuals with a profile of rapid progression. The first CD4+ T cell count determination (black symbols) and the last CD4+ T cell count determination (red symbols) (connected by dashed lines) in individuals, defined by the progression to fewer than 350 CD4+ T cells (denoted by the gray area) in fewer than 3 years after seroconversion. Only values beyond the 3-month window after seroconversion are considered.

Notably, at the time of analysis, VNPs had higher levels of viral replication (set point HIV RNA, 5.4 log10 cp/ml; interquartile range [IQR], 5.1–5.5 log10 cp/ml]) compared with those of RPs (set point HIV RNA, 4.7 log10 cp/ml [IQR, 4.3–5.2 log10 cp/ml]). Transcriptome analysis also included 9 elite/viremic controllers (ECs) and 5 chronic progressors, as previously defined [75]. Patient characteristics are detailed in Table 4. The HLA and KIR alleles were determined in all individuals, compared across clinical groups and compared to the allele frequencies of 1,609 participants of the SHCS (Supplemental Figures 1 and 2, Addendum). Protective alleles were underrepresented, and risk alleles were more common in RPs compared with the general population. In contrast to HLA alleles, there was no

depletion of protective KIR alleles or KIR/HLA combinations in RPs (Supplemental Figure 3, Addendum).

Table 4. Demographic characteristics of study participants.

	VNP (n=6)	RP (n=66)	EC (n=9)	CP (n=5)
Male gender, n(%)	5 (83)	54 (82)	5 (56)	3 (60)
White ethnicity, n(%)	5 (83)	56 (89)	9 (100)	5 (100)
Mode of transmission, n (%)				
Heterosexual	1 (17)	17 (26)	2 (22)	1 (20)
MSM	5 (83)	29 (44)	3 (33)	1 (20)
Intravenous drug use	0 (0)	14 (21)	3 (33)	3 (60)
Other	0 (0)	6 (9)	1 (88)	0 (0)
Median (IQR) age, years	38 (34-39)	37 (34-40)	31 (27-36)	36 (30-38)
Set point HIV RNA, log_{10} cp/ml	5.4 (5.1-5.5)	4.7 (4.3-5.2)	0 (0-2)	4.2 (3.9-4.7)
Viral subtype (n)	B(5), F(1)	B(51), A(2), 01_AE(1). 02_AG(1), BF(1), BD(1), C(2), G(1), NA(6)	B(3), 11_CPX(1), NA(5)	B(2), 01_AG(1), NA(2)

IQR: interquartile range; MSM: Men who have sex with men, CP= chronic progressors

HLA alleles of VNPs are shown in Supplemental Table 1 (Addendum). To determine whether any common variants of very large effect could be implicated in mediating rapid progression, the study was completed with a genome-wide association across an approximately 500,000-loci study that included 66 RPs and 757 participants of the SHCS. No SNPs reached genome-wide significance (Supplemental Figure 4 and Supplemental Table 2, Addendum), likely due to the limited power to detect anything other than very large effect sizes. A previous

genome-wide association study of rapid progression [93] identified 8 SNPs that passed the study-wide false discovery rate (FDR) cutoff of 25%. These failed confirmation in our study (Table 5).

Table 5: Results for SNPs previously reported to be associated with rapid progression. Reported frequencies and OR are with respect to the A1 (minor) allele.

SNP	Chr	A1	A2	Case freq	Control freq	OR previous	OR current	P previous	P current
rs4118325	1	A	G	0.18	0.17	0.24	1.12	6.09E-07	6.39E-01
rs1522232	12	T	C	0.46	0.48	0.45	1.17	1.80E-06	3.87E-01
rs1360517	9	A	G	0.07	0.07	3.09	0.99	3.27E-06	9.67E-01
rs10800098	1	A	G	0.05	0.06	3.29	0.99	3.86E-06	9.80E-01
rs10494056	1	A	C	0.20	0.17	0.27	1.34	4.29E-06	2.01E-01
rs12351740	9	T	C	0.05	0.04	3.46	0.97	6.63E-06	9.45E-01
rs1020064	2	T	G	0.21	0.22	0.34	0.94	7.04E-06	7.65E-01

Transcriptome analysis in CD4+ T cells

To investigate differences at the transcriptome level between RPs and VNPs, we performed microarray analysis on purified CD4+ cells from 27 RPs, 5 VNPs, 5 chronic progressors, and 9 ECs (Table 6). RPs, with and without transcriptome analysis, were similar with regard to CD4+ T cell counts and HIV viral load. The median CD4+ T cell counts at baseline were 440 cells/μl (IQR, 350–506 cells/μl) and 382 cells/μl (IQR, 315–497 cells/μl) for those with and without transcriptome analysis, respectively; the median baseline HIV viral loads were 4.8 cp/ml (IQR, 4.1–5.5 cp/ml) and 4.9 cp/ml (IQR, 4.3–5.1 cp/ml). During follow-up, the median CD4+ T cell counts were 263 cells/μl (IQR, 197–313 cells/μl) and 223 cells/μl (IQR, 186–299 cells/μl), and median HIV viral loads were 4.8 cp/ml (IQR, 4.3–5.4 cp/ml) and 5.0 cp/ml (IQR, 4.4–5.2 cp/ml) ($P > 0.4$ for all comparisons). Thirteen (20%) individuals had an AIDS-defining event within 3 years of seroconversion.

Principal component analysis identified 4 outliers that were excluded from further analysis. Various parameters were assessed as covariates (clinical center, gender, age, CD4+ T cell viability and laboratory date, and microarray chip batch); we

retained chip batch as a statistically significant covariate. To contrast specific patient profiles, we applied a Bayesian approach to the analysis of gene expression [407]. Analysis of RPs versus ECs identified 14 differentially expressed genes at a FDR-adjusted P value of less than 0.05. Interferon-stimulated genes (ISGs) are well known to be upregulated in patients with progressive HIV disease. Consistent with this knowledge, 6 ISGs, IFI44 (and its ligand IFI44L), MX1, EIF2AK2, IFI6, LY6E, TRIM22, were upregulated in RPs. Other upregulated genes included SYNCRIP that encodes a nuclear ribonucleoprotein (hnRNP-Q) associated with the APOB mRNA editosome complex that may modulate the posttranscriptional C to U RNA-editing PRIC285 that encodes a helicase acting as a transcriptional coactivator for a number of nuclear receptors, EPSTI1 and MRPS18B. Genes downregulated in RPs included TRK1, which encodes a kinase, and FOXJ2, a transcriptional activator. Next, we specifically searched genes uniquely associated with the VNP profile by contrasting this profile with that of RPs or chronic progressors. This analysis failed to identify FDR-adjusted differentially expressed genes.

Table 6. Viral load and CD4 T cell count at transcriptome day.

Median (IQR)

	samples	VL at sample date	CD4 at sample date	VL setpoint
RP	28	4.7 (4.3-5.1)	346.5 (280.3-461.8)	4.8 (4.3-5.2)
EC	9	0 (0-0)	858 (655-1140)	0 (0-2)
CP	5	4 (3.6-4.7)	932 (345-1289)	4.2 (3.9-4.7)
VNP	5	5.1 (4.9-5.2)	556 (490.5-923)	5.3 (5-5.5)

Transcriptome analysis in CD8+ T cells

We also performed microarray analysis on purified CD8+ T cells derived from the same PBMC samples used for CD4+ T cell transcriptome analysis. Expression analysis was successfully completed for 25 RPs and 5 VNPs as well as 5 chronic progressors and 8 elite and viremic controllers (Table 6). No outliers were

identified, and all samples progressed to further analysis. As above, we retained microarray chip batch as covariate in all definitive analyses.

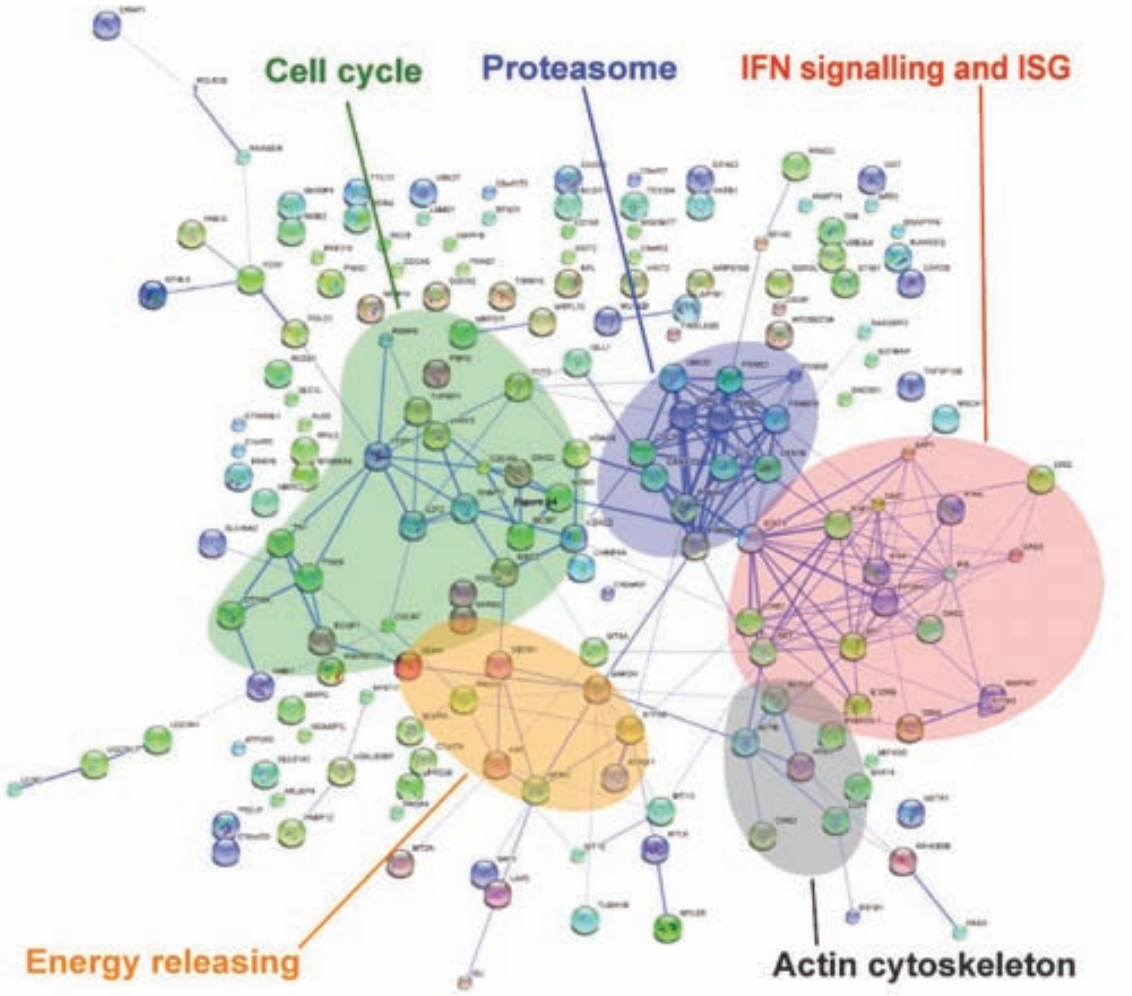


Figure 19. Predicted interaction networks of genes differentially upregulated in CD8+ T cells during HIV-1 infection. Genes (n=180) upregulated in RP compared to elite/viremic controllers (FDR adjusted p-value<0.05) are shown, (although due to size issues the names of each specific gene are not readable in the printed version, but can be found on the online version of the paper): links have been predicted using STRING (http://string.embl.de/). Interactions are depicted according to the strength of evidence obtained from direct (physical) and indirect (functional) associations (the thickness of the connecting blue line indicates the strength of confidence). Information is derived from four sources: genomic context, high-throughput experiments, conserved co-expression, and previous knowledge from literature.

Using the same sensitive Bayesian approach as for the CD4+ T cell analysis [407], contrasting of RPs and ECs yielded 317 differentially expressed genes at a FDR-adjusted P value less than or equal to 0.05 (Supplemental Table 3, Addendum). Among the 180 genes upregulated in RPs, prominent groups of genes included multiple members of the proteasome and interferon-induced immunoproteasome, ISGs, and cell cycle, cell division, and metabolic genes indicating cell proliferation (Figure 19). No apparent mechanisms were deduced from the collective analysis of 137 genes downregulated in RPs by using EMBL Search Tool for the Retrieval of Interacting Genes/Proteins (STRING), Ingenuity Pathway Analysis 7.0 (IPA), and KEGG pathway analysis (see Methods). As for the CD4+ T cells, we specifically searched genes uniquely associated with the VNP profile by contrasting this profile with that of RPs or chronic progressors. Given power limitations, this analysis failed to identify FDR-adjusted differentially expressed genes. Thus, we proceeded to the analysis of specific pathways and of the genes identified in primate studies of nonpathogenic SIV infection [96, 97].

Analysis of genes of the interferon response

Recent publications [95-99] highlight a distinctive downregulation of the interferon response after SIV infection of natural host species, such as sooty mangabeys and African green monkeys. In contrast, SIV infection of the pathogenic models of rhesus or pig-tailed macaque is characterized by persistence of deregulated interferon responses. Consistent with the primate model of natural infection, we observed a lower level of expression of ISGs (see Methods for the specific ISGs) in CD8+ T cells of individuals with a VNP profile in comparison with that of individuals with a RP profile (Figure 20) (difference of the means, median –0.21 [IQR, –0.05 to –0.40]; paired t test, P = 0.014). However, these differences were not observed in CD4+ T cells, (difference of the means, 0.01 [IQR, 0.13 to –0.04]; P = 0.59). As expected, more profound differences in expression of ISGs were found in the comparison between ECs and RPs (median –0.36 [IQR, –0.13 to –0.59], P = 2.5 × 10–5 in CD4+ T cells, and median –0.33 [IQR, –0.21 to –0.59], P = 3.8 × 10–6 in

CD8+ T cells) (Figure 20). The expression of SOCS1, involved in a negative feedback loop in the regulation of signal transduction through the JAK/STAT5 pathway, was higher in CD4+ T and CD8+ T cells of VNPs and ECs compared with that of RPs; the differences were statistically significant for the comparison of ECs and RPs in CD4+ T cells (P = 0.02) (Figure 20). This trend was not observed for a second regulator, ADAR.

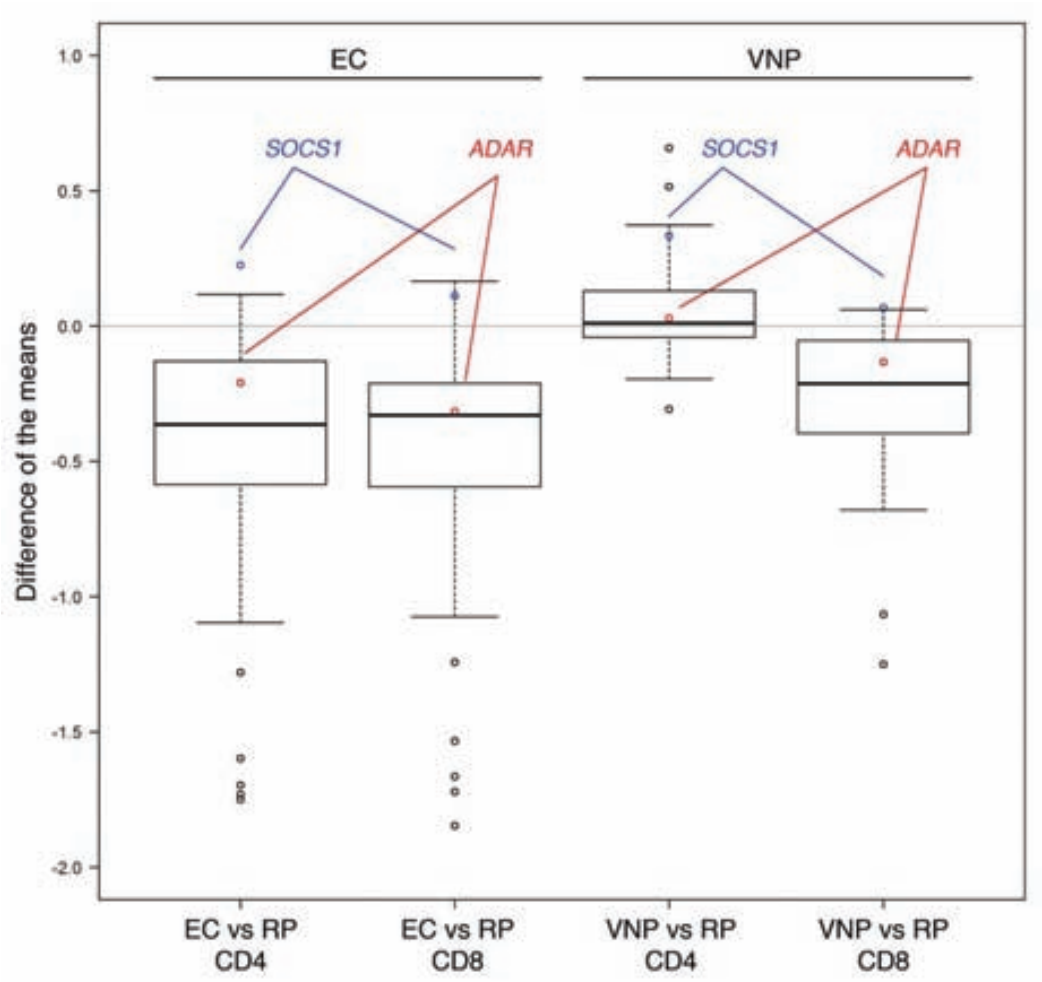


Figure 20. Analysis of differential expression of ISGs. Consistent with the primate natural infection model, a representative set of ISGs (n = 29) had lower expression levels in $CD8^+$ T cells from VNPs than in those from RPs. The box-and-whisker plot indicates that the differences are more pronounced for the comparisons of ECs and RPs. The horitzontal bars indicate the median values, the boxes indicate the 25th to 75th percentiles, and whiskers indicate extremes. Each dot represents the difference in expression value for a given gene across groups. The profiles of the inhibitor of interferon response, SOCS1, and of ADAR are highlighted (blue and red, respectively).

Gene set enrichment analysis of human VNPs and SIV-infected sooty mangabeys

To examine whether the phenotype maintained by VNPs and natural host species was due to a shared, underlying molecular mechanism, we used gene set enrichment analysis (GSEA) of the human transcriptome data sets with gene sets derived from the analysis of sooty mangabeys and rhesus macaques [96]. GSEA tests the relative position of a collection of genes ("query gene set") within an independent, ranked data set ("reference gene set"). Because GSEA relies on an additive signal of multiple genes within a data set, it is less dependent on arbitrary cutoffs, such as fold change of specific P values, making its ability to detect an underlying process within transcriptome data potentially more sensitive than a "single-gene" approach using traditional statistics. The use of rank data rather than absolute intensity measurements in GSEA also affords greater flexibility to make comparisons between diverse gene-expression data (i.e., between tissues, species, or array platforms) [411]. As presented in Table 7 and in Figure 21, the query set of ISGs identified as differentially expressed in the rhesus macaque was associated with enrichment in human RPs, although the P values were only consistent with a statistical trend. The CD8+ T cell expression data was particularly enriched for the ISGs; the data set comprised 15,879 non-redundant genes, and the lowest-ranked ISG was at position 10,562, well above the phenotype threshold at position 7,556, below which genes demonstrated higher expression in VNPs, and 12 out of 20 queried ISGs were higher than position 14,500 (Figure 21).

Genes found to be correlated with immune activation in rhesus macaques were also enriched in the RP phenotype in humans in both CD4 and CD8+ T cell data (Table 7 and Figure 21). The enrichment of immune activation genes in RPs would indicate that VNPs have reduced cellular activation/proliferation relative to RPs. Taken together, these data suggest that VNPs, at least at the transcriptional level, are able to reduce the chronic immune activation seen in pathogenic HIV/SIV infection and that this attenuation largely overlaps with comparisons between sooty mangabeys and rhesus macaques.

Table 7. Analysis of gene sets of the primate model.

	VNP vs. RP CD4 pre-ranked data set		VNP vs. RP CD8 pre-ranked data set	
Gene set	**ES**	***P* value**	**ES**	***P* value**
ISGs (RM)	−0.70	0.156	−0.85	0.089
IA (RM)	−0.69	0.141	−0.83	0.075
SM > RM chronic phase	0.98	0.010	−0.30	0.786
Random	0.28	0.910	−0.44	0.667

Enrichment of genes upregulated in sooty mangabeys (SMs) or rhesus macaques (RMs) after SIV infection was analyzed by GSEA in expression data sets derived from contrasting human HIV-infected RPs with VNPs. Positive enrichment scores (ES) indicate enrichment in the VNP phenotype, and negative scores indicate enrichment in the RP phenotype. "SM > RM" denotes genes that were upregulated in sooty mangabeys to a higher degree than in rhesus macaques. IA, immune activation genes.

Because the human VNP and RP samples were obtained from the postacute phase of infection, we reasoned that genes found to be differentially expressed between sooty mangabeys and rhesus macaques during chronic infection may be enriched in the VNP phenotype. When we performed GSEA using genes found to be significantly higher in sooty mangabeys than rhesus macaques during chronic infection against the human data sets, we found that there was no significant enrichment in either phenotype in CD8+ T cells, but that there was significant enrichment in the VNP phenotype of CD4+ T cells (Table 7). The enrichment was largely driven by a single gene, SV2A, which ranked extremely high in the VNP phenotype. Taken together, these results suggest that sooty mangabeys and VNPs share some similarities in expression during chronic SIV/HIV infection; however, these similarities were not statistically significant.

Detailed analysis of genes identified in nonpathogenic primate models of natural infection

We extended the above analysis to examine in detail a list of genes reported by Bosinger et al. [96]. We used a heuristic approach to inform this list (see Methods) by assessing (a) the consistency and direction of the association (downregulation

or upregulation) between the primate model and the human expression profile, (b) the general correlation between CD4+ T cell and CD8+ T cell observations, and (c) the statistical support for the different associations in this subanalysis. Six genes fulfilled the criteria; genes CASP1, CD38, LAG3, and TNFSF13B presented lower expression levels in VNPs and in the nonpathogenic animal model, and SOCS1 and EEF1D presented greater expression levels in VNPs and in the nonpathogenic animal model of infection (Fig. 21).

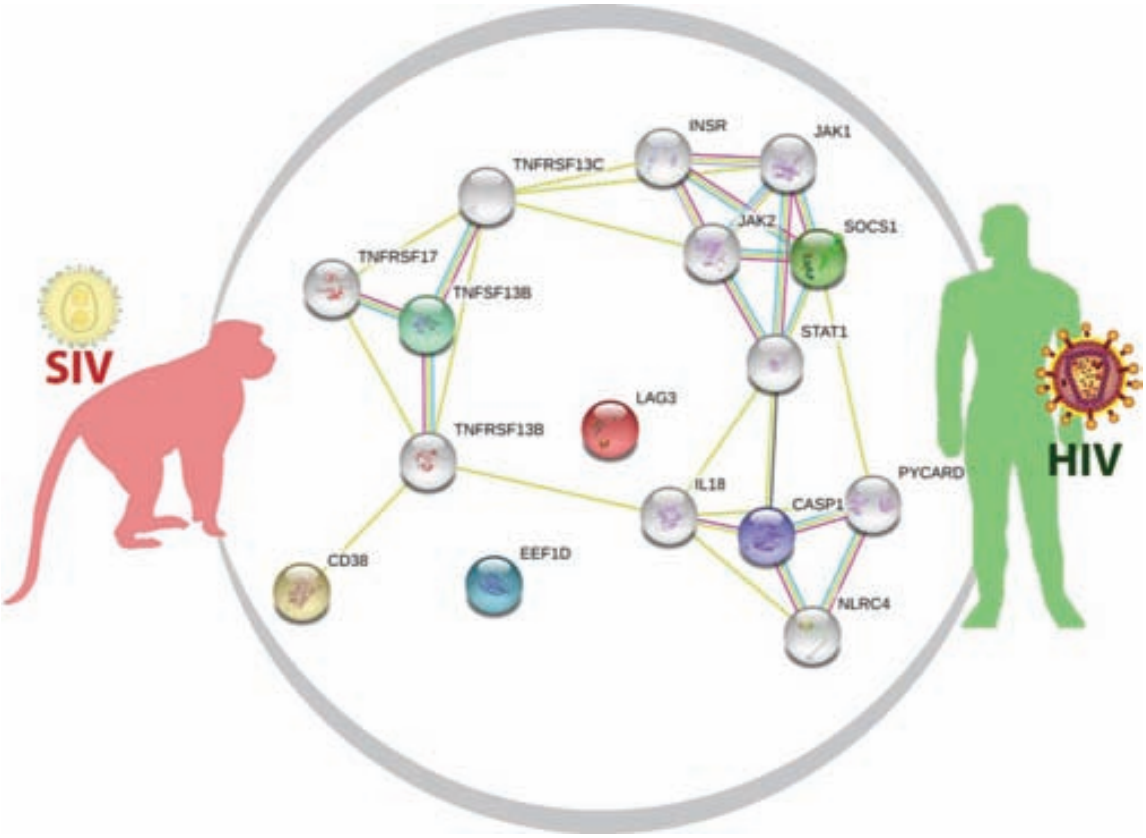


Figure 21. Differentially expressed genes. Six genes were differentially expressed in RPs vs. VNPs, in correlation with their homologous non-human primate model (pathogenic vs. nonpathogenic, respectively).

The short list of genes was constituted into a signature to be evaluated in an independent set of data. For this, we used the large data set of CD4+ T cell expression [408] to assess the association of the signature genes with viral load and with progression of immunosuppression (as defined by time to fewer than 350 CD4+ T cells/μl). In unadjusted regression, the following genes showed statistically

significant association with time to progression to fewer than 350 CD4+ T cells/μl: CASP1, LAG3, CD38, TNFSF13B, and EEF1D (Figure 22).

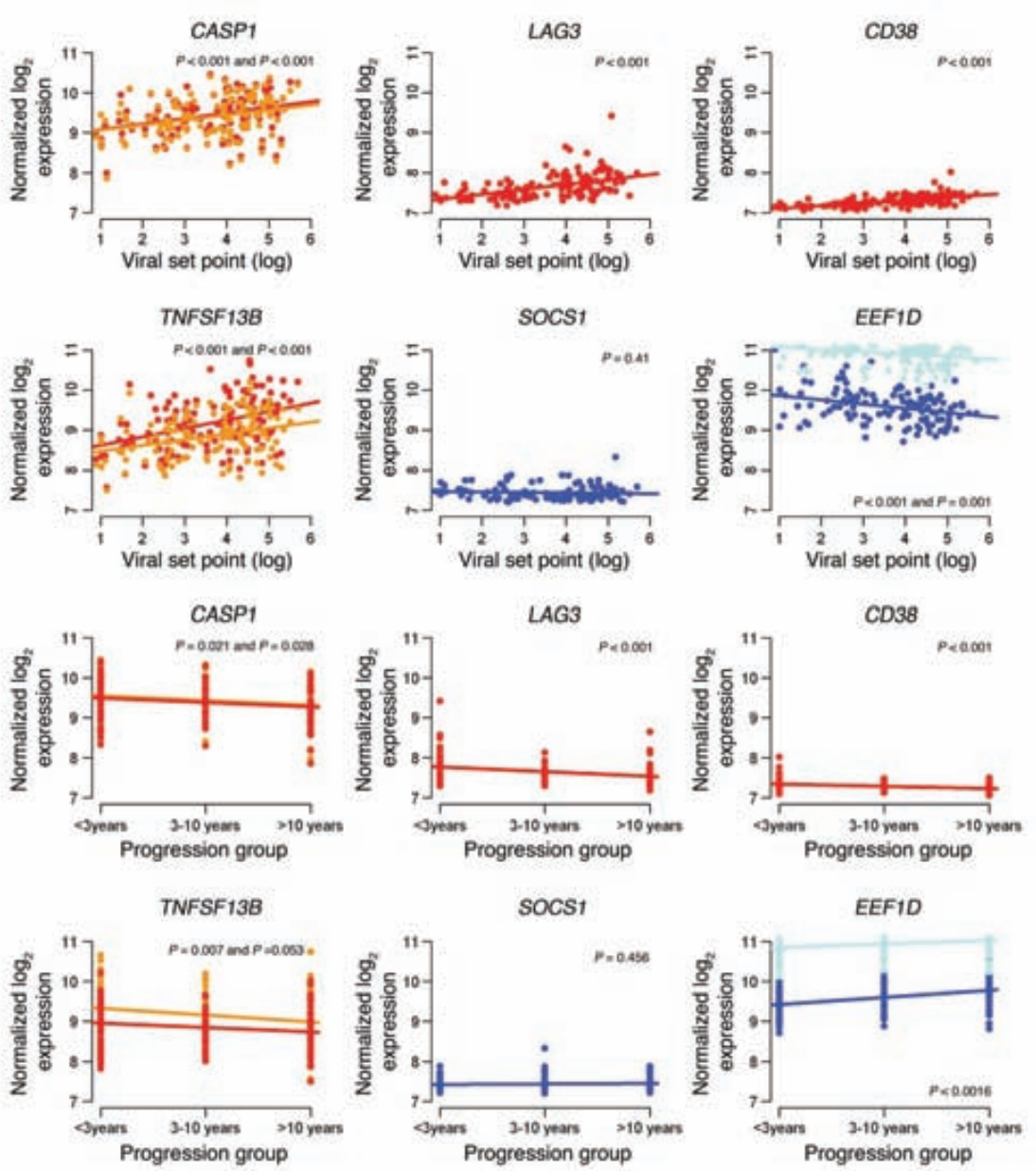


Figure 22. Analysis of the candidate VNP signature in an independent CD4+ T cell expression data set. The signature associated with the VNP profile upon transcriptome analysis in humans and nonhumans was tested in an independent validation set of 153 individuals, contributing CD4+ T cell expression data across all levels of viral set point after seroconversion. Correlations with individual gene expression levels and viral set point after seroconversion are shown in the 6 top panels. Correlations with disease progression, as indicated by time to CD4+ T cell count depletion to fewer than 350 cells/μl, are shown in the 6 bottom panels. Multiple probes for 1 gene are shown in different colors: orange/red is used for genes differentially upregulated in RPs, and blue/light blue is used for genes differentially upregulated in the VNPs. Where there are 2 P values, the first value represents the red/blue lines, and the second value represents the orange/light blue lines. Each dot represents an individual. The regression lines from the linear models are shown.

A multigene model explained 19.5% of the variance in disease progression (P = 0.0003). Inclusion of viral load in the model improved the proportion of variance explained to 26% (P = 4.8 × 10–7). However, there was significant colinearity with viral load and, after its inclusion in the model, only EEF1D remained as an independent variable (P = 0.013).

Association of soluble CD14 levels with clinical groups. To further assess whether the differences between RPs and VNPs reflected differences in mechanisms of pathogenesis, we assessed plasma levels of soluble CD14 (sCD14), which is produced by monocytes on becoming activated by LPS. Thus, plasma sCD14 levels reflect the host response to translocated bacterial products and are a significant independent predictor of mortality in HIV infection [52, 412]. We analyzed samples from 24 RPs and 4 VNPs collected within 3 years after seroconversion. To contextualize these data, we measured plasma sCD14 levels in healthy volunteers and from chronic progressors.

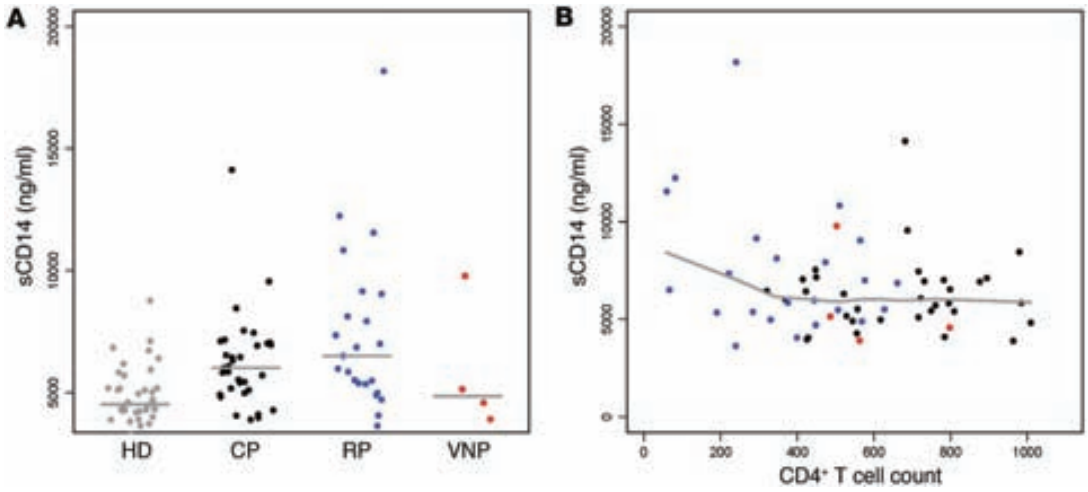


Figure 23. Analysis of sCD14 plasma levels. (A) sCD14 levels were measured during the 3-year period after seroconversion and/or transcriptome analysis in RPs (n = 24) and VNPs (n = 4). Chronic progressors (CP; n = 39) and healthy donors (HD; n = 38) contributed reference data. The gray line represents the median values. (B) RP sCD14 levels were higher at lower CD4+ T cell counts. The gray line represents the LOWESS curve fitted to the sample population. Each dot represents an individual.

sCD14 levels were significantly higher in the plasma samples from RPs than in samples from chronic progressors, healthy donors, and for 3 out of 4 VNP samples analyzed (median 6,235 ng/ml [IQR, 5,069–8,808 ng/ml], median 6,065 ng/ml [IQR, 4,973–7,043 ng/ml], median 4,516 ng/ml [IQR, 3,972–5,304 ng/ml], and median 4,852 ng/ml [IQR, 4,069–8,612 ng/ml]); the differences between RPs versus healthy controls and chronic progressors versus healthy controls were significant ($P < 0.0001$) (Figure 23A).

Additional plasma samples of the fourth VNP were consistently elevated. There was a trend toward increasing levels of sCD14 for individuals sampled at the time of advanced immunosuppression, with CD4+ T cell counts of below 350 cells/µl (Figure 23B).

DISCUSSION

The current study defines 2 presentations of HIV infection that share a similar level of high viral replication but differ in the degree of immunological damage and in the pattern of clinical evolution, i.e., RPs and VNPs. The proportion of individuals with rapidly progressive disease was estimated in the SHCS [413]. In this nationwide and representative cohort, 7.9% of HIV-infected individuals with a known seroconversion date fulfilled the criteria of RPs. Severity of the disease, rapid initiation of treatment, and the need for precise knowledge of the seroconversion window hampered recruitment of RPs into clinical cohorts and research protocols in the past. VNPs constitute a group of individuals that sustain prolonged periods of high viral load, in the range of 100,000 copies/ml, while maintaining stable CD4+ T cell counts. VNPs represent a very uncommon pattern of disease progression; prevalence estimates in the SHCS indicate that only 0.1% of HIV-infected individuals would fulfill the strict definition of VNPs used in the current work. However, the selected individuals likely represent the extreme of the distribution of VNPs, and relaxed criteria compared with those used in the present study will lead to different estimates of frequency.

The various genomic analyses in this study associate rapid progression with an enrichment for HLA alleles linked to adverse prognosis and a depletion of protective alleles. This pattern validates the phenotypic set of criteria elaborated to define rapid progression. In contrast, we found no association of the RP cohort with KIR alleles or KIR/HLA combinations previously related to disease progression or viremia [414]. The genome-wide association study was conducted to exclude a major impact of common variants and to assess the candidates from a previous study of similar power [93] that could not be validated here. The transcriptome profile did confirm the deregulation of the ISGs in CD8+ T cells in RPs, as previously documented for CD4+ T cells [65, 408, 415] and in lymphatic tissue [416]. It also identified a characteristic pattern of upregulation in CD8+ T cells of RPs for genes involved in cell proliferation and cell division as well as in the immunoproteasome. RPs shared a number of features with the chronic SIV infection of rhesus macaques, in particular the prominent expression of a ISG and of immune activation markers. The absence of persistent immune activation during chronic SIV infection is a key characteristic of natural host species, such as the sooty mangabeys [48], and the presence of proliferation/activation markers on CD4+ and CD8+ T cells is an accurate predictor of disease in HIV-infected individuals [417]. The immune activation gene set assessed in the present study was originally identified as being correlated with CD8+ T cells expressing the activation marker Ki67 in SIV-infected rhesus macaques but was not expressed in SIV-infected sooty mangabeys [96].

More remarkable were the observations in VNPs. While the study did not have the power to allow a discovery that was not a priori, it permitted the assessment of a number of characteristics that have been previously described in SIV-infected sooty mangabeys. Individuals with the VNP profile display a limited deregulation of the ISG when compared with RPs, particularly in CD8+ T cells. It should be stressed that these differences were present despite greater levels of viremia among VNPs than in RPs. In addition, to assess whether VNPs demonstrated lower immune activation and/or chronic interferon responses relative to RPs, we ranked the CD4+ and CD8+ expression data sets according to the significance

value determined by the Bayesian analysis and used GSEA to test the relative position of ISGs/immune activation genes and genes differentially expressed in SIV-infected sooty mangabeys and rhesus macaques. This analysis supported the notion that the human profile of VNPs shares common features, at the transcriptome level, with the nonpathogenic model of SIV infection in the natural host. Reduced ISG expression is a consistent feature of natural host infection and not due to temporal fluctuation [97]. Although the observation of reduced ISGs in VNPs in the current study is cross-sectional, it was consistent in showing ISG reduction relative to RPs. How differences in transcription levels of the ISGs translate into protein and the mechanisms of regulation should be the focus of future research [418].

We investigated in detail a set of genes identified through a comparative analysis of human and nonhuman primate transcriptome data; CASP1, CD38, LAG3, and TNFSF13B were upregulated in rhesus macaque and in human RPs; SOCS1 and EEF1D were upregulated in sooty mangabeys and in human VNPs. The shared expression pattern between VNPs and sooty mangabeys supports their role in lentiviral pathogenesis. Caspase-1 precursor (CASP1) is a well-known intermediate of the inflammatory processes and apoptosis. The lymphocyte differentiation antigen CD38 is associated with immune exhaustion during immune activation and with adverse prognosis [419-421]. LAG3 negatively regulates the expansion of activated T cells, and T cell homeostasis and is required for maximal regulatory T cell function [422] and has been demonstrated to associate with immune dysfunction/exhaustion of CD8+ T cells in LCMV infection [346]. Tumor necrosis factor ligand superfamily member 13B (TNFSF13B) is a receptor involved in the stimulation of B and T cell function and the regulation of humoral immunity. Suppressor of cytokine signaling (SOCS1) is involved in a negative feedback loop in the regulation of cytokine signal transduction signaled through the JAK/STAT5 pathway. Although SOCS1 was downregulated in RPs compared with ECs and VNPs, its expression levels did not exhibit a significant association with viral set point or disease progression in the validation data set of CD4+ T cell transcription data [408].

We completed the study by the analysis of a biomarker of compromised intestinal mucosal barrier, the monocyte-expressed LPS receptor sCD14 [412]. Our data show higher plasma levels among RPs, in particular during advanced immunosuppression, than for other clinical progression groups. Although only 4 VNPs could be tested, 3 presented low sCD14 plasma levels, a pattern fitting other observations of lesser immunopathogenesis in these individuals. The transcriptome and biomarker data thus complement the work of Choudhary et al. [404] on VNPs that presented less extreme viral loads. They identified a lower percentage of activated HLADR+ CD38+CD4+ and CD8+ T cells and lower levels of proliferating Ki67-expressing CD4+ and CD8+ T cells in VNPs compared with those of progressors. In contrast, viral isolates from VNPs and progressors replicated to similar levels and shared the capacity to deplete CD4+ thymocytes or CD4+ T cells in secondary lymphoid tissue and were equally cytopathic.

Future studies should extend analyses to plasmacytoid dendritic cells, as they are key activators of the immune system in HIV and SIV infection. Assessment of this cell population is limited by the low percentage of these cells in fresh blood, in particular, in the infected individual [423]. The study has limited power due to the rarity of the study phenotypes and inherent limitations in recruitment. However, this work highlights the importance of 2 poorly understood clinical patterns of disease progression that have been minimally studied in the past and provides working definitions that should help identifying additional individuals to allow greater power in future genomic and functional studies. In addition, this report of a strong phenotypic similarity between nonpathogenic SIV infection of sooty mangabeys and a subset of HIV-infected individuals emphasizes the importance of studying natural SIV infection as a model to better understand HIV/AIDS pathogenesis.

Chapter 3c

STUDY III

In–Depth characterization of viral isolates from plasma and cells compared with plasma circulating quasispecies in early HIV-1 infection

This chapter corresponds to the manuscript:

Dalmau J.*, Codoñer F.M.*, Erkizia I., Pino M., Pou C., Paredes R., Clotet B., Martinez-Picado J.[#], Prado J.G.[#]. ***In–Depth characterization of viral isolates from plasma and cells compared with plasma circulating quasispecies in early HIV-1 infection***. *PLoS ONE*. 2012; 7(2): e32714.

**Equal contribution, [#] Corresponding authors*

PRESENTATION

The next study of factors of rapid progression included the analysis of whole viral primary isolates from RP in comparison to that of SP. However, PBMCs and/or plasma samples were not available for all the patients at the early timepoint. Our laboratory has developed protocols for viral isolation from plasma and from PBMCs, and it was a need to analyze if the isolates obtained from both techniques might be comparable. Therefore, a deep analysis was performed to compare the viral isolates obtained from both sources, in order to make sure that they might be used indistinctly in our experiments. The results of this study are shown in the present section.

INTRODUCTION

Human immunodeficiency virus (HIV-1) exhibits a high degree of genetic diversity particularly difficult to characterize due to the complexity of the RNA viral populations. This complexity is associated with factors such as the lack of proof-reading activity of HIV-1 polymerase, the high rate of generation of viral particles, and the recombination and hypermutagenesis process favored by host cellular proteins [200, 202, 424-428]. Consequently, the HIV-1 population is composed of a swarm of genetically related variants, known as viral quasispecies, which grant the virus with the ability to quickly adapt to various selective pressures. Examples of the rapid adaptive machinery of HIV-1 are the selection of mutations enabling escape from the humoral and cellular host immune responses [351, 429-431] and the selection of mutations generating resistance to currently available antiretroviral drugs [432]. Therefore, to define the composition of HIV-1 quasispecies and identify virus diversity or variability within a single infected subject or at the population level it is essential to understand the pathogenesis of HIV-1 and design optimal antiretroviral treatments and vaccines.

Some studies associated pathogen diversity with poor prognosis [433-435], and increased diversity of HIV-1 has been related to disease progression [215, 436]. As a result, the maintenance of virus population structures in primary isolates is a key

feature for the accurate study of specific viral biological traits, such as fitness and co-receptor usage, which are central to completing our understanding of the HIV-1 pathogenesis. The recent development of a new generation of massively parallel sequencing technologies has enabled us to carry out comprehensive studies of the genotypic characteristics of viral populations, genetically comparing thousands of sequences and increasing our chances of identifying minority variants. Deep Pyrosequencing (DPS) technology has made possible to describe the complexity of viral dynamics during immune escape, to quantify the presence of minority drug resistance variants, and to define virus co-receptor use for the management of CCR5 antagonists [437-441].

This study aims to investigate with the use of DPS technologies whether viral isolates from biological samples preserves the variability of circulating viruses and the phenotypic features found *in vivo*. For that reason, we compared paired HIV-1 isolates obtained from plasma and cells with total plasma viral RNA in four recently HIV-1-infected subjects. We combined multiple-amplicon DPS covering gag, protease, integrase, and env-V3 with *in vitro* replicative capacity and virus co-receptor use assays in order to address the genetic and phenotypic associations between HIV-1 isolates and viral quasispecies.

MATERIALS AND METHODS

Study subjects and Ethics Statement. The study sample comprised four treatment-naïve HIV-1-infected subjects. Epidemiological and clinical data are summarized in Table 8. Virus subtype was assigned based on gag, pol, and env-V3 sequences using the REGA HIV-1 Subtyping tool. The study was approved by the institutional review board of Hospital Germans Trias i Pujol, and all four subjects gave their written informed consent to participate.

Cell lines. The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: TZM-bl from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc; U87CXCR4 and U87CCR5 from Dr. HongKui Deng and Dr. Dan R. Littman as previously described [381, 442].

Plasma virus isolation. Viral isolates were obtained from plasma samples using anti-CD44 beads following the manufacturer's protocol (Miltenyi Biotec, Germany) with minor modifications as previously described [443]. Briefly, before virus extraction, PBMCs from three HIV-1-seronegative donors were isolated and CD8+ T cells depleted using the RosetteSep human CD8+ depletion cocktail (Stemcell Technologies, France). Pooled CD8+-depleted PBMCs were then stimulated under three different conditions ('363' method, Miltenyi Biotech). After 72 hours, cells were mixed to a final concentration of 106 cells/ml in R10 supplemented with IL-2 (100 U/ml) (Roche, Spain), and 200 ml of the extracted virus was added to the culture. Cultures were fed weekly with 106 cells/ml fresh 363-stimulated cells. Viral growth was monitored weekly using p24 enzyme-linked immunosorbent assay (ELISA) (Innogenetics, Spain). Virus isolates were harvested when the p24 concentration in the supernatant reached at least 100 ng/ml and then stored at 280ºC.

Cell virus isolation. Viral isolates from cryopreserved cells were obtained by coculture of PBMCs from each HIV-1-infected subject with a pool of PBMCs from three HIV-1-seronegative subjects that had been previously stimulated with phytohemagglutinin (PHA) (3 mg/ml) and IL-2 (10 U/ml) for 72 hours. Viral growth was monitored weekly by p24 ELISA and cultures were fed weekly with fresh cells. Viral stocks were harvested and stored at 280ºC.

PCR amplification and amplicon preparation. Total viral RNA was extracted (QIAamp Viral RNA Mini KitTM, QIAGEN, CA) from plasma (2 ml), plasma viral isolates (1 ml), and cell viral isolates (1 ml) in order to carry out PCR amplification. gag, pol, and env-V3 were amplified using one-step reverse transcriptase polymerase chain reaction (RT-PCR) (Super-ScriptH III One-Step RT-PCR System with PlatinumH Taq High Fidelity, Invitrogen, Carlsbad, CA, USA) based on a primer set containing 59-GCA GAA TGG GAT AGA TTG CAT CCA-39 (1,417R1,440, HXB2) and 59-CCT TGT TAT GTC CTG CTT GAT ATT CAC-39 (5,438r5,464, HXB2), and 59-TAG AGC CCT GGA AGC ATC CAG GAA G-39 (5853R5877, HXB2) and 59-TTG CTA CTT GTG ATT GCT CCA TGT-39 (8,913r8,936, HXB2) for gag, pol, and env-V3, respectively. Amplification

conditions were as follows: 30 minutes at 52ºC during reverse transcription, 2 minutes at 94ºC, 30 seconds at 94ºC, 30 seconds at 55ºC, and 4 minutes at 68ºC for 25 cycles. A final polymerization step of 5 minutes at 68ºC was applied. The enzyme used for the RT-PCR was the Super-Script III one-step PCR (Invitrogen, USA). Amplicons for QDS were generated using carried 454 adaptor A and subject-specific multiple identifiers; pyrosequencing was unidirectional. The conditions for the enzyme were 5 minutes at 94ºC, 30 seconds at 52ºC, and 1 minute at 68ºC for 25 cycles. A final polymerization step of 5 minutes at 68ºC was applied. The enzyme used was Platinum High Fidelity (Invitrogen, USA). The specific primer set was composed of the forward primers 59-CAG GAT TTA AAC ACC ATG CTA AA-39 (1,333R1,355 HXB2), 59-AAT TTG CCA GGA AGA TGG-39 (2,361R2,378 HXB2), 59-TTA AGG CCG CCT GTT G-39 (4,606R4,621 HXB2), and 59-TGG CAG TCT AGC AGA AGA AG-39 (7,010R7,029 HXB2), and the reverse primers 59-TAT CCA TCT TTT ATA GAT TTC TCC-39 (1,564r1,587 HXB2), 59-CAA TAG GAC TAA TGG GAA AA-39 (2,546r2,565 HXB2), 59-TTT TGT AAT TTG TTT TTG TAA TTC-39 (4,863r4,886 HXB2), and 59-CTG GGT CCC CTC CTG AGG-39 (7,315r7,332 HXB2) for gag, protease, integrase, and env-V3, respectively. All PCR reactions were performed in triplicate to reduce amplification bias and the founder effects. Triplicate amplifications were pooled before the purification procedure. Reactions were purified using the Agencourt AMPure Kit (Beckman Coulter, Germany) to eliminate the primer-dimers produced. The number of molecules was quantified by fluorometry using the Quant-iT PicoGreen dsDNA assay kit (Invitrogen, USA). When concentrations were below 5 ng/ml, amplicon quality was assessed by spectrometry using BioAnalyzer (Agilent Technologies, USA). Quantitative multiple amplicon DPS was performed in a 454 Genome Sequencer FLX (454 Life Sciences/Roche, USA) using FLX chemistry. A pNL4.3 clone was sequenced to assess the likelihood of errors during DPS. Discrepancies between data obtained by DPS and Sanger sequencing of pNL4.3 clone were attributed to the process.

Multiple amplicon DPS data clean-up and phylogenetic analysis. Data were cleaned in order to increase the quality of the sequences for down-stream analysis after multiple-amplicon DPS. The first step was to retrieve those sequences with a similarity >70%, when compared with HXB2 from the sequencing run. We then manually corrected the homopolymer tracks, since these are the most common sequencing errors produced by the technique. Sequences with stop codons within the open reading frame of the protein were removed from the analysis, and sequences containing gaps were maintained and included in the analysis, rather than being removed using a conservative bias towards an unknown nucleotide at this position. Identical sequences were collapsed into a single unique sequence or haplotype. Haplotypes with less than 1% presence in the population were removed from the analysis. A summary of the number of reads after the various filtering steps and the final number of haplotypes is represented in Table 9. Phylogenetic trees were built on the nucleotide alignment for the total unique reads collapsed into unique haplotypes. The best phylogenetic model was inferred using jModeltest v0.1.1 [444] for each HIV-1 protein in all subjects. Phylogenetic trees were constructed taking into account the inferred model in PhyML over 1000 bootstrap replicates (www.HIV-1.lanl.gov) were: for gag (TrN), protease (HKY+G), integrase (HKY) and env-V3 (TrN). Newick trees were exported and edited with MEGA4 [445].

Population variability per HIV-1 protein and sample type. To study and reproduce the variability according to sample type and among HIV-1 proteins, we simulated a viral population taking into account the sequences obtained in the sequencing run as a sample of the real population. The percentage of each sequence, based on the sequencing run, was used to create a population of 100 sequences where each haplotype was represented as many times as indicated by the percentage of the sequence in the sequencing run. This population of 100 sequences was used to infer variability among populations in the same patient and among HIV-1 proteins. We measured pairwise intra- and inter-population variability using the best model found by jModeltest v0.1.1, as implemented in MEGA4.

Replicative capacity experiments. Viral isolates obtained from plasma and cells were titrated in the TZM-bl immortalized cell line. Replicative capacity experiments were carried out using PBMCs from three seronegative individuals; previous infection PBMCs were stimulated for 72 hours with PHA (3 mg/ml) and IL-2 (10 U/ml). Stimulated PBMCs were then infected in triplicate with an equal multiplicity of infection of each viral variant at 37ºC for 2 hours. Pellets were washed twice with phosphate-buffered saline (PBS) and cultured at 37ºC and 5% CO_2 in R20 supplemented with IL-2 (20 U/ml) (Roche, Spain) [382]. Viral growth was measured by p24 ELISA in supernatants over 10 days (Perkin Elmer, Spain). Replicative capacity was calculated by fitting a linear model to the log10-transformed data of p24 production and comparing the slopes as previously described [446].

Determination of virus co-receptor use. Viral tropism from VP and VC was measured in U87 immortalized cell lines expressing CCR5 or CXCR4, as previously described [381, 403]. Briefly, 5,000 cells were plated on a 96-well plate and infected with 2 ng of p24 for each viral variant overnight. The next day, virus was washed 3 times with 200 ml of PBS and fresh media added to a final volume of 200 ml. Five days after infection, virus growth was identified microscopically by observation of syncytium formation, and the results were corroborated by p24. Furthermore, virus tropism was assessed in plasma samples at similar time-points using the Enhance Sensitivity Trofile Assay (ESTA, with a detection limit of 0.3% for non-R5 variants). In addition, two algorithms were used to infer virus co-receptor use based on env-V3 loop sequences from DPS: PSSM (http://indramullins.microbiol.washington.edu/webpssm) and geno2pheno (g2p) (http://www.geno2pheno.org/) with a false positive rate of 10%. Cut-off values to define non-R5 using sequences were 24.75 for PSSM and #3.5 for g2p [440].

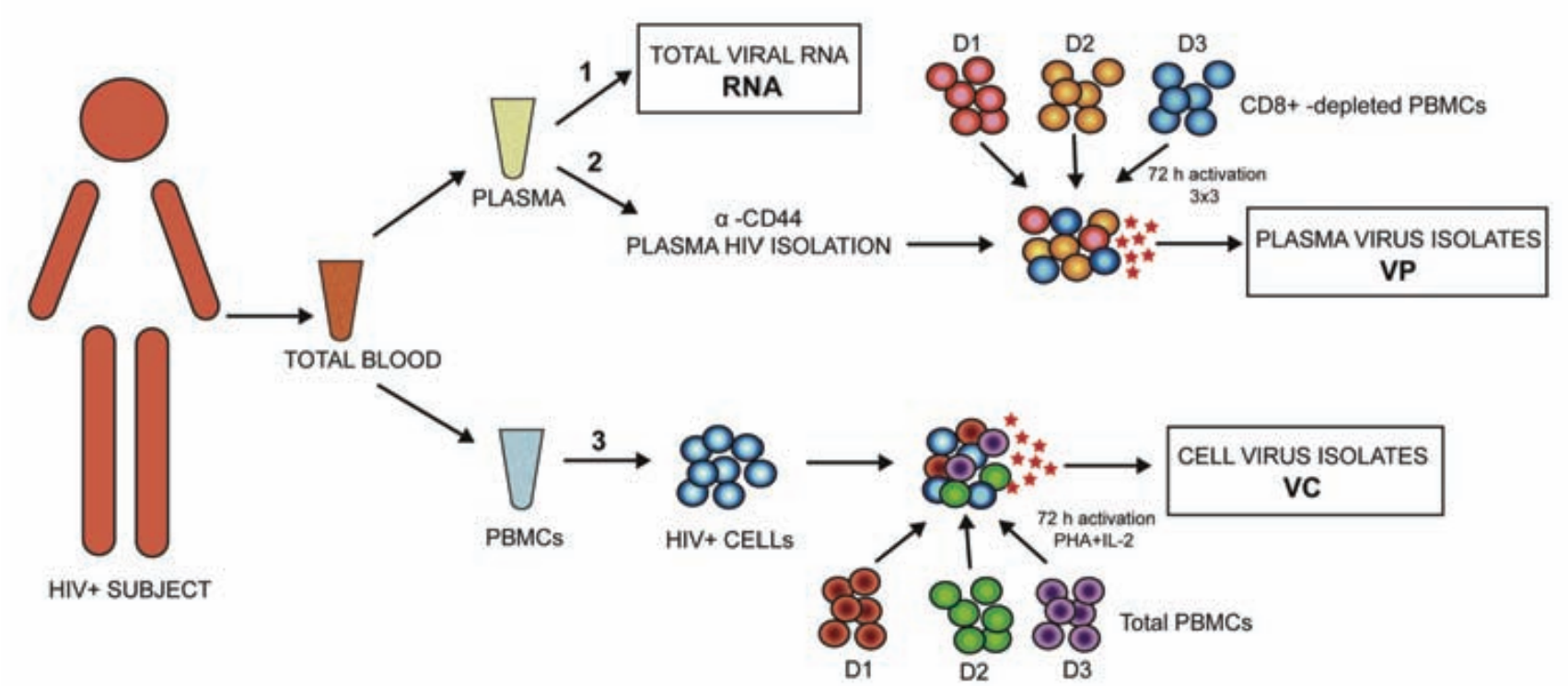


Figure 25. Schematic representation HIV-1 sample types analyzed for comparative purposes per study subject. Total blood was separated into plasma and PBMCs for the following: 1. Total viral RNA extraction (RNA) used for DPS and virus tropism. 2. Plasma virus isolation (VP) used for DPS, virus tropism and Replicative Capacity (RC): VP isolates were obtained by mixing plasma extracted anti-CD44 HIV-1 particles with a pool of CD8+-depleted PBMCs from three seronegative-donors (D1, D2, and D3) and culture during 2 to 3 weeks for virus in vitro expansion. 3. Cell virus isolation (VC) used for DPS, virus tropism and RC. VC isolates were obtained by co-culture of HIV+ cells with a pool of total PBMCs from three seronegative-donors (D1, D2, and D3) and culture during 3 to 4 weeks for virus in vitro expansion. Colored red, orange and dark blue circles represent cells from CD8+ depleted seronegative donors D1, D2 and D3 respectively. Colored red, green and purple circles represent cells from seronegative donors D1, D2 and D3 respectively. Light blue circles represent HIV+ cells. Red stars indicate virus production.

RESULTS

Efficiency of HIV-1 recovery correlates with sample viral load for both plasma-derived and cell-derived viral isolates

In order to compare the efficiency of the methods used to obtain primary HIV-1 isolates from plasma or peripheral blood mononuclear cells (PBMCs), we analyzed a total 94 samples from different subjects at unique time-points, with the exception of the four included in the study; 56 plasma samples and 38 PBMCs samples with viral loads ranging from 10 to $>10^6$ copies/ml.

Of those, we recovered a total of 63 primary isolates (34 from plasma samples and 29 from PBMCs). After stratification of samples by viral load, we observed an increase in the efficiency of virus recovery concomitant with the increase in viral load for both plasma and PBMCs HIV-1 isolation methods, Fig. 24.

Furthermore, the categorization of viral load ranges into linear values demonstrated the existence of a direct correlation between sample viral load range and efficiency of virus recovery (Plasma: $r = 0.94$, $p<0.016$; PBMCs: $r = 0.94$ p,0.016 [Spearman correlation test]). Therefore, overall efficiency of the HIV-1 isolation methods used was similar and correlated to sample viral load.

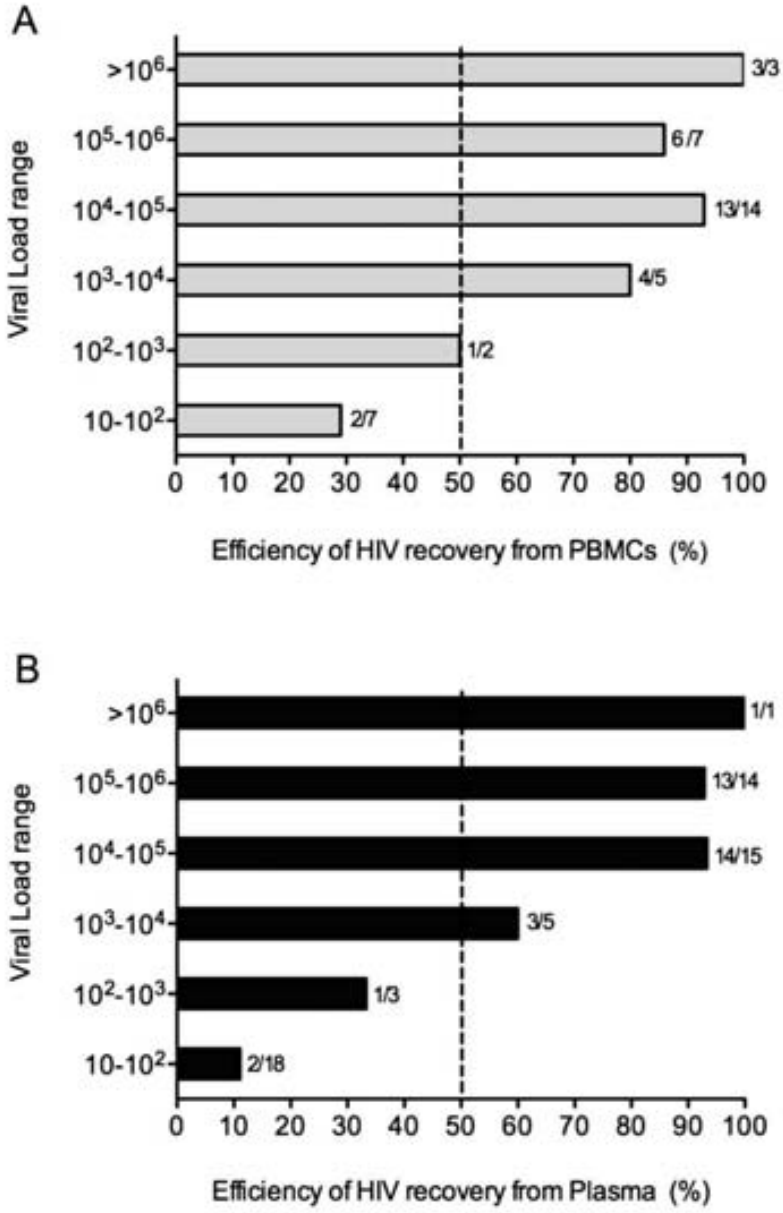


Figure 24. Comparison of the efficiencies of HIV-1 isolation methods from PBMCs or plasma samples. To determine the efficiency of HIV-1 isolation from PBMCs and plasma, we compared virus recovery from 56 plasma samples and 38 PBMCs samples with viral load ranging from 10 to $>10^6$ copies/ml. (A) Efficiency of HIV-1 recovery from PBMCs in percentages per viral load range. (B) Efficiency of HIV-1 recovery from plasma samples in percentages per viral load range. Bars represent mean values. Numbers next to the bars indicate (number of positive samples/total number of samples tested). Analysis of sCD14 plasma levels. (A) sCD14 levels were measured during the 3-year period after seroconversion and/or transcriptome analysis in RPs (n = 24) and VNPs.

Phylogenetic analysis of multiple-amplicon DPS reveals clusters of interspersed variants between cell virus isolates, plasma virus isolates, and plasma viral RNA

Four naïve, recent HIV-1-infected subjects were enrolled in the study. A summary of their clinical and epidemiological characteristics is shown in Table 8.

Table 8. Epidemiological and clinical data of study subjects.

Subject	Sex	Virus[a] Subtype	Time after seroconversion (months)	Viral Load at sample collection (HIV-1 RNA copies/ml)	CD4 T-cell count at sample collection (cells/µl)	Nadir CD4 T-cell count (cells/µl)
P20	Male	B	5.5	69,000	190	144
P21	Male	B	13.4	320,000	181	181
P22	Male	BF	4.2	27,000	576	242
P23	Female	B	1.3	170,000	627	197

[a] Virus subtype was determined based on sequences from *gag*, pol, and *env*-V3 using the REGA HIV-1 Subtyping tool. BF denotes the recombinant BF HIV-1 form.

Three sample types from a unique blood sample were obtained per subject, as represented in Fig. 25, for comparative purposes: 1.Total plasma viral RNA (RNA); 2. Plasma virus isolates (VP) after HIV-1 capture from plasma and virus *in vitro* expansion and; 3. Cell virus isolates (VC) obtained from PBMCs co-culture and virus *in vitro* expansion.

VP and VC primary isolates were expanded *in vitro* for a period of 2 to 3 weeks and 3 to 4 weeks respectively. Afterwards, virus were harvested for further genotypic (DPS) and phenotypic characterization (Tropism and Replicative Capacity) Fig. 25. Multiple-amplicon DPS was carried out in the three samples types RNA, VP, and VC, thus covering the gag, protease, integrase, and env-V3 regions with an average number of reads per nucleotide of 4039, 4193, 3629, and 4488, respectively. Data extracted using DPS were corrected for sequencing errors, filtered to a final number of unique reads, and merged into haplotypes (unique sequences represented in ≥1%), a resume of the sequences obtained after the various filtering steps is represented in Table 9.

Final haplotypes were used to build phylogenetic trees based on the best-inferred model for conserved regions gag, protease and integrase as well as variable regions env-V3 of the HIV-1 proteome. As shown in Fig. 26, the phylogenetic trees for gag, protease, and integrase did not show segregation of clusters between VC, VP, and RNA variants, with low genetic distances between sample types and preservation of major variants after *in vitro* culture.

Table 9. Number of sequences obtained for each subject and sample type by DPS.

Subject	Protein	Sample	Total Reads[a]	Valid Reads[b]	Unique Haplotypes[c]
P20		RNA	2,979	786	4
	Gag	VC	4,812	197	17
		VP	4,842	1,383	3
		RNA	3,18	2,321	2
	PR	VC	2,374	1,8	10
		VP	2,541	1,726	5
		RNA	6,898	4,271	3
	IN	VC	2,97	2,102	4
		VP	1,64	1,266	5
		RNA	1,823	1,535	3
	Env-V3	VC	4,776	2,951	3
		VP	3,101	2,701	3

cont.

Subject	Protein	Sample	Total Reads[a]	Valid Reads[b]	Unique Haplotypes[c]
P21		RNA	2.425	1.213	2
	Gag	VC	3.85	89	12
		VP	3.875	2.541	4
		RNA	6.503	5.115	2
	PR	VC	5.385	4.315	5
		VP	3.443	2.806	2
		RNA	3.931	2.702	4
	IN	VC	212	120	6
		VP	1.484	899	9
		RNA	3.073	2.511	2
	Env-V3	VC	5.723	3.594	2
		VP	4.265	3.545	1
P22		RNA	2.96	2.37	3
	Gag	VC	5.284	2.238	1
		VP	3.91	3.146	3
		RNA	2.582	2.138	3
	PR	VC	7.605	3.063	3
		VP	1.812	1.443	2
		RNA	8.334	4.527	6
	IN	VC	6.593	4.143	8
		VP	1.301	1.07	5
		RNA	2.698	2.412	1
	Env-V3	VC	10.669	3.476	2
		VP	5.14	4.335	4
P23		RNA	5.707	4.255	4
	Gag	VC	4.585	2.802	7
		VP	3.244	2.748	3
		RNA	4.189	3.64	2
	PR	VC	2.694	2.282	5
		VP	8.019	6.565	2
		RNA	5.752	4.746	4
	IN	VC	2.906	2.387	2
		VP	1.527	1.289	4
		RNA	2.417	2.083	1
	Env-V3	VC	5.534	3.298	4
		VP	4.639	4.18	1

a Total reads is the total coverage of sequences obtained after direct DPS. **b** Valid reads are those sequences obtained after cleaning the total reads by selecting unique sequences with >70% homology to HXB2 and manual correction of homopolymer tacks. **c** Unique haplotypes are defined by similar sequences represented as a proportion □1% from the total unique reads. DPS, deep pyrosequencing; PR, protease; IN, integrase; RNA, plasma viral RNA; VC, cell virus isolates; VP, plasma virus isolates.

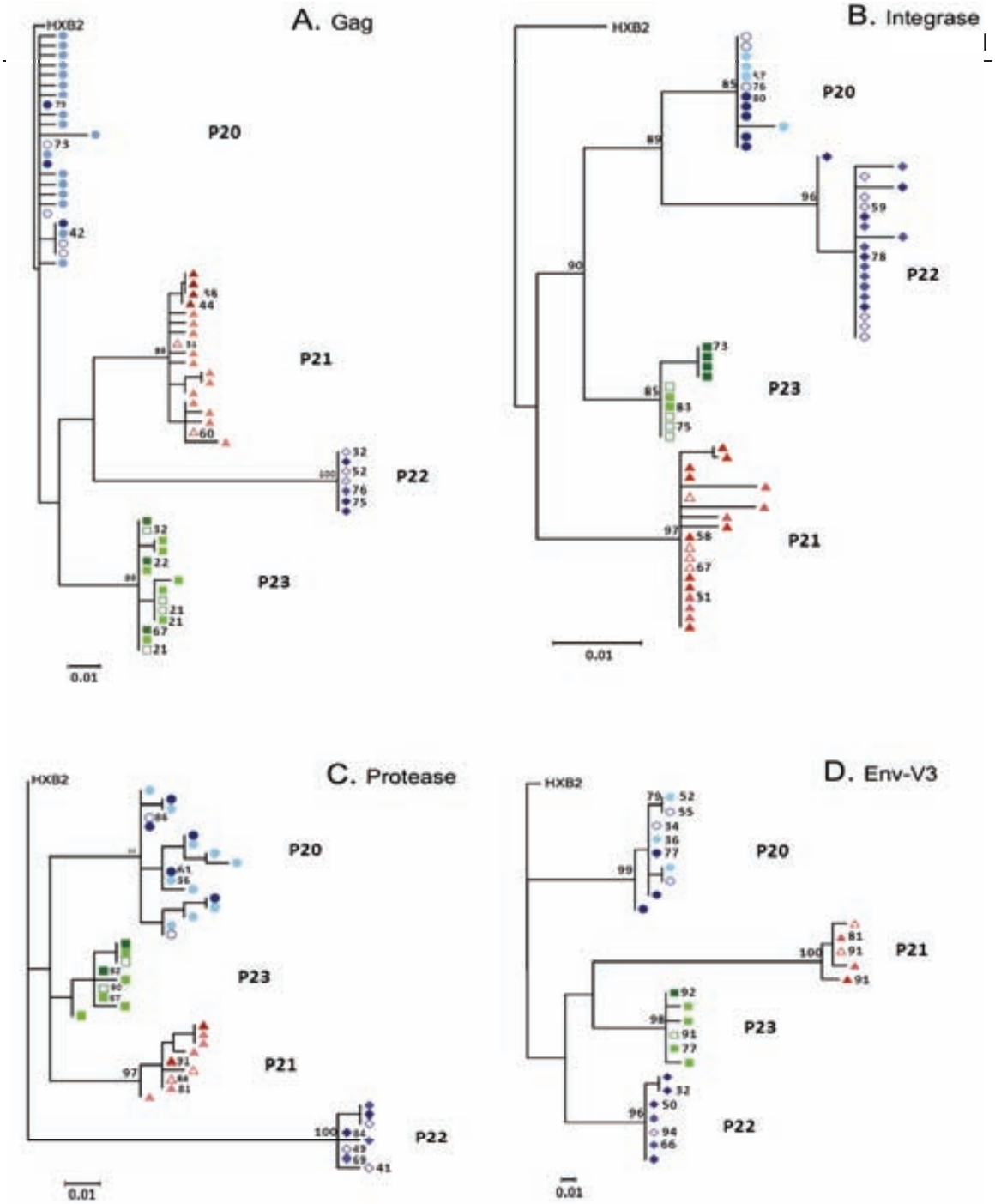


Figure 26. Phylogenetic trees for HIV-1 gag, protease, integrase and env-V3 sequences extracted from VC, VP, and RNA using DPS. Symbols represent unique haplotypes extracted from DPS for subjects P20 #, P21 n, P22 e, and P23 %, according to sample type: VC (light symbols), VP (dark symbols), and RNA (empty symbols). Numbers next to symbols indicate haplotype frequencies obtained from the total reads; only values above 20% are indicated in the figure. Node numbers indicate bootstrap values over 75%. (A) gag maximum-likelihood phylogenetic tree based on the TrN model. (B) Integrase maximum-likelihood phylogenetic tree based on the HKY model. (C) Protease maximum-likelihood phylogenetic tree based on the HKY+ G (a = 0.565) model. (D) env-V3 maximum-likelihood phylogenetic tree based on the TrN model.

Low intra- and inter-population variability for VC, VP, and RNA variants among HIV-1 proteins

To define in detail VC, VP, and total viral RNA populations, we calculated intra- and inter-population variability, defined as the tendency for individual genomes to vary from one to another in a population. For that purpose, we simulated a viral population by considering the sequences obtained in the DPS run as a sample of the real population. We measured pairwise intra- and interpopulation variability according to sample type for each HIV-1 protein and subject. We found low intra-population variability, with values close to zero for VC, VP, and RNA populations in all subjects and genetic regions (Table 10).

Table 10. Comparison of intra- and inter-population variability for each subject, HIV-1 protein, and sample type (RNA, VC, or VP).

		Π^{a}_{intra}			Π_{inter}		
Subject	Protein	Π_{int} RNA	Π_{int} VC	Π_{int} VP	RNA vs VC	RNA vs VP	VC vs VP
P20	Gag	0.0011	0.0110	0.0008	0.0010	<0.0001	0.0009
	PR	0.0005	0.0075	0.0041	0.0022	0.0033	0.0002
	IN	0.0005	0.0013	0.0004	<0.0001	<0.0001	<0.0001
	Env-V3	0.0051	0.0050	0.0018	<0.0001	0.0037	0.0035
P21	Gag	0.0240	0.0300	<0.0001	0.0037	0.0023	0.0040
	PR	0.0006	0.0010	<0.0001	<0.0001	<0.0001	<0.0001
	IN	0.0009	0.0025	0.0014	<0.0001	<0.0001	<0.0001
	Env-V3	0.0002	0.0006	<0.0001	<0.0001	0.0194	0.0194
P22	Gag	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	PR	0.0033	0.0018	0.0009	0.0013	0.0012	<0.0001
	IN	0.0009	0.0016	0.0005	<0.0001	<0.0001	0.0002
	Env-V3	<0.0001	<0.0001	0.0437	<0.0001	0.0033	0.0033
P23	Gag	0.0023	0.0036	<0.0001	<0.0001	0.0007	0.0012
	PR	0.0002	0.0029	0.0010	<0.0001	<0.0001	<0.0001
	IN	0.0004	0.0002	0.0006	<0.0001	0.0042	0.0042
	Env-V3	<0.0001	0.0012	<0.0001	<0.0001	<0.0001	<0.0001

[a] Average number of nucleotide differences per site between sequences. PR, protease; IN, integrase; RNA, plasma viral RNA; VC, cell virus isolates; VP, plasma virus isolates.

Additionally, interpopulation analyses comparing RNA with VC, RNA with VP, and VC with VP (Table 10) demonstrated a similar pattern of low variability. These results indicated that VC, VP, and plasma viral RNA populations were composed of HIV-1 variants with low intra- and inter-population variability.

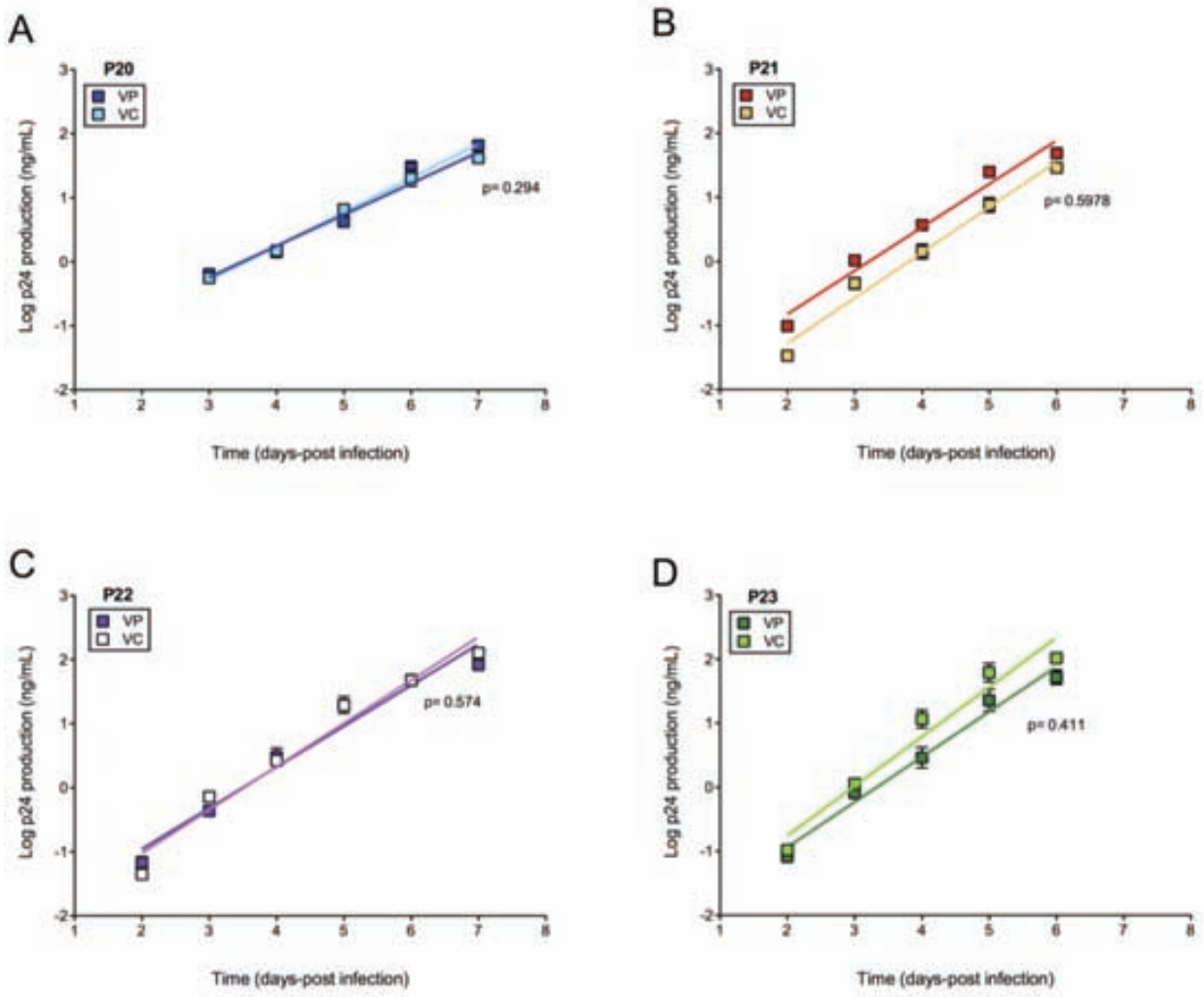


Figure 27. Replicative capacity of VC and VP HIV-1 isolates in primary cells. PBMCs stimulated from seronegative donors were infected in triplicate with each viral variant, and virus growth was monitored by p24 production over one week. Slopes were compared for VC and VP pairs, and p,0.001 was considered significant. Squares represent mean values and bars represent the standard error of the mean. Light squares correspond to VC and dark squares correspond to VP. (**A**) P20, (**B**) P21, (**C**) P22, and (**D**) P23.

VC and VP isolates display similar in vitro replicative capacity in primary cultures

In certain cases, heterogeneity in the distribution of quasispecies during *in vitro* passage of HIV-1 modified virus fitness in the absence of changes in the consensus sequences [447]. In order to test whether minor genetic changes

described in our populations (synonymous changes, differences in the number of unique variants) affected the phenotypic properties of VC and VP isolates, we measured replicative capacity for VC and VP isolates in primary cells. After infection, viral growth was monitored by p24 production for one week and the log-transformed data on the exponential growth phase used to calculate the virus growth rate (slope of the linear regression) for each type of isolate.

As shown in Fig. 27, VC and VP pairs display similar replication kinetics with no differences in replicative capacity per pair in any of the study subjects. Thus, in spite of minor genetic differences in quasispecies composition, our data revealed no differences in replicative capacity between VC and VP isolates for each subject.

Phenotypic determination and genotypic prediction of co-receptor usage in VC isolates, VP isolates, and plasma RNA

HIV-1 co-receptor use is a key determinant of viral pathogenesis; the presence of CXCR4 using strains has been related to disease progression, and detection of minor CXCR4 variants has a clear clinical interest in the management of CCR5-antagonists [144, 440, 448, 449].

Therefore, in order to understand the relationship between VC, VP and plasma RNA, we compared virus coreceptor use by means of U87 cells in VC and VP isolates and by means of ESTA in plasma RNA. Furthermore, for genotypic prediction of virus co-receptor, we used the PSSM and g2p algorithms in env-V3 loop from the most frequent haplotypes, with cut-off values of -4.75 and ≤3.5, respectively. These results are summarized in Table 11.

Phenotypic data show a concordance of 100% between U87 and ESTA results. Moreover, genotypic prediction of co-receptor usage with PSSM was 75% (9/12) concordant with g2p. In spite of minor discrepancies between the methods used, plasma RNA, VC, and VP isolates exhibited good matching in terms of virus co-receptor per study subject and sample type.

Table 11. Phenotypic and genotypic prediction of co-receptor usage from total plasma RNA, VC and VP primary isolates.

Sample	Phenotype		Genotype	
	U87	**ESTA[a]**	**PSSM[b]**	**g2p[b]**
P20 RNA	-	X4/R5	R5	Non-R5
VC	X4/R5	-	R5	Non-R5
VP	X4/R5	-	R5	Non-R5
P21 RNA	-	R5	R5	R5
VC	R5	-	R5	R5
VP	R5	-	R5	R5
P22 RNA	-	R5	R5	R5
VC	R5	-	R5	R5
VP	R5	-	R5	R5
P23 RNA	-	R5	R5	R5
VC	R5	-	R5	R5
VP	R5	-	R5	R5

[a] ESTA, Enhance Sensitivity Trofile Assay. This assay has a detection limit of 0.3% for non-R5 variants. [b] Cut-off values to define non-R5 using sequences were –4.75 for PSSM and ≤3.5 for g2p, respectively. Non-R5 sequences include X4 and X4/R5 dual tropic virus. RNA, plasma viral RNA, VC, cell virus isolates; VP, plasma virus isolates. – Indicates non-determined.

Additionally, a more detailed prediction of coreceptor use was made in VC, VP, and RNA by inference of g2p and PSSM scores in the unique env-V3 sequences extracted from DPS. We observed a cluster of combined variants from VC, VP, and RNA with low intra-patient deviation and preferential R5 use, with the exception of p20 Fig. 28. In the case of P20, g2p and PSMM scores from DPS sequences suggest the presence of a homogeneous population of X4R5 dual tropic virus when compared to previously defined R5+X4R5 or X4R5 HIV-1 isolates Fig. 28 [403]. Therefore, inference of phenotypic and genotypic tropism in VC and VP pairs and plasma viral RNA demonstrated concurrence in virus co-receptor usage among sample types for each study subject.

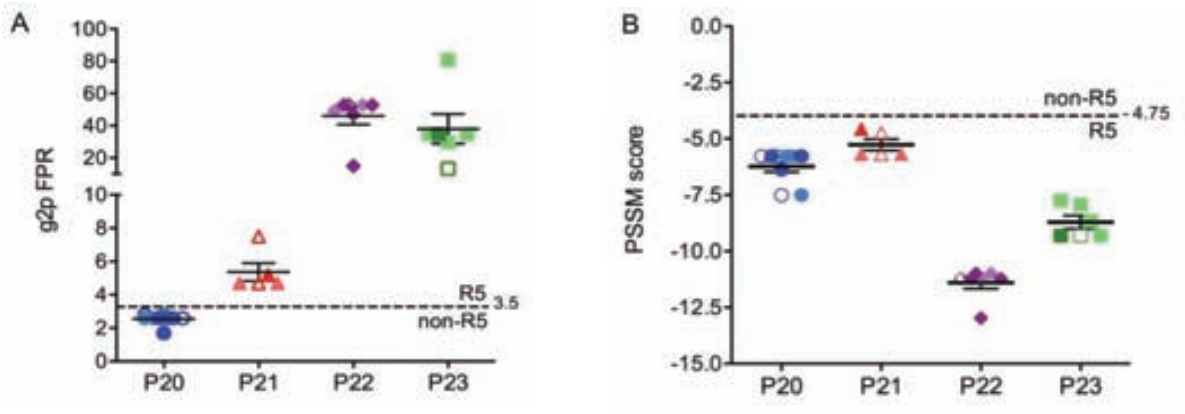


Figure 28. Genotipic predicition of co-receptor use in: DPS sequences from VC, VP and total plasma RNA. Unique sequences obtained from the DPS of the env-V3 loop region were used to run PSSM and g2p algorithms to infer virus co-receptor use per sample type VC (ligth symbols), VP (dark symbols) and RNA (empty symbols) and subject. For comparative purposes env-V3 loop sequences from virus with dual mix (R5+R5X4) and dual (R5X4) co-receptor use were included. (A) Prediction of co-receptor use based on g2p algorithm with a false positive rate of 10%. Dashed line represents cut-off values (3.5) to infer R5 and non-R5 use. (B) Prediction of co-receptor use based on PSSM scores. Dashed line represents cut-off value (24.75) to infer R5 and non-R5 use. (TIF)

DISCUSSION

Primary viral isolates play a key role in our understanding of the HIV-1 pathogenesis and are a common approach for various *in vitro* studies such as antibody neutralization, drug testing, or virus co-receptor use assays. Furthermore, the relevance of using primary isolates for an accurate description of virus phenotype has been highlighted by differences in replicative capacity found between recombinant viruses and full isolates [443, 450].

Previous studies in the HIV-1 field have determined whether viral populations from primary isolates were representative of *in vivo* findings with contradictory results. Some of them report a decrease in HIV-1 gp120 diversity in isolates [451], while others support the maintenance of major variants in blood after coculture with PBMCs [452]. Additionally, most of these studies are limited by the number of samples analyzed, the number of clonal sequences obtained, and their focus on comparing proviral DNA to primary isolates recovered from co-cultured PBMCs. In

order to overcome previous experimental limitations, we carried out multiple-amplicon DPS to genetically compare thousands of sequences in four regions of the HIV-1 genome and clearly define phylogenetic relationships between primary isolates obtained from VP, VC after *in vitro* HIV-1 expansion and plasma circulating quasispecies (RNA) *in vivo*. Our results demonstrate a structured population of interspersed major VC, VP, and RNA variants with fluctuations in low frequency unique sequences in most of the HIV-1 genes studied (gag, protease, integrase, and env-V3) and among subjects but with no significant differences in the total numbers of unique haplotypes (data not shown). The presence of major variants in similar frequencies for VC and VP primary isolates, when compared to *in vivo* RNA, demonstrates the maintenance of high frequency variants after *in vitro* expansion in both VC and VP isolates. Furthermore, the low intra- and interpopulation variability, with values close to zero, reflects homogeneous populations both within HIV-1 proteins or sample types. Nevertheless, relative homogeneous viral populations have been reported in both proviral HIV-1 DNA and plasma HIV-1 RNA during early infection [215, 453]. As a consequence, the low level of genetic variability found among primary viral isolates and total RNA, could be related to the short time after seroconversion in our samples, where homogeneous viral populations will be present before diversification at later stages of disease [215]. On the other hand, recent studies on founder virus evolution support early variation in the HIV-1 genome after transmission and accumulation of changes over the first year after infection [26]. In this context, our results suggest an adequate representation of RNA circulating quasispecies after HIV-1 *in vitro* expansion. However, these results should be viewed with caution until they are confirmed in chronically infected samples.

RNA virus populations are composed of a swarm of closely related genotypes or quasispecies in which viral evolution operates as a unit and adaptation is the result of cooperative interactions between multiple genomes [435]. Various studies have demonstrated how minor genetic differences in composition and quasispecies heterogeneity can modulate HIV-1 fitness in the absence of changes in population sequence [447, 454]. Additionally, genetic similarities in studied regions cannot be

extrapolated to the whole viral genome. Therefore, similarities in virus genotype might not take the form of similarities in virus phenotype. In this context, our data revealed no differences in terms of virus replicative capacity in paired VC and VP isolates, regardless of minor differences in genotypic composition of the viral quasispecies studied. However, our approach is limited by the short-term *in vitro* culture of the replicative capacity experiments and presence of antiretroviral drugs, neutralizing antibodies, cytotoxic T lymphocytes, or other selective pressures may induce unpredictable fluctuations in closely related viral populations, which are not capture in this study.

Together with replicative capacity, HIV-1 co-receptor use is an essential trait when defining HIV-1 pathogenesis. The presence of CXCR4-using HIV-1 variants is associated with disease progression [144, 448, 449], and detection of minor CXCR4 HIV-1 populations has become a key marker for the management of CCR5 antagonists [440, 455]. A previous study showed high concordance of co-receptor usage in paired plasma and PBMCs samples during primary infection [456]. In agreement with this observation, we found concordance in co-receptor use between VC isolates, VP isolates, and plasma RNA as measured both by ESTA and U87. Comparable results were obtained by genotypic inference of virus co-receptor use in DPS env-V3 sequences with g2p and PSSM. Regardless of small differences in the methods applied intra-subject, co-receptor use was very homogeneous.

Many studies have described the use of DPS in combination with genotypic algorithms in the env-V3 variable region as a key tool when detecting minor CXCR4 populations for the management of CCR5 antagonists. We used the same approach to compare VC isolates, VP isolates, and circulating plasma quasispecies. We found clusters of mixed sequences from VC, VP, and RNA sequences with homogeneous populations and preferential R5 use. These data contrast with those of previous studies, where DPS revealed the presence of more heterogeneous populations in proviral quasispecies [455], but argue in favor of homogeneous replication-competent populations obtained after *in vitro* expansion in VC primary isolates, regardless of the heterogeneity in proviral DNA.

In summary, our study provides the first direct comparison of viral isolates with plasma circulating quasipecies using DPS in recently HIV-1 infected subjects. Our data demonstrated that VC and VP share genotypic characteristics with HIV-1 quasispecies and maintain the presence of major variants after virus *in vitro* expansion. In spite of minor genetic differences, phenotypic data reveal similarities in paired VP and VC isolates with regard to replicative capacity and co-receptor use. Our data support the potential use of VP or VC primary isolates as a reliable tool to characterize the circulating quasispecies. Nevertheless, further comparisons will help to clarify whether our findings also apply to later stages of the disease.

Chapter 3d

STUDY IV

Highly Pathogenic Adapted HIV-1 Strains and Limited Immune Responses Dictate Rapid Disease Progression

This chapter corresponds to the manuscript:

Dalmau J.*, Rotger M.*, Erkizia I., Rauch A., Reche P., Pino M., Esteve A., Palou E., Brander C., Paredes R., Phung P., Clotet B., Telenti A.#, Martinez-Picado J.#, Prado J.G.#; The CoRP Study Group. ***Highly Pathogenic Adapted HIV-1 Strains and Limited Immune Responses Dictate Rapid Disease Progression.*** *Submitted for publication*

**Equal contribution, #Corresponding authors*

PRESENTATION

Once we corroborated that both samples' source, plasma and PBMCs, could be used for the isolation of virus from our patients, we obtained the primary isolates and started the study of the characteristics of the viral strains of RPs, in comparison to that of SPs. Furthermore, an exhaustive study of cellular and neutralizing immune responses was carried out. Finally, immunogenetics and adaptive/escape profiles were also analyzed. The results of these sections of the project are showed in the present chapter.

INTRODUCTION

Humans show a remarkable variation in the clinical outcome following HIV-1 infection. In absence of antiretroviral therapy, the median time for development of AIDS has been estimated to vary from 7.7 to 11.0 years depending on age at seroconversion [28]. Nevertheless, there are marked variations from these estimates: while some individuals are able to control HIV-1 replication for longer periods such as HIV-1 Controllers (HIC) or Elite Controllers (EC), others progress quickly to AIDS or meet criteria for antiretroviral treatment initiation within the first few years after seroconversion (Rapid Progressors, RP). Thus, the studies of extreme phenotypes provide a unique opportunity to dissect the contribution of viral and host factors to HIV-1 disease outcome [73, 74, 403, 457].

To date, most of the studies on HIV-1 extreme phenotypes have focused on HIC or EC as a model to define the correlates of protective immunity and virus control [73, 74]. By contrast, the study of RP has proved particularly challenging because the identification of this selected disease phenotype requires a reliable estimation of the seroconversion date in a small window of clinical observation [457]. These limitations are still more remarkable if biological samples for multiple analyses need to be taken. Consequently, a number of constraints have hampered the accurate interpretation of this extreme clinical phenotype. This is reflected in the very low number of core studies and the heterogeneity of the reported data [75, 87,

403]. However, to uncover the factors contributing to rapid disease progression is crucial to improve the clinical management of these individuals.

The present study comes from a previous case-report study on HIV-1 extremely severe progression [403] in order to broadly characterize RP in functional terms, including viral and host factors. The aim of this work was to bring new insights into the causes of rapid progressive disease through the recruitment of a large group of RP never before identified and compared to HIV-1 infected subjects with average disease progression. Our criteria for rapid progression based on the reduction of CD4+ T-cell counts below 350 cells/µl within three years after documented seroconversion in the absence of antiretroviral treatment, accounts for an accurate selection of RP, as previously validated by our genomic studies [457].

Our findings reveal a specific profile of HIV-1 traits such as coreceptor usage, replicative capacity and virus variability common to RP. Additional differences between groups were observed in cellular and humoral immune profiles and HLA-class I immunogenetics.

MATERIALS AND METHODS

Cohort description and Study Groups. In a collaborative effort between the AIDS Research Institute, IrsiCaixa, (Barcelona, Spain) and the Swiss HIV Cohort Study (SHCS), retrospective and prospective recruitment of HIV-1 seroconvertors allowed us to establish two progression cohorts. The cohorts were defined based on a HIV-1 seroconversion window of less than a year with documented negative and positive serology. Disease progression stage was established in the period of 3 years after HIV-1 seroconversion. The Cohort of Rapid Progressors (CoRP, n=106) were clinically defined by 2 or more CD4+ T-cell counts bellow 350 cells/µl with no subsequent increase in the absence of antiretroviral treatment and/or antiretroviral treatment initiation for clinical criteria and CD4+ T-cell counts bellow 350 cells/µl after treatment initiation. Individuals with average progression or Standard Progressors (CoSP, n=84) maintained CD4+ T-cell counts above 350 cells/µl, over the same period of time in absence of antiretroviral treatment (**Fig**

29). Based on clinical data and availability of biological materials we selected two study groups for comparative analyses: the RP (n=46) and the SP (n=46) (**Fig 29**).

Table 12. Characteristics of rapid and standard progressors included in the study

	RP (n=46)	SP (n=46)	P-value
Male gender, n (%)	35 (76.1)	43 (93.5)	0.02
Age at HIV-1 diagnostic, median (IQR), years	31 (26-37)	30 (26-35)	0.48
White ethnicity, n (%)	39 (84.8)	32 (71.1)	0.08
Mode of transmission, n (%)			0.002
Heterosexual	12 (26.1)	5 (10.9)	
MSM	20 (43.5)	37 (80.4)	
IVDU[a]	10 (21.7)	1 (2.2)	
Other & NA	3 (8.7)	3 (6.5)	
B viral subtype[b], n (%)	38 (86.4)	43 (93.5)	0.11
Baseline CD4 count[c], median (IQR)	385 (245-508)	658 (442-804)	<0.0001
Baseline Viral Load[d], log_{10} copies/ml, median (IQR)	4.88 (4.48-5.51)	4.35 (3.75-4.99)	0.02
Viral Load setpoint[e] log_{10} copies/ml, median (IQR)	4.73 (4.28-5.21)	4.10 (3.22-4.37)	<0.0001
CD4 count mean in the absence of ART[f], median (IQR)	317.3 (216.1-393.7)	615.3 (518.8-713.6)	<0.0001

[a] Intravenous Drug Users, [b] Viral subtype was available for 44 RP and 46 SP, [C] Baseline CD4 count was available for 37 RP and 30SP, [d] Baseline Viral Load was available for 34RP and 28SP, [e] Viral Load Setpoint was available for 35RP and 37SP, [f] CD4 count mean was available for 43RP and 39SP, NA not available; RP, rapid progressors, SP, standard progressors; IQR, interquartile range

The samples were selected at baseline (BL) at the earliest timepoint available after seroconversion (median ≤ 1 year) and after a median of 1.5 years POST-baseline (P). Of note, for the RP study group, despite a window of 3 years to define HIV-1 rapid progression, individuals were equally distributed across severity groups over the 3-years period after seroconversion, with a 63% (29/46) of RP progressing in less than a year and a remaining 37% in the range of progression between 1 and 3 years after seroconversion. Groups' characteristics included in the study are summarized in **Table 12**.

Together with differences in viral load set point, baseline CD4 count and mean CD4 count between groups obtained by clinical definition of RP. We observed additional differences in the mode of HIV-1 transmission with an increase of intravenous drug users (IVDU) in the RP group. Therefore, to discard any potential bias in our results due to overrepresentation of IVDU in RP, sensitivity analyses were carried out excluding IVDU for all data sets. The robustness of sensitivity analyses and the consistency of the results regardless the presence of IVDU in RP, validate the maintenance of the IVDU in the RP to balance the number of individuals in the study groups.

Ethics Statement. The project was approved by the institutional review board at Hospital Germans Trias i Pujol and at the University Hospital of Lausanne; all subjects gave their written informed consent to participate.

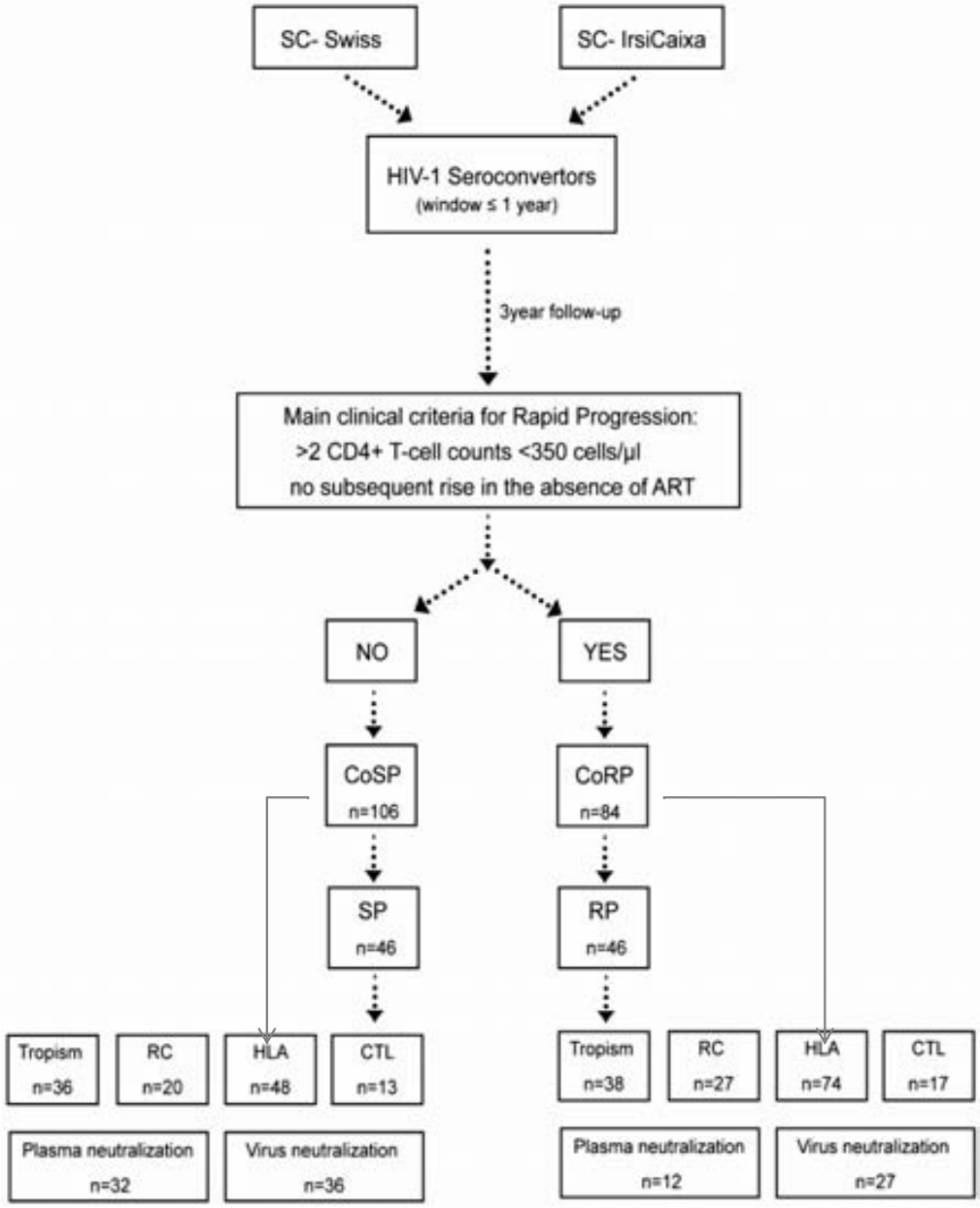


Figure 29. Flow- diagram on selection criteria and cohort description for RP and SP. Subjects were recruited from the Spanish and Swiss cohorts based on available documented seroconversion in a window of 1 year between the first negative and the first positive sample. The main clinical criteria from inclusion in the Rapid Progressors Cohort (CoRP) was CD4+ T-cell counts below 350 (cells/ml) within the first three years after documented seroconversion as represented in the diagram. Patients that do not meet inclusion criteria were included in the Standard Progressors Cohort (CoSP). For the present study 46 patients from each group (SP and RP) were selected based on clinical data and sample availability early after HIV-1 seroconversion and a median of 1.5 years from initial sampling. Sample distribution among study groups and experiments is represented at the bottom of the figure (Tropism; RC, replicative capacity; HLA; CTL or CD8+ T-cell responses, plasmas neutralization and virus neutralization).

HIV-1 isolation from plasma and PBMC and co-receptor usage. Viral isolates were obtained from plasma samples using anti-CD44 beads following the manufacturer's protocol (Miltenyi Biotec, Germany) with minor modifications as previously described [443, 446, 458]**.** In addition, viral isolates from cryopreserved PBMC were obtained by co-culture of HIV-1 infected PBMC for each individual with a pool of PBMC from three HIV-1 seronegative subjects, as described in [443, 458]. Viral isolate growth was monitored weekly by p24 ELISA and virus were harvested at a p24 concentration >50 ng/ml, and stored at –80°C. Tropism from viral isolates was measured in U87 immortalized cell lines expressing CCR5 or CXCR4, as previously described [443] in 74 samples (38 RP and 36 SP). Virus tropism was assessed in parallel in plasma samples using the Enhanced Sensitivity Trofile Assay (ESTA, with a detection limit of 0.3% for non-R5 HIV-1 variants).

Virus replicative capacity. Viral isolates obtained from plasma and cells were titrated in the TZM-bl cells. Replicative capacity experiments were carried in a pool of PBMC from three seronegative individuals, previously stimulated for 72 h with PHA (3 μg/ml) and IL-2 (10 U/ml). Stimulated PBMC were then infected in triplicate at a multiplicity of infection of 0.001 of each viral variant for 2 h, subsequently washed twice with phosphate-buffered saline (PBS), and cultured in R20 supplemented with IL-2 (20 U/ml) (Roche, Spain) [382]. Viral growth was measured by p24 ELISA in supernatants over 10 days (Perkin Elmer, Spain). Replicative capacity was calculated by fitting a linear model to the $\log_{10}$-transformed data of p24 production in the exponential growth phase to calculate the slope for each virus [446]. Slope values were standardized in percentage to the slope of HIV-1_{NL4-3} reference virus as 100%.

HLA typing and assessment of HIV-1-specific CD8+ T-cell responses. High-resolution HLA class I-typing for alleles A, B and Cw was performed from all patients by sequence-based typing methods. HIV-1-specific CD8+ T-cell responses were assessed at BL and P timepoints. Clinical parameters and sampling times are detailed in **Table 13.** Comprehensive HIV-1 epitope screening of optimal responses was carried out by Interferon-γ (IFN-γ) ELISpot assay as previously described [85]. Wells were considered positive if containing at least 50 spot forming cells (SFC) per 10^6 PBMC calculated based on (2x Mean + 3x STD) above the background level. Frequency of HIV-1-specific responses was calculated as: (number of positive responses/number of optimal peptides tested) x 100.

Table 13. Clinical parameters of the study subgroups selected for the assessment of HIV-1 specific CD8+ T cell responses**.**

Subjects	Sample for ELIspot [b]	VL[a] (HIV_{RNA} copies/ml)	CD4 count[a] (cells/μl)	HLA-I molecules A	B	C
RP46	P	69,000	190	A*33:01/34:02	B*08:01/38:01	Cw*07:01/12:03
RP47	BL, P	25,000	375	A*29:02/11:01	B*44:03/35:01	Cw*04:01/16:01
RP48	BL, P	110,000	59	A*02:01/68:01	B*35:03/44:02	Cw*03:04/05:01
RP51	BL, P	370	473	A*11:01/30:02	B*18:01/44:01	Cw*03:04/05:01
RP52	BL, P	na	627	A*01:01/02:01	B*44:03/58:01	Cw*04:01/07:01
RP53	BL, P	320,000	181	A*24:02/68:02	B*15:03/40:02	Cw*02:10/03:04
RP54	P	<50	725	A*02:01/02:17	B*15:40/35:02	Cw*03:03/04:01
RP55	P	<50	419	A*01:01/24:02	B*07:02/08:01	Cw*07:01/07:02
RP58	BL, P	27,000	576	A*02:01/24:02	B*44:02/44:03	Cw*05:01/16:02
RP60	BL	<50	535	A*02:01/02:01	B*44:02/44:02	Cw*05:01/14:02
RP61	BL, P	<50	673	A*01:01/2902	B*44:03/58:01	Cw*03:02/16:01
RP62	BL, P	3300	339	A*02:01/33:01	B*14:02/15:01	Cw*03:03/08:02
RP69	P	<50	565	A*29:02/29:02	B*44:03/35:03	Cw*04:01/16:01
RP74	P	2,000,000	1	A*02:01/03:01	B*07:02/18:01	Cw*07:02/12:03
RP75	na	29,400	108	A*02:01/11:01	B*35:03/40:01	Cw*03:04/04:01
RP76	na	na	na	A*24:02/33:01	B*08:01/14:02	Cw*07:01/08:02
RP77	na	40,000	32	A*30:01/30:02	B*18:01/50:02	Cw*04:01/05:01
SP1	BL, P	8,800	806	A*01:01/24:02	B*14:02/51:08	Cw*16:02/16:02
SP2	P	5,100	516	A*01:01/24:02	B*18:01/57:01	Cw*05:01/07:01
SP3	BL	801	na	A*02:01/29:02	B*27:05/44:02	Cw*02:02/05:01
SP6	BL, P	2,900,000	244	A*24:02/32:01	B*49:01/51:01	Cw*02:02/07:01
SP7	BL, P	280,000	420	A*11:01/24:02	B*35:01/35:02	Cw*04:01/04:01
SP8	BL	160,000	660	A*24:02/30:02	B*18:01/51:01	Cw*05:01/15:02
SP12	BL	<50	1,198	A*01:01/29:02	B*44:03/57:01	Cw*07:01/16:01
SP14	BL, P	8,700	731	A*02:01/30:02	B*18:01/27:05	Cw*01:02/05:01
SP15	P	160,000	422	A*31:01/02:01	B*08:01/18:01	Cw*07:01/12:03
SP17	na	39,000	979	A*02:01/02:01	B*44:02/44:02	Cw*05:01/14:02
SP18	BL, P	690	798	A*01:01/23:01	B*27:05/51:01	Cw*02:02/02:02
SP19	na	7,000	1,099	A*26:01/29:02	B*14:01/44:03	Cw*08:02/16:01
SP20	BL, P	34,000	1,009	A*11:01/25:01	B*15:01/55:01	Cw*03:03/03:03

[a] Viral load (VL) and CD4 values corresponding to baseline samples; BL , Baseline sample; P, Post-baseline sample, na, not available

HIV-1 genome sequencing. HIV-1 full-length genome sequencing was performed in 9 RP and 8 SP. Briefly viral RNA was obtained from 2ml of plasma after ultracentrifugation and extraction (Qiamp Viral RNA MiniKitTM, Qiagen). Total viral RNA was retrotranscribed to cDNA with oligodT primers and use as a template for a first round PCR amplification with HIV-1 specific primers; 5´-AAATCTCTAGCAGTGGCGCCCGAACAG-3´ (208 → 212HXB2) and 5´-GGCAAGCTTTATTGAGGCTTAAGC-3´ (9624 → 9601HXB2). Second round nested-PCR was carried in order to amplify two HIV-1 hemigenomes using specific primers as follow; for the 3´END 5´- TCTGGAAAGGTGAAGGGGCAGTAG-3´ (4954 → 4977HXB2) and 5´- GGTCTAACCAGAGAGACCCAGTACAG-3´ (9557 → 9532HXB2), and for the 5´END primers: 5´-CAGGACTCGGCTTGCTGAAGC-3´ (692 → 712HXB2) and 5´-CCCTAGTGGGATGTGTACTTCTGAAC-3´ (5220 → 5195HXB2). Additionally, partial HIV-1 genome sequencing was carried out for Gag and Pol genes in total of 40 subjects (22RP and 18SP) from plasma samples. Gag and Pol genes were amplified from viral RNA by one-step RT-PCR and nested-PCR. Virus subtype was assigned using the REGA HIV-1 Subtyping tool (dbpartners.stanford.edu/RegaSubtyping/) in Gag and Pol sequences.

Frequency of HIV-1 variation at optimally defined CD8+ T cell epitopes. HIV-1 optimal CD8+ T-cell epitopes were chosen base on the best-defined CD8+ epitope summary from Los Alamos Molecular Immunology Database (http://www.HIV.lanl.gov/content/immunology). HIV-1 variation at optimal CD8+ T-cell epitopes, was determined using full and partial length viral sequences from RP and SP subjects with HLA class I with four digits type resolution. HIV-1 variation at optimal epitopes was defined using the epitope-sequence mapping tool EPIMAP, that allowed for a fuzzy matching with up to 3 substitutions per HIV-1_{HXB2} epitope and their HLA-I restriction element at high definition level. Insertions and deletions were not allowed. This epitope mapping tool was built using the *String::Aprox* - Perl module and is available upon request. Frequency of virus variation at HLA-I match-epitopes was determined by the ratio between the number of total mismatches found at optimal epitopes/number of total optimal epitopes found per sequence.

Determination of plasma HIV-1 neutralizing activity and virus susceptibility to neutralization. Heterologous neutralizing activity of plasmas was tested against a standard panel of HIV-1 isolates (**Table 14**). Reference panel virus NL4-3, SF162 and JR –CSF were included as positive control and amphotropic murine leukemia virus (aMLV) was used as specificity control. Neutralizing activity was also tested in a panel of autologous and heterologous Env-pseudotyped recombinant virus obtained from plasma samples, as previously described [459]. In order to determine virus sensitivity to neutralization in RP and SP, Env-pseudotyped virus from both groups were tested against a standard panel of sera (309194, Z1679, Z1641, Z23) and a panel of bNAbs (b12, 4E10, 2F5, 2G12) [460]. Neutralization experiments were performed with the use of the PhenoSense™ HIV-1 neutralization assay in full titers curves [460]**.** Neutralizing antibody titres in plasma samples were calculated as the reciprocal of the plasma dilution conferring 50% inhibition (IC50) [461]**.** For the bNAbs panel, neutralizing values are expressed as the concentration of IgG that reduces infectivity by 50% (IC50).

Table 14. Standard panel of HIV-1 reference strains to measure neutralizing activity**.**

Virus panel	HIV stage	Subtype	Level of Neutralization Sensitivity to Subtype B HIV+ plasma	bNAbs Sensitivity	Sensitivity group
94UG103	Chronic	A	Moderately Resistant	b12, 4E10	Moderate
92BR020	Chronic	B	Moderately Resistant	b12, 2G12	Moderate
93IN905	Chronic	C	Moderately Resistant	b12, 4E10	Moderate
MGRM -C-026	Chronic	C	Resistant	b12, 4E10	Resistant
92TH021	Chronic	CRF01-AE	Moderately Resistant	b12, 2F5, 4E10	Moderate
MGRM-SC-B-006	Acute	B	Resistant	b12, 2G12, 4E10, 2F5	Resistant
MGRM-D-009	Chronic	D	Moderately Sensitive	4E10, 2F5	Moderate
MGRM- AG -002	Chronic	CRF02-AG	Resistant	4.00E+10	Resistant
1196	Early	B	Sensitive	b12, 2G12, 4E10, 2F5	Sensitive
SF162	Control	B	Very Sensitive	b12, 2G12, 4E10, 2F5	Sensitive
JRCSF	Control R5	B	Very Sensitive	b12, 2G12, 4E10, 2F5	Sensitive
NL4-3	Control X4	B	Very Sensitive	b12, 2G12, 4E10, 2F5	Sensitive
aMLV	Negative	---	Resistant	---	Resistant

Statistical analysis. For clinical groups comparisons p-values were calculate using Mann-Whitney U test for continuous variables (age, CD4-BL, VL-BL, VL set-point and CD4 mean) and Fisher's exact test for categorical variables (gender, ethnicity, mode of transmission and virus subtype). Spearman's correlation coefficient was calculated between CD4+ T cell count and virus replicative capacity. P values of <0.05 were considered significant. Statistical significance of

the HLA-class allelic distribution in the study groups were reported correcting for multiple comparisons using the Benjamini and Hochberg procedure. All analyses were performed using SAS 9.3 (Statistical Analysis Software, Cary, NC, USA).

RESULTS

Viral strains in rapid progressors have an increased frequency of X4/DM coreceptor usage and overall greater replicative capacity

HIV-1 coreceptor usage and virus Replicative Capacity (RC) are key for the establishment of a productive infection upon transmission bottleneck and to determine the rapidity of CD4+ T-cell depletion at the early stages of the disease. These viral traits have been associated with HIV-1 pathogenesis and disease outcome [448, 456, 462, 463] and are crucial to be evaluated in the context of HIV-1 rapid progression.

Thus, we assessed virus coreceptor usage and RC in RP in comparison to Standard Progressors (SP), at the earliest time-point available after seroconversion (median ≤1 year). In terms of virus coreceptor usage, we tested a total of 74 samples (38 RP and 36 SP) using the Enhanced Sensitivity Tropism Assay (ESTA) and the U87 phenotypic assay. Our results demonstrated a significant difference in the proportion of X4/DM virus between groups (**Fig 30A**), with an unexpected increased of X4/DM coreceptor usage in association with rapid progression (26.3% RP vs. 2.8% SP; p=0.006). In spite of the increased in X4/DM distribution, a remaining 70% of RP carried R5 variants. These R5 variants were equally associated to higher plasma viremia (p<0.001, **Fig 30B**), indicating additional particularities in the pathogenesis of R5-using virus in RP.

In agreement with this observation, we decided to investigate the overall contribution of virus RC to rapid progression. We measured *in vitro* RC in primary isolates from RP (n=27) and compared to SP (n=20). Our results revealed a significantly higher viral RC in isolates from RP (median 81.5% vs. 67.9%; p=0.025, **Fig 30C**). Moreover, such an increased in virus RC was independent of

the coreceptor usage (**Fig 30C**) and demonstrated the greater pathogenic potential of the HIV-1 variants present in rapid disease progression.

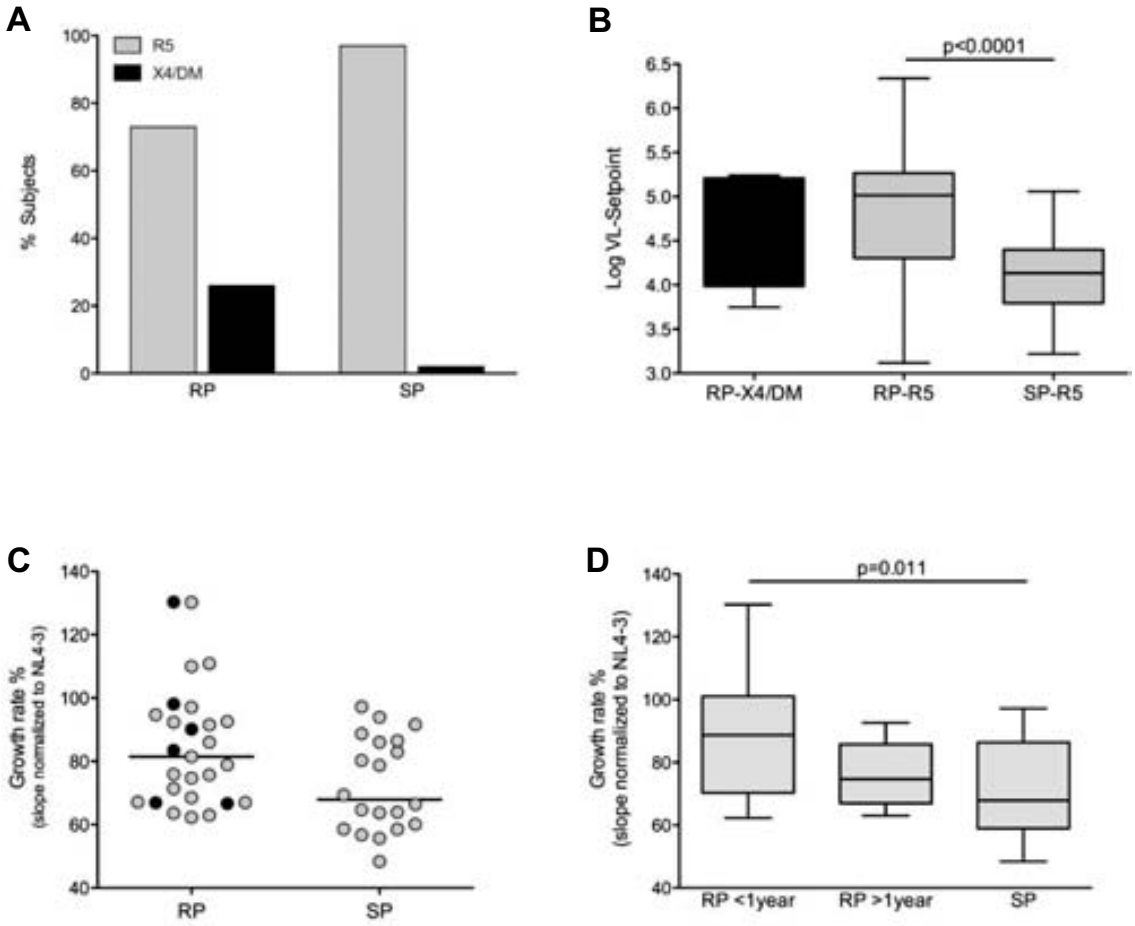


Figure 30. HIV-1 co-receptor usage and replicative capacity in rapid and standard progressors. A. Total frequency of R5 and X4/DM virus in Rapid Progressors (RP) and Standard Progressors (SP). Grey bars represent R5 virus and black bars X4/Dual Mix (X4/DM) tropic virus. **B**. Viral load set-point stratified by virus coreceptor usage. Box and whiskers graph show median values and 10-90 percentiles. The p–values were calculated using the Mann-Whitney U Test. Only significant p-values ($p<0.05$) are represented. **C.** Virus replicative capacity from primary isolates in RP and SP. Horizontal line represents median values (the p–value was calculated by Mann-Whitney U Test). Grey dots indicate R5 variants and black dots X4/DM variants. **D.** Correlation between baseline CD4+ T-cell counts and replicative capacity. Lines indicate the fitting of the data to linear regressions per study group. The nonparametric Spearman correlation coefficient (r) and the associated two-tailed p-value were calculated per group and shown in text..

We then examined the correlation between RC and clinical parameters as predictors of rapid progression. We found an inverse correlation between RC and baseline CD4+ T-cells counts in the overall study group, as supported by previous studies [464, 465]. Though, our subanalysis by disease status in both groups demonstrated how these correlations are led by highly replicative virus present in RP but not in SP (**Fig 30D**, RP; r=-0.51, p=0.002). These results evidence the contribution of specific viral factors including X4/DM coreceptor usage and replicative capacity to CD4+ T-cell depletion and rapid disease progression.

Rapid progressors present a cellular and humoral dysfunctional immune phenotype

Once established the viral traits that prevail in HIV-1 RP, we sought for the contribution of host immune factors to rapid disease outcome. Therefore, we extended our comparative analyses to cellular and humoral functional markers in RP and SP. We focused in virus specific CD8+ T-cell responses, virus susceptibility to neutralization and plasma neutralizing activity as important components of adaptive immune responses against HIV-1.

We measured HIV-1 specific CD8+ T-cells responses in a subgroup of 17 RP and 13 SP to a comprehensive panel of HLA-class I optimally defined HIV-1 CD8+ T-cell epitopes. Our results demonstrated weak or even absent HIV-1 specific CD8+ T-cell responses in RP when compared to SP at the baseline (BL) (mean 2.58% RP vs. 10.45% SP, p= 0.004) **Fig 31A**. These differences in early infection were maintained when responses were tested later in infection (post-baseline (P) mean 1.34% RP vs. 8.29% SP, p=0.005) **Fig 31A**, with no improvement or recovery despite normalization of CD4+ T-cell levels after treatment introduction in RP.

In addition, we evaluated humoral responses in RP by measuring virus susceptibility to neutralization and plasma neutralizing activities across groups. No differences between RP and SP were observed regarding to the sensitivity of HIV-1 Env-pseudotyped virus to neutralization, against broadly neutralizing antibodies (**Fig 31B**).

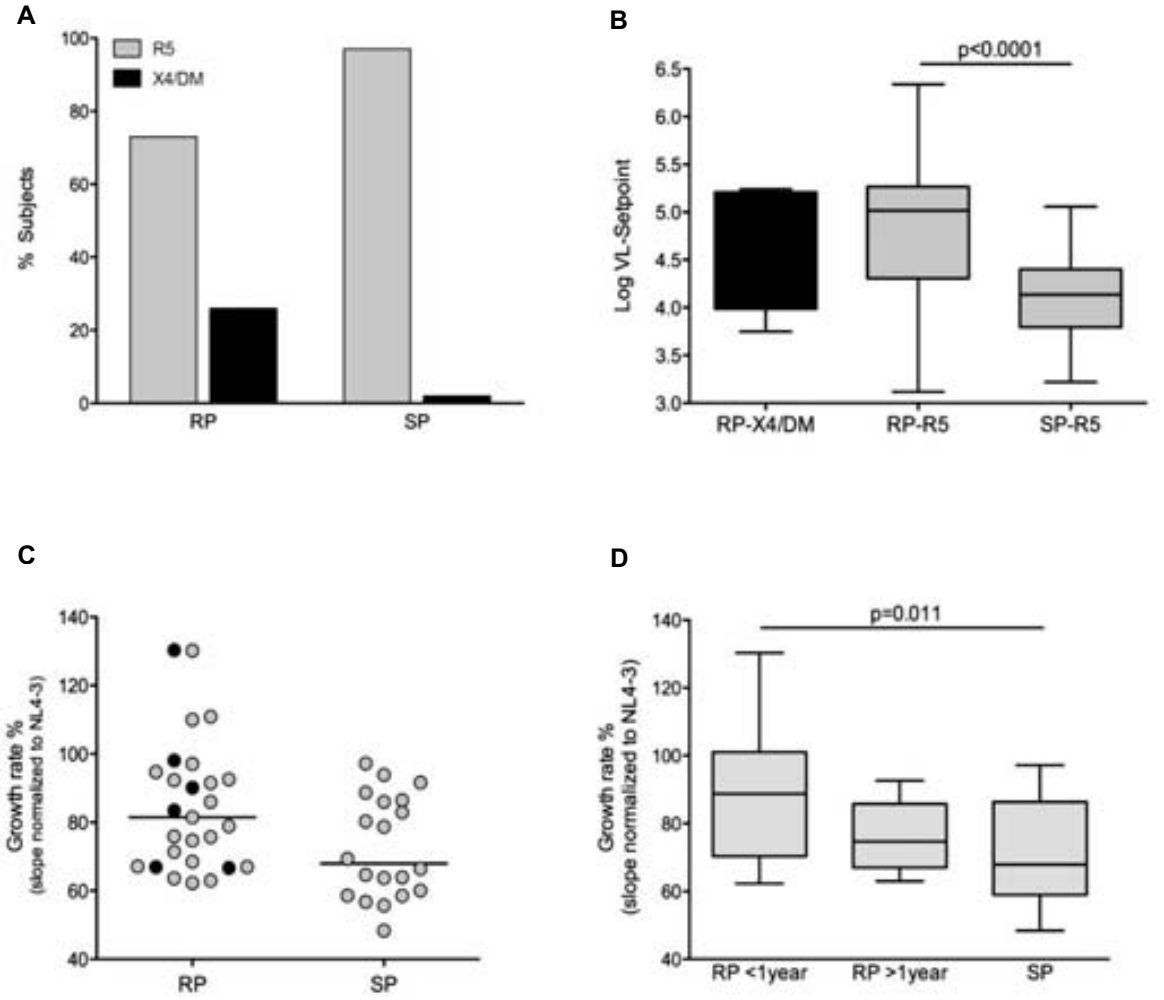


Figure 31. HIV-1 specific CD8+ T-cell responses, virus sensitivity to neutralization and plasma neutralizing activity in rapid and standard progressors. A. Frequency of IFN- HIV-1 specific CD8+ T-cell responses at Baseline (BL) and Post (P) were measured in PBMCs against a panel of HLA class I optimally defined HIV-1 epitopes. Box and wishkerswhiskers graph show median values and 10-90 percentiles. The p–values were calculated using the Mann-Whitney U Test. Only significant p-values ($p<0.05$) are represented. **B**. Sensitivity to neutralization of Env-pseudotyped virus derived from plasma of RP and SP against a panel of broadly Neutralizing Antibodies (bNAbs) including b12, 4E10, 2F5 and 2G12. **C**. Heterologous plasma neutralizing activity at baseline (BL) against standard panel of viral isolates from various subtypes and with various ranges of susceptibilities to neutralization; sensitive, moderate and resistant. **D**. Heterologous cross-reactive virus neutralizing activity in plasma samples, at baseline (BLp) and POST (Pp), against a panel of baseline patient derived env-pseudotyped virus (BLv). **E**. Autologous neutralizing activity in plasma and Env-pseudotyped virus pairs. BLv (Baseline virus), BLp (Baseline plasma), Pv (POST virus), Pp (POST plasma). All p–values were calculated by Mann-Whitney U Test. Only significant p-values ($p<0.05$) are represented.

Moreover, plasma neutralizing activities in 12 RP and 32 SP were evaluated against a panel of viral isolates with a wide range of susceptibilities to neutralization (**Table 14**). Even though a consistent trend was observed in RP towards lower IC_{50} values, results did not reach statistical significance (**Fig 31C**). Similarly, no differences were found between groups in total heterologous plasma neutralizing activity (**Fig 31D**, and **Table S5** (Addendum)).

Additionally, autologous plasma neutralization was generally low against contemporaneous virus only increased in SP overtime (RP vs SP; p=0.019) **Fig 31E**. By contrast, autologous neutralizing titers were generally higher in both groups against ancestral virus [RP (p=0.024) and SP (p<0.0001)] but with a modest increase in the RP within the same period of time (**Fig 31E**).

Overall, these data suggest an early and long-term defect of host immune responses in HIV-1 rapid progression. Weak or absent specific CD8+ T-cell responses together limited neutralizing activity define a common dysfunctional immune phenotype in RP.

Presence of adapted variants in early infection and enrichment in common HLA-class I alleles are associated to HIV-1 rapid disease progression

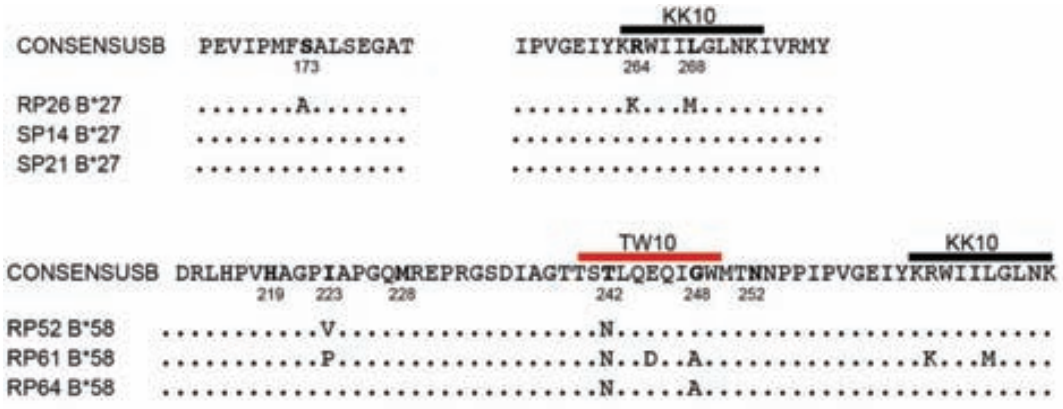
One of the major limitations to broken equilibrium between virus and host immunity found in the RP study group, can be related to the rate of initial virus adaptation/escape to particular HLA class I immunogenetic profiles [256]. Transmission of HLA adapted viral variants has been previously described in rapid progression [403]. Additionally, individuals with protective alleles progress rapidly after transmission of HLA-matched viral strains [190, 379].

Consequently, to initially define the contribution of HLA adapted strains to rapid progression, we focused in subjects with protective HLA class I alleles (HLA-B*58 and B*27) and rapid progression. We found one HLA-B*27 and three HLA-B*58 RP and evaluated the presence of adapted strains to the early KK10 and TW10 Gag protective responses. Escape variants at positions K264R of the KK10 epitope and T242N of the TW10 epitope were confirmed in all RP early in infection. By contrast,

no immune escape was observed in two HLA-B*27 SP within the same period of time (**Fig 32A**). In addition, escape mutations in Gag at positions K264R and T242N, known to incur in viral replication defects [170, 196], occurred together with replicative capacity compensatory changes at Gag positions S173 for R264K and I223/G248 for T242N (**Fig 32A**). Development of such a complex mutational escape patterns appear only late in infection [263, 264, 379], consistent with the possibility of transmission of HLA-adapted variants and supported by the presence of KK10 escape mutants in a non-B*27 RP (**Fig 32A**). Moreover, clonal viral sequences obtained 12 months after seroconversion through deep sequencing revealed homogeneous populations of TW10 escape mutants in HLA-B*58 RP52. This observation is in agreement with the low level of viral genetic variability described in recent infections and likely represents HIV-1 transmitted variants before diversification at later stages of infection (**Fig 32B**).

To generalize these findings of HIV-1 HLA-adaptation to a specific immunogenetic profile common to rapid progressors, we carried out an explorative analysis of HLA distribution in 122 HIV-1 infected individuals (74 RP and 48 SP). Our data showed an enrichment in the proportion of common Caucasian HLA haplotypes including HLA-A*02:01, B*07:02 and B*08:01 (75.6% RP vs. 50% SP) in RP in detriment of protective ones (6.76% RP vs. 22.9% SP). In addition, only a modest trend was observed towards an increase on risk alleles **Fig 33A**. Similar trends across data analysis were supported by individual HLA type analyses (**Table S6**, Addendum).

A



cont.

B

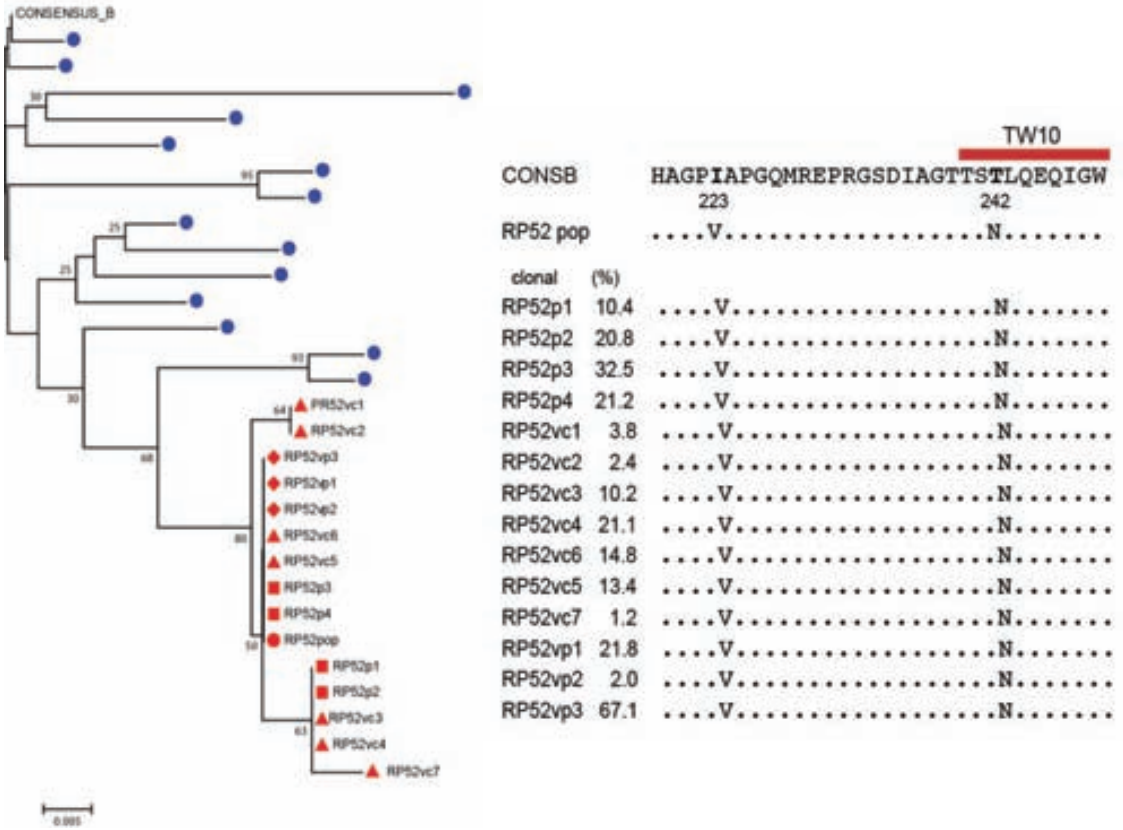
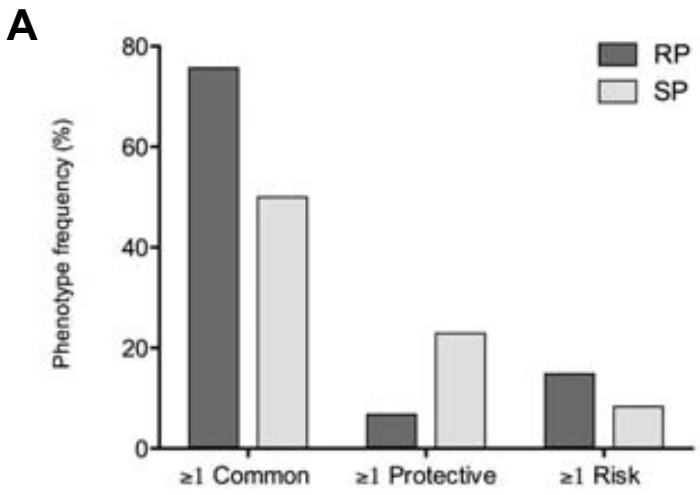
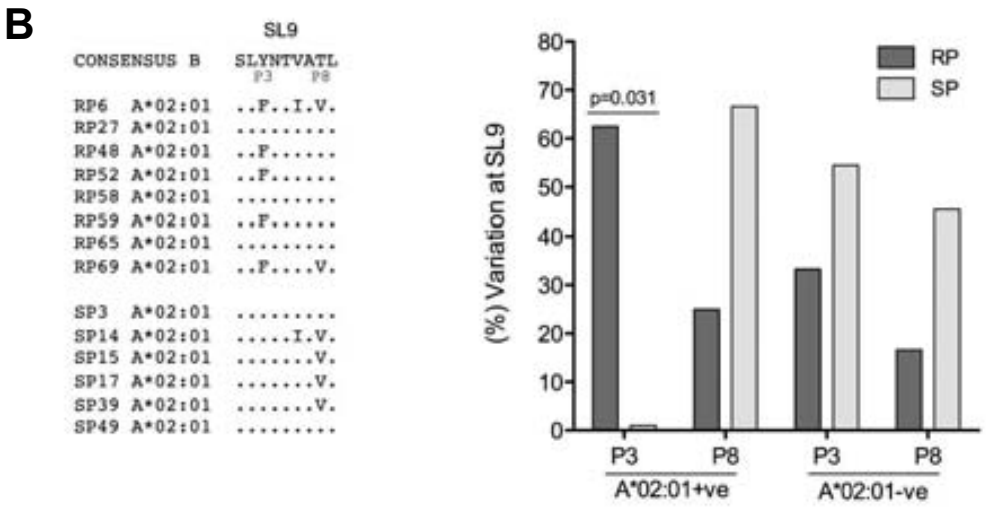
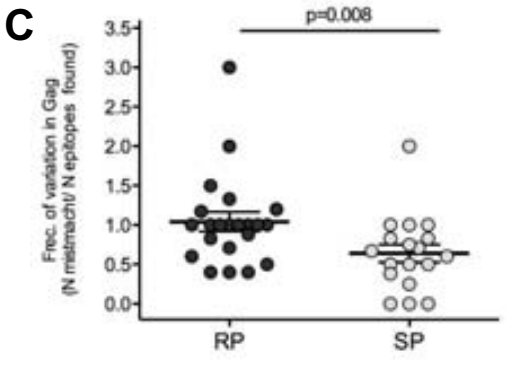


Figure 32. HLA adapted HIV-1 strains to Gag TW10 and KK10 protective responses in RP with HLA-B*58 and HLA-B*27 alleles. Gag viral sequences were obtained from plasma samples in a HLA-B*27 (RP26) and three HLA-B*58 (RP52, RP61 and RP64) subjects with rapid progression. For comparative purposes, Gag sequences were obtained from two HLA-B*27 SP (SP14, SP21). **A**. Comparison of HIV-1 Gag amino acid sequences at KK10 and TW10 epitopes. Epitopes are marked at the top of the sequence with a black line (KK10) or red line (TW10). Letters in bold indicate residues associated with escape in KK10 (264, 268) and replicative capacity compensatory sites (173). Similarly, bold letters indicate escape in TW10 (242) and replicative capacity compensatory sites (219, 223, 248, 252). Amino acid numbering is based on HXB2 sequence. **B**. Gag phylogenetic tree in HLA-B*58 RP52 covering the TW10 epitope and the 223 compensatory site. The tree were built by the Neighbor-joining method taking over 1000 bootstrap replicates and tree was rotted to the consensus B Gag sequence and edited with MEGA.5. Number at the nodes indicates bootstrap support. Blue dots indicate Gag population sequences from additional RP. Red symbols indicate RP52 Gag sequences. Red dot represent the population sequence (RP52pop). Squares, diamonds and triangles represent clonal viral sequences. Red triangles and diamonds are clonal viral sequences obtained from primary viral isolates from cells (RP52vc) or plasma (RP52vp). Red squares are clonal viral sequences directly obtained from total plasma (RP52p). The alignment next to the tree shows the clonal populations of TW10 escape variants in RP52. Next to each clonal sequence the frequency of that variant in the total of the population is indicated in percentatges.


A
Phenotype frequency (%)
80
60
40
20
0
RP
SP
≥1 Common
≥1 Protective
≥1 Risk


B
SL9
CONSENSUS B SLYNTVATL
P3 P8
RP6 A*02:01 ..F..I.V.
RP27 A*02:01 ....
RP48 A*02:01 ..F.....
RP52 A*02:01 ..F.....
RP58 A*02:01 ....
RP59 A*02:01 ..F.....
RP65 A*02:01 ....
RP69 A*02:01 ..F....V.
SP3 A*02:01 ....
SP14 A*02:01 .....I.V.
SP15 A*02:01 ....V.
SP17 A*02:01 ....V.
SP39 A*02:01 ....V.
SP49 A*02:01 ....
(%) Variation at SL9
p=0.031
RP
SP
P3
P8
P3
P8
A*02:01+ve
A*02:01-ve


C
Frec. of variation in Gag
(N mismachV N epitopes found)
p=0.008
RP
SP

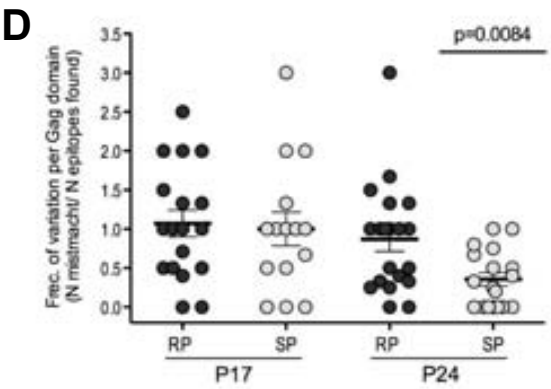

D
Frec. of variation per Gag domain
(N mismachV N epitopes found)
p=0.0084
RP
SP
RP
SP
P17
P24

Figure 33. HLA class I immunogenetics and HIV-1 variation at HLA-matched epitopes in RP and SP. A. Phenotypic frequency (number of subjects with a define haplotype/ number of total individuals in the group x 100) in pooled analyses for HLA class I alleles. Subjects in groups (74 RP and 48 SP) were included for having at least one common allele (A*02:01, B*07:02 and B*08:01), a protective allele (B*57:01, B*14:01 and B*27:05) or a risk allele (B*35:02, B*35:03, B*35:04 and B*53:01). Only those alleles with a frequency above 3% in the overall population were taken into account for data representation. **B.** HIV-1 SL9 epitope alignment in HLA-A*02:01+ve RP and SP; P3 and P8 indicate amino acid sites in SL9. Bar graph show the frequency of epitope variation at P3 and P8 sites in HLA-A*02:01+ve and HLA-A*02:01-ve SP and RP. **C.** Frequency of epitope variation in HIV-1 Gag. Partial sequencing was carried out in a total of 40 subjects (22 RP and 18 SP). **D.** Frequency of HLA-matched epitope variation in HIV-1 Gag per functional domains p17 and p24. For C and D, graph represents the mean and SEM. The p–values were calculated by Mann-Whitney U Test. Only significant p-values ($p<0.05$) are represented.

To define the role of adapted variants to this particular HLA profile of common alleles in RP, we focused on the SL9-Gag epitope restricted by HLA-A*02:01, the commonest allele in our RP study group. Thus, we compared HIV-1 SL9-Gag epitope variation in HLA-A*02:01+ve and HLA-A*02:01-ve infected individuals early after seroconversion **Fig 33B**. Our data demonstrated preferential variation at position 3 (Y3F) of the SL9-Gag epitope in A*02:01+ve RP when compared to A*02:01+ve SP (62.5% RP vs. 0 % SP p=0.031) (**Fig 33B**).

These differences were restricted to A*02:01+ve individuals and not to the background A*02:01-ve population, identifying the specific increase of SL9 escape variants in RP. Moreover, to extend these results to other epitopes in the HIV-1 proteome, we determined total virus variation at HLA-matched Gag and Pol epitopes in our study groups. Our data indicated a significant increase in total Gag epitope variation in viral strains from RP when compared to SP (1 RP vs. 0.63 SP, p=0.008) (**Fig 33C**) but not in pol. However, these differences were not equally distributed across functional domains in Gag but significantly accumulated in p24 region (**Fig 33D**).

These results support the association between HLA immune adapted/escape HIV-1 variants and rapid disease progression. This association is marked by a high prevalence of common Caucasian haplotypes among rapid progressors.

DISCUSSION

The duration of HIV-1 infection is bounded to the temporal equilibrium between viral and host factors. Such complex and dynamic interplay can be broken and presented as extreme disease representation that either contribute to prolonged periods of HIV-1 control or drive rapid disease progression. In this context, rapid progression remains one of the least understood extreme phenotypes in HIV-1 infection but its understanding is crucial to tackle the epidemic. Thus, this work overcome previous studies limitations in RP through a systematic assessment of viral and host factors in a large study group. For the first time, we provide evidences on the contribution of specific viral and host traits to rapid HIV-1 disease progression at the study group level.

In terms of viral factors, our data demonstrate the convergence of highly pathogenic viral strains in RP. These strains have an increase of X4/DM coreceptor usage (>25%) and overall high RC, uncommon at the onset of HIV-1 infection. The high prevalence of X4/DM virus contrasts with the previously reported low frequency in primary infection [456, 466]. Moreover, despite that X4/DM virus are more pathogenic than R5 variants [144], no association was observed between X4/DM coreceptor usage and higher RC. These findings may illustrate the unpredicted role of very replicative HIV-1 R5 virus in rapid disease outcome.

Together with viral traits, the quality of the host immune responses should be taken into account in the establishment of the infection and disease outcome. Our data support an irreversible damage of the immune system in rapid progressors early in infection. We observed defective HIV-1 specific CD8+ cellular responses and no major role of neutralizing antibodies, as previously reported [350, 467]. This generalized static immune dysfunction in RP will be established at the early stage of infection through the dramatic loss of CD4+ T-cells that irreversibly affect CD4+ homeostasis. Consequently, the generation of CD4+ T helper responses will be also impaired, interfering with the maintenance of long-term memory CD8+ T-cell specific responses [468] and B cell function [469, 470].

Our data underline the early disruption of the virus-host balance in HIV-1 infection leading to rapid progression. One of the important contributors to this imbalance is the degree of initial virus adaptation/escape to particular HLA class I molecules. HIV-1 adapts to the most frequent alleles in a population [256]. Therefore, the probability of transmission of adapted viruses will be increased among common HLA carriers limiting the breadth of the immune responses [255, 256]. Our data revealed a consistent trend towards the accumulation of common Caucasian alleles including HLA-A*02:01, B*07:02 and B*08:01 in around 75% of RP. These data confirm and extent previous studies in which common A2 and B7 supertypes were disadvantageous for HIV-1 control [255, 471]. According to the immunogenetics of RP, the likelihood of having adapted or escaped HIV-1 variants should be higher among those. This hypothesis is in line with the presence of well-adapted and well-compensated virus in HLA-B*57, HLA-B*27, and reinforced by the widespread of SL9 escape variants in HLA-A*02:01+ve rapid progressors. Moreover, higher number of variation in Gag epitopes in RP strains supports the pre-existence of these changes against the rapid viral evolution early in infection. Despite we cannot directly associate amino acid variation in Gag to virus immune escape, multiple changes at epitopes sites may interfere with the priming or expansion of protective Gag CD8+ T-cell responses [73, 472-474].

Although our study indicated a dominant role of specific viral factors in the absence of immune responses in rapid disease progression, we cannot establish conclusively the causative agent/s of this extreme disease presentation. Thus, earlier sampling or identification of transmission pairs with rapid progression will be needed to unravel the causality dilemma. Nevertheless, some of our data, including the presence of virus with complex mutational patterns only found late in infection [263, 264, 379] and the homogeneous populations of escape mutants in early infection, advocate for transmission events as the origin of these pathogenic variants. Whether such particular strains are indicative of a source of transmission and rapid progression will be key to confront the epidemiology of HIV-1 infection. As previously reported, a portion of new infections originates from chronically infected individuals [475]. In addition, sexual intercourse with individuals presenting

AIDS defining symptoms has been associated to rapid disease progression [476]. Thus, source individuals at late stages of HIV-1 infection will increase the probability of transmission of X4/DM or pathogenic R5 variants [477] fully replicative and fully adapted to host HLAs through chronic infection.

In conclusion, our findings define the association of highly pathogenic variants with specific patterns of HLA adaptation/escape together with a dysfunctional immune phenotype to the lack of HIV-1 control. Ultimately, these data provide new insights into the mechanism of rapid progression, crucial to identify new roads into the correlates of HIV-1 disease outcome.

Chapter 4

DISCUSSION & PERSPECTIVES

The natural course of HIV-1 infection varies between infected individuals. Up to 80% of infections have an average progression profile, taking between 7.7 and 11 years to develop AIDS in the absence of antiretroviral therapy [28]. The extremes of the progression spectrum, however, are of remarkable interest in research. HIV controllers, especially elite controllers, have been extensively studied to find correlates of protection, and the data obtained continuously provide relevant insights into the pathogenesis of HIV [73, 74, 300, 401, 402]. The other extreme of the spectrum, RPs, has received much less attention, probably due to the difficulty in identifying and recruiting individuals with this phenotype.

The present thesis originates from a comprehensive study of 2 contemporaneous cases of extremely severe HIV-1 infection in young individuals. The interesting results obtained prompted us to turn our attention to the RP phenotype. Our findings revealed clear convergence of viral and host factors in the clinical severity of primary HIV-1 infection. We also observed that patients infected with highly replicative, dual-tropic viruses might be more prone to develop AIDS-defining symptoms during acute infection if they are unable to mount humoral and cellular HIV-1–specific immune responses. In addition, the presence of concordant HLA supertypes in both the source and the recipient was also suggested to facilitate preferential transmission of HLA-adapted viral variants and thus further accelerate disease progression. Despite the results obtained from our study and available information from specific case reports [87-92] little was known about RPs at the time the reports were published. Although many hypotheses for the factors behind this extreme phenotype had been put forward, no studies with large well-defined cohorts had been performed and no rigorous standard definition of RPs established. Therefore, we decided to define this particular set of special individuals by generating clear inclusion criteria and an unprecedentedly large cohort of RP (CoRP), and collecting relevant clinical data and biological samples. We also established comparative control cohorts, including a cohort of SPs and a cohort of VNPs, who, despite high levels of viremia, are able to maintain $CD4^+$ T-cell counts for many years in the absence of ART.

We then designed a comprehensive project to evaluate the factors involved in rapid progression; the results from the large dataset generated are presented in the thesis. The first part of the study compared the genomic and transcriptomic traits of RPs with those of other phenotypes, especially VNPs. Moreover, a sensitivity analysis was performed on a set of genes from studies in rhesus macaque and sooty mangabey, which are the respective phenotypes of RPs and VNPs in SIV infection. The most remarkable findings include the absence of common genetic variants associated with these phenotypes, probably in part owing to the limited number of study candidates (genome-wide association studies generally require a very large number of individuals before conclusions can be drawn on common gene variants). Reduced sample size was probably also behind the lack of association between the RP cohort and KIR alleles or KIR/HLA combinations previously associated with progression or viremia. Furthermore, the transcriptomic profile of $CD4^+$ and $CD8^+$ T cells in RPs was similar to that of rhesus macaques, whereas the VNP profile exhibited lower expression of interferon-stimulated genes and was comparable to the sooty mangabey profile. Remarkably, the study of the set of genes identified through a comparative analysis of human and nonhuman primate transcriptome data showed that *CASP1*, *CD38*, *LAG3*, and *TNFSF13B* were upregulated in RPs, and *SOCS1* and *EEF1D* were upregulated in VNPs, also in parallel to the respective SIV models. Indeed, when evaluated in an independent set of $CD4^+$ T-cell expression data, the individual expression levels of these 6 genes correlated significantly with viral setpoint and with time to $CD4^+$ T-cell count depletion to <350 cells/µl. Further studies are required to investigate the role of these specific genes in progression phenotypes and to elucidate the mechanisms underlying their effect in progression of HIV infection. Some groups have started to investigate specific genes, for example, *CASP-1*, which is activated by the host DNA sensor IFI16 (involved in progressive depletion of quiescent "bystander" $CD4^+$ T cells, a principal driver of AIDS) [478] and by action of the NLRP3 inflammasome [479], which contributes to $CD4^+$ T-cell depletion and AIDS progression.

In addition to the genomic and transcriptomic profiles, we compared the different viral and host factors that might be involved in the RP profile with those of SPs. The most remarkable finding was that RPs are more often infected by more pathogenic strains, with a high percentage of X4/DM-tropic variants in early infection and high replicative capacity (independently of co-receptor usage). Furthermore, they presented complex profiles of adaptation/escape mutations, which are typical of late stages of the disease [263, 264, 379]. This observation suggests that such variants are more likely transmitted than generated within the recipient, although further investigations are needed to confirm this hypothesis. Interestingly, different reports have associated the presence of X4 variants with higher virulence, accelerated disease progression, and a rapid fall in $CD4^+$ T-cell count [393-395]; however, no previous studies have shown such a high incidence of X4/DM strains in early infection. Moreover, the role of these strains in the first stages of rapid disease progression has not yet been demonstrated. The HLA genotype was also found to play a role in rapid progression through the presence of common HLA alleles at the population level, the accumulation of risk alleles, and the absence of protective alleles which were shown to further accelerate disease progression. Finally, the difficulty in mounting HIV-specific humoral and cellular responses and the inability to recover them even after stabilization of viremia through antiretroviral therapy were also found to be determinant in rapid progression to AIDS. Indeed, the profile of cytotoxic lymphocyte responses in RPs is rare, and only a few previous reports have identified individuals with such a low number of responses during primary HIV-1 infection [329, 400].

Therefore, the infecting viral strains were demonstrated to play a critical role in the RP phenotype. Interestingly, extreme phenotypes (i.e., elite controllers *vs.* RP) were shown to be driven by divergent factors. Specifically, the observation that viral factors seem to have a higher specific weight in RPs than in other phenotypes raises questions about the spread of pathogenic strains in the population. Furthermore, concerns have been voiced about the increased virulence of HIV in the infected population. Despite recent reports of a higher number of RPs in different cohorts, lower post-seroconversion $CD4^+$ T-cell counts, and shorter

periods until $CD4^+$ T-cell counts fall below 350 cells/mm^3, some investigators have associated increased virulence with more frequent and improved identification of seroconverters during this period, with lower seroconversion windows being observed in the last few years [480]. Some studies have reported a decrease in $CD4^+$ T-cell counts [481-484], whereas others found no evidence for such changes [485, 486]. The most recent analysis associated this variation in the results with methodological differences and different lengths of study periods. In fact, after correction of the multiple statistical issues in previous analyses, the findings of Gras *et al.* published in 2013 point to a trend toward a faster decline in $CD4^+$ T-cell counts after seroconversion during the latter part of the study period [487]. These results warrant further clarification, as an increase in rapid progression or the possible spread of more virulent variants has serious implications for the HIV pandemic.

Of note, our studies and the analysis of increasing virulence at the population level focus mainly on subtype B strains of HIV-1. Thus, further studies on different HIV subtypes should be carried out in order to investigate the factors involved in disease progression and pathogenicity of other variants. For example, the findings recently published by Palm *et al.* [488] show remarkable differences in disease progression between A-like subtypes and CRFs, with the highest observed progression rate among subjects infected with the A3/02 recombinant virus.

The observations and concerns presented above emphasize the need to intensify efforts aimed at early identification of RPs. First, our results indicate that specific treatment guidelines should be developed for this specific phenotype. Patients must be treated as early as possible in order to avoid severe loss of $CD4^+$ T cells and subsequent difficulties in generating effective HIV-specific immune responses, especially because of the lack of immune recovery after stabilization of viremia through ART. Second, early identification and treatment of RPs is important at the population level, since these patients harbor more pathogenic variants, whose spread should be contained. Finally, the development of successful antiretroviral therapy in the last decade has improved both the survival and the quality of life of HIV-infected individuals. However, the overwhelming efficacy of antiretroviral

therapy in the last 15 years has indirectly contributed to a relaxed awareness of the need for prevention under the assumption that HIV-1 infection is no more than a treatable chronic disease. Other economic, clinical, and social considerations aside, we show that rapid progression is undesirable and should be prevented at all costs.

The present thesis describes a series of factors that contribute to rapid progression. However, the role of transmitted variants in RPs warrants further evaluation through recruitment at earlier timepoints after infection and investigation of more transmission pairs. Other factors currently being analyzed include plasma cytokine profiles, immune phenotypes of cellular populations, and the clinical long-term effect of antiretroviral therapy in RPs. The ultimate objective of the project is to develop a global model of rapid progression that will eventually enable us to predict the RP phenotype in newly detected infections. Also worthy of study are the differences between genders, the mechanics of pathways involved in rapid progression, and the specific characteristics of other viral proteins such as Nef.

Chapter 5

CONCLUSIONS

1. RPs constitute an extreme phenotype of disease progression in HIV infection that has important implications for pathogenesis. Definition and recruitment of RPs is challenging because of the need for a reliable estimation of the seroconversion date within a small window of clinical observation.

Convergence of viral and host factors contributes to the clinical severity of primary HIV-1 infection and to rapid progression:

2. The presence of concordant HLA supertypes might facilitate preferential transmission of HLA-adapted viral variants, further accelerating disease progression.
3. The fact that RPs are enriched in HLA alleles associated with adverse prognosis and depleted of protective alleles validates the phenotypic criteria developed to define rapid progression. In addition, the prevalence of common HLA alleles at the population level and the incidence of HLA-adapted/escaped HIV-1 strains are higher in RPs.
4. The transcriptomic profile of RPs was similar to that observed in chronic SIV infection of rhesus macaques, whereas VNPs showed similarities with the profile of sooty mangabeys, the natural host of SIV infection. Specifically, the transcriptomic profile of RPs is characterized by deregulation of interferon-stimulated genes, which are further deregulated in VNPs, and upregulation of genes involved in cell proliferation, cell division, and the immunoproteasome, in $CD8^+$ T cells.
5. *CASP1*, *CD38*, *LAG3*, and *TNFSF13B* are upregulated in rhesus macaques and in human RPs, whereas *SOCS1* and *EEF1D* are upregulated in sooty mangabeys and in human VNPs.
6. RPs have higher plasma levels of the monocyte-expressed LPS receptor sCD14 than other groups, in particular during advanced immunosuppression, thus demonstrating higher levels of microbial translocation and immune activation.

7. Primary viruses isolated from both peripheral blood mononuclear cells and plasma from HIV-1–infected individuals showed similar genotypic and phenotypic characteristics, thus enabling them to be used indistinctly in subsequent experimental designs.

Viral factors play an important role in rapid progression in the absence of host immunity, with an unexpected convergence of highly pathogenic HIV-1 strains, thus raising awareness of the role of these virulent variants in the epidemiology of HIV-1 infection.

8. RP viral variants are characterized by a higher frequency of X4/DM coreceptor usage, and greater viral replication than SPs, independently of viral tropism. In addition, RP strains show specific viral HLA adaptation/escape profiles that further contribute to their pathogenicity.
9. Compared to SPs, RPs show a very limited or no capacity to mount functional HIV-1–specific cellular responses, probably owing to the dramatic loss of $CD4^+$ T cells.
10. Neutralizing antibodies might not play a central role in rapid progression, since no differences in viral susceptibility or plasma neutralizing capacity are found in comparisons with SPs. RPs only show a trend toward diminished maturation of autologous responses over time, compared with SPs.
11. It would be advisable to develop strategies for early identification of RPs, specific clinical guidelines, and strong prevention policies in order to provide adequate care and to prevent the spread of highly pathogenic viral strains in the population.

Chapter 6

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Chapter 7

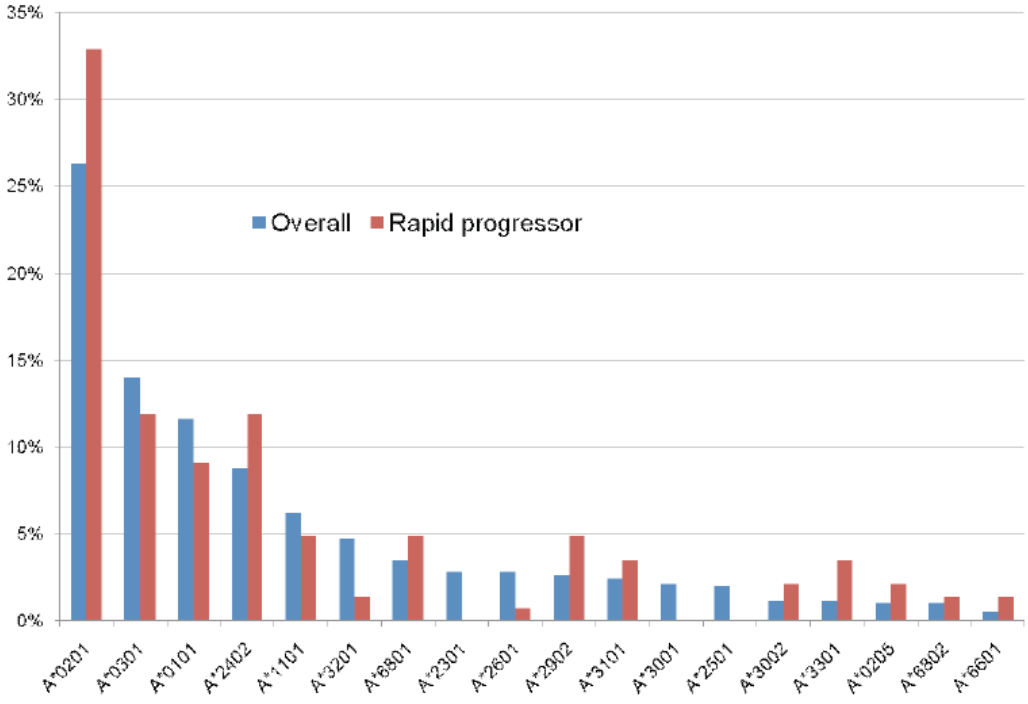
ADDENDUM
SUPPLEMENTAL MATERIALS

CHAPTER 3b. Comparative transcriptomics of extreme phenotypes of human HIV-1 infection and SIV infection in sooty mangabey and rhesus macaque. *SUPPL MATERIALS.*

Immunogenetics. The carriage frequencies of HLA-A, -B and -C alleles in individuals with rapid HIV disease progression were compared to the allele frequencies of 1609 participants of the Swiss HIV Cohort Study (SHCS) (Figure S1). HLA alleles previously associated with lower HIV RNA levels and/or slower HIV disease progression in Caucasians were classified as protective, those with unfavorable effects as risk alleles, as summarized by R. Kaslow (http://retroconference.org/2010/Abstracts/ 39871.htm). Protective alleles were clearly underrepresented in RP compared to the overall HIV-infected population. The proportion of individuals carrying at least one protective HLA allele was significantly lower in RP compared to the general HIV infected population (17.2% vs 30.7%, p=0.02). The depletion of protective alleles was particularly evident for the two strongest protective markers HLA-B*57:01 and HLA-B*27:05. There was an increase in the prevalence of HLA-B*58:01 in RP, this may be explained by the low frequency of the allele, or by the fact that the original description of this allele as protective was in individuals with HIV-1 clade C infection. Risk alleles were more common in RP compared to the general population, however this difference was not statistically significant (23.3% vs 14.9%, P=0.2). Among VNP, one individual carried HLA-B*57:01; a comparative analyses of HLA allele frequencies for VNP was not possible due to the rarity of this profile (Supplementary Table S2).

In contrast to HLA alleles, there was no depletion of protective KIR alleles or KIR/HLA combinations in RP. The distribution of protective and risk alleles in RP was very similar to the overall HIV-infected population (Figure S2). The only significant difference was found in the prevalence of the KIR3DL1-B*57 combination due to the absence of HLA*B57:01 in RP.

A


35%
30%
25%
20%
15%
10%
5%
0%
Overall
Rapid progressor
A*0201
A*0301
A*0101
A*2402
A*1101
A*3201
A*6801
A*2301
A*2601
A*2902
A*3101
A*3001
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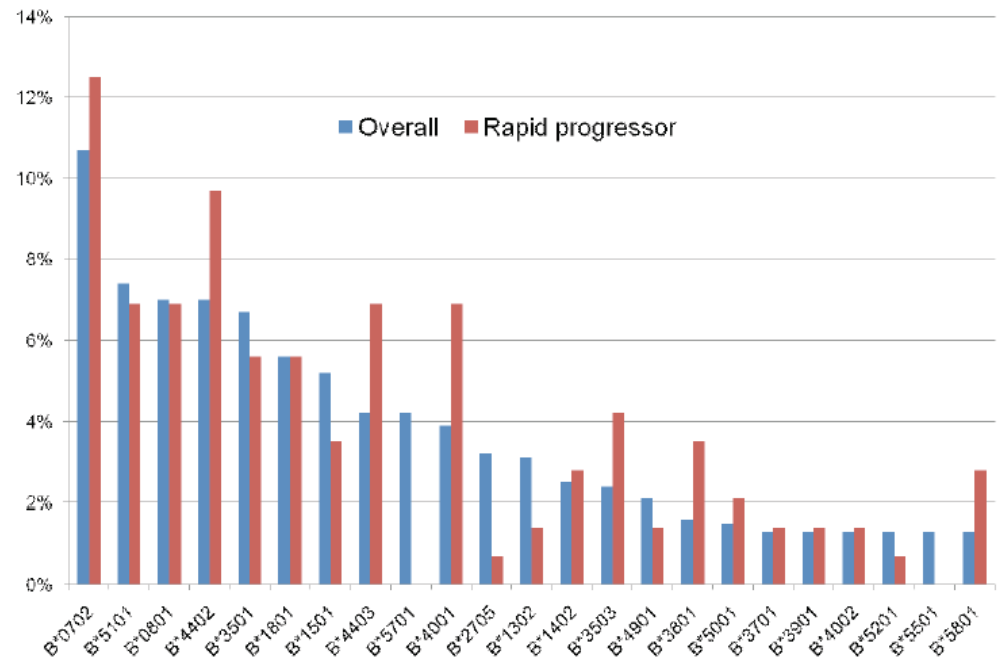
A*3002
A*3301
A*0205
A*6802
A*6601

B


14%
12%
10%
8%
6%
4%
2%
0%
Overall
Rapid progressor
B*0702
B*5101
B*0801
B*4402
B*3501
B*1801
B*1501
B*4403
B*5701
B*4001
B*2705
B*1302
B*1402
B*3503
B*4901
B*3801
B*5001
B*3701
B*3901
B*4002
B*5201
B*5501
B*5801

cont.

C

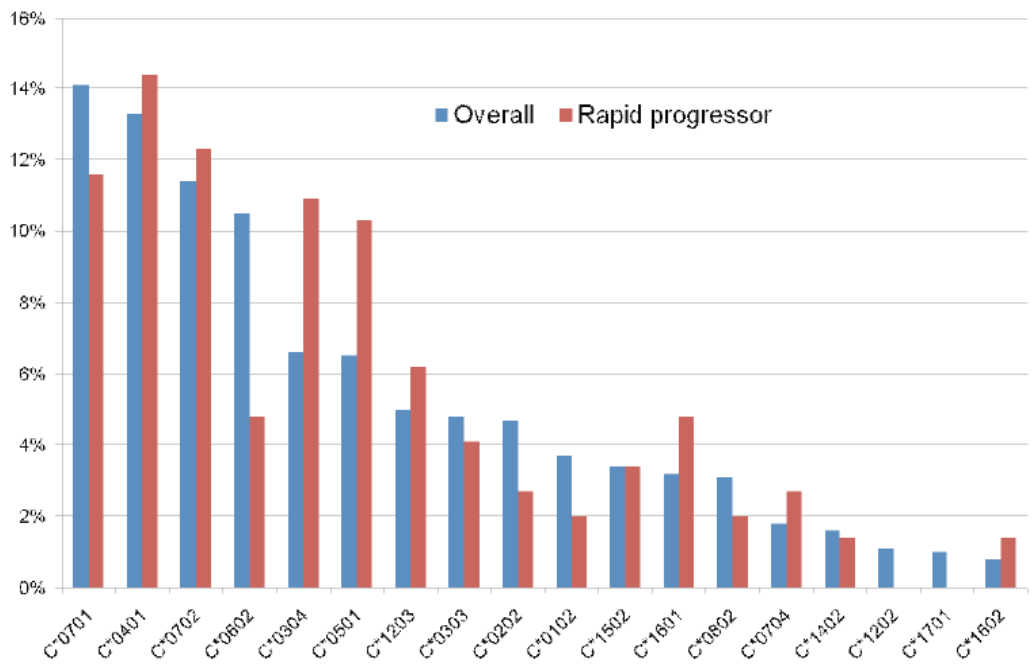


Figure S1: *HLA* allele frequencies in individuals with rapid progression (n=66) and the general HIV infected population (n=1609). Only *HLA* alleles with allele frequencies ≥1% are shown. Panel A, HLA-A; Panel B, HLA-B; Panel C, HLA-C.

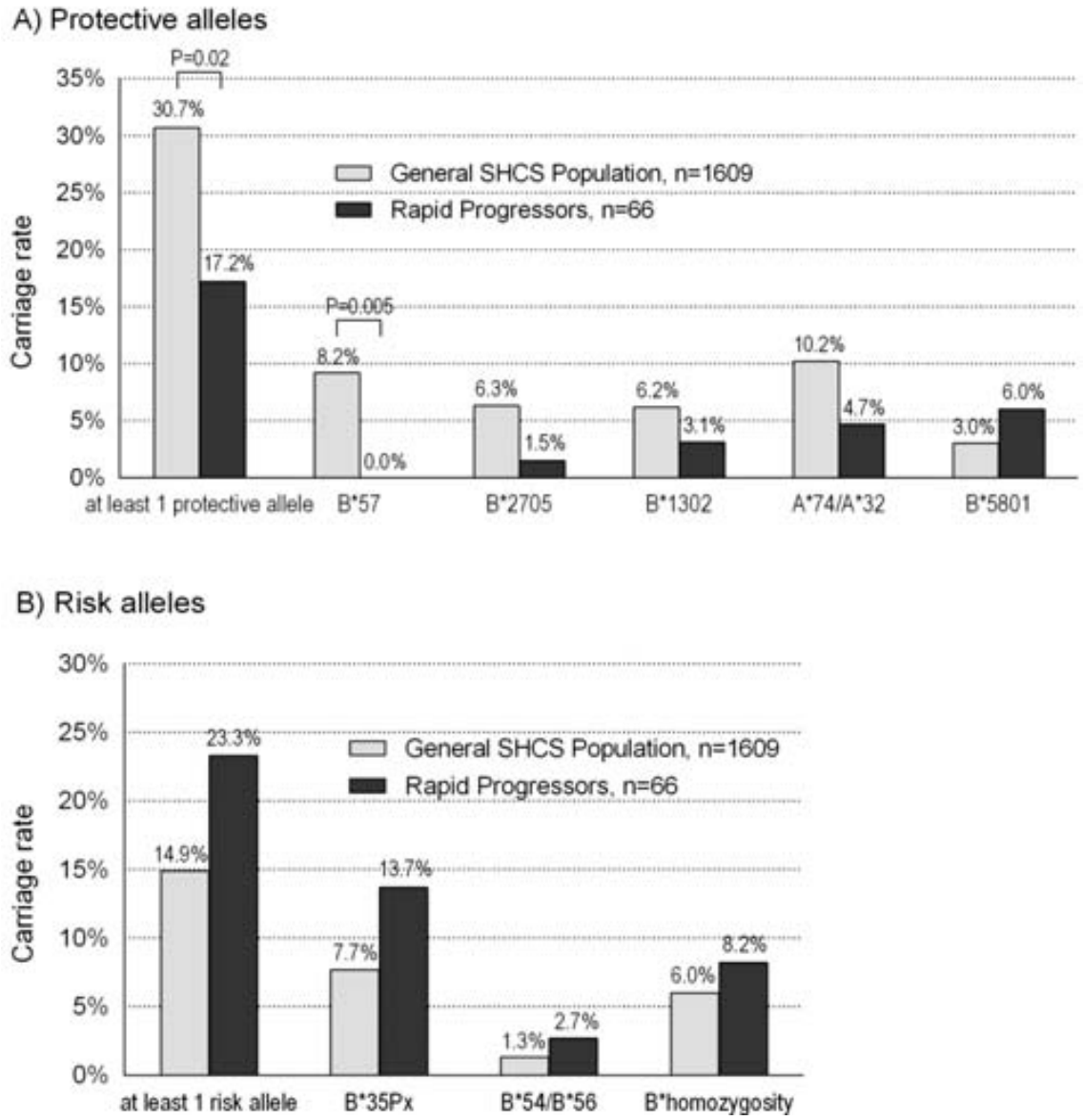


Figure S2: Frequency of selected protective and risk HLA alleles in individuals with rapid progression (n=66) and the general HIV infected population (n=1609). The indicated effect (protective vs risk alleles) is based on published references. Only significant P-values are shown.

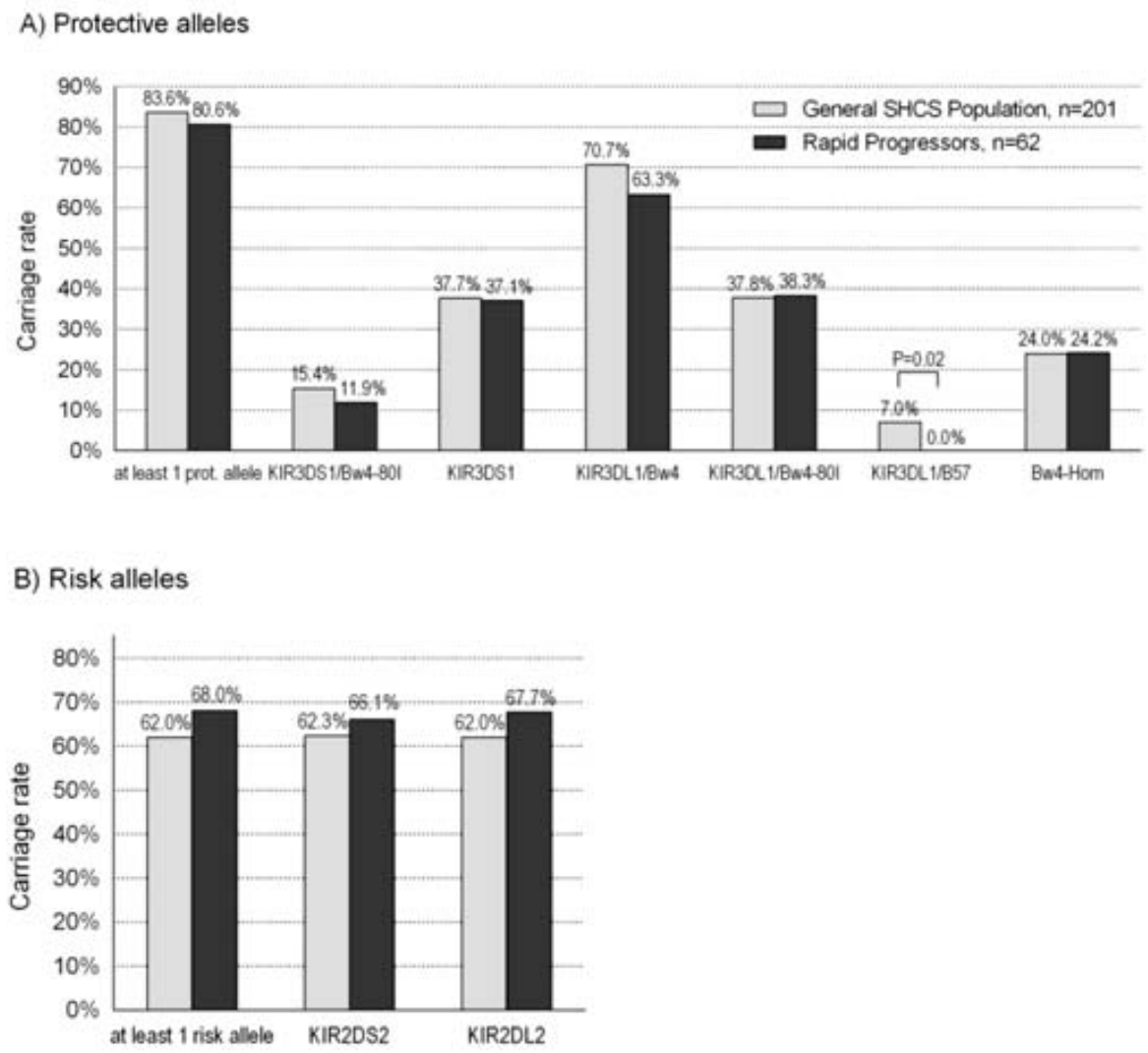


Figure S3: *KIR* allele frequencies and HLA ligand pairs in individuals with rapid progression, and in the general HIV infected population. The indicated effect (protective vs risk alleles) is based on published references. Only significant P-values are shown.

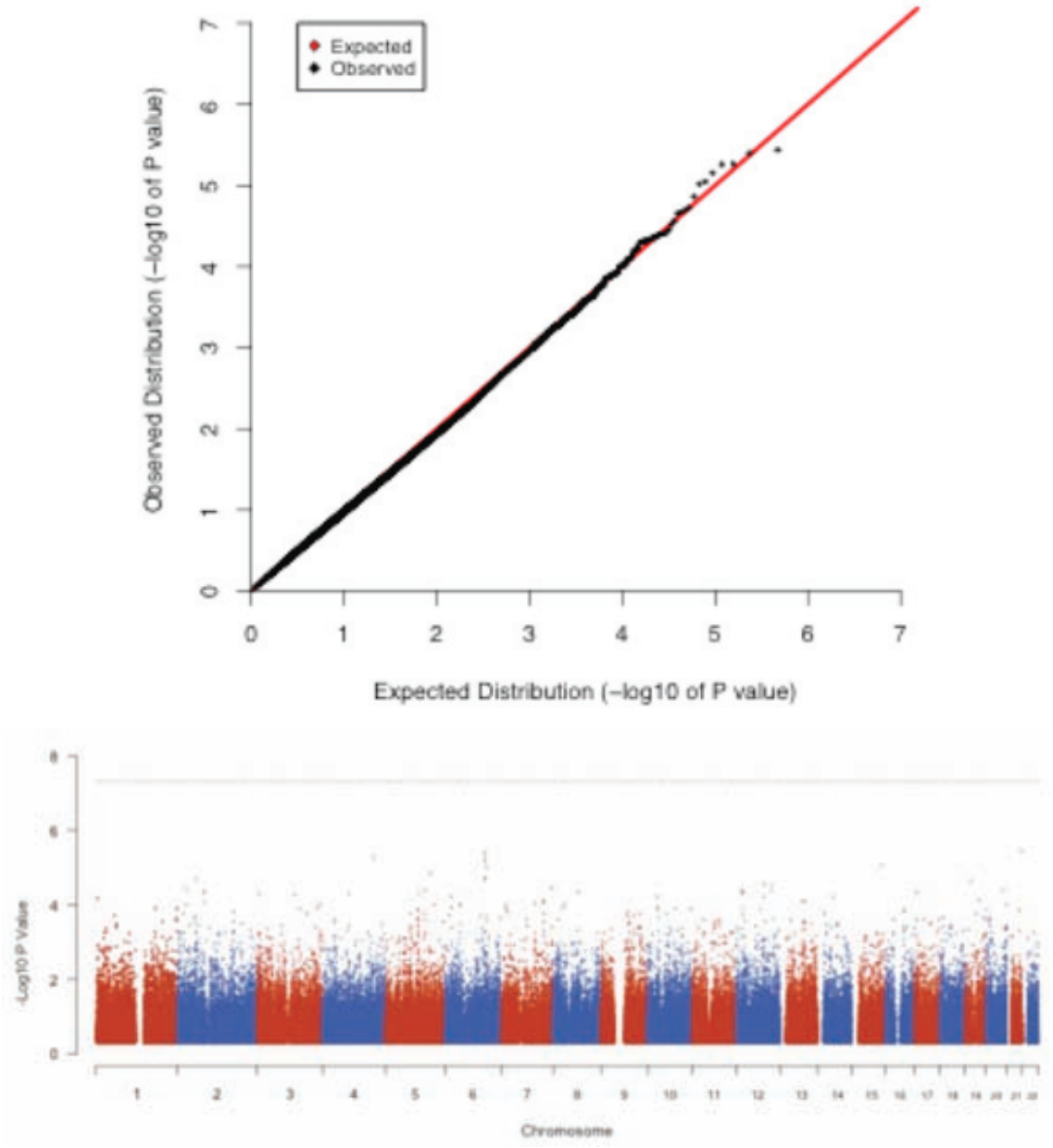


Figure S4: Genome-wide association results comparing rapid progressors to controls. Top panel shows the p-value distribution across the data set. The data fall along the null distribution, showing no evidence for confounding due to population structure (lambda = 1). Bottom panel shows Manhattan plot of p-values genome-wide. No SNPs pass the genome-wide threshold of $p<5x10^{-8}$ (dotted line).

Table S1: *HLA* alleles of viremic non progressors.

ID	HLA_AI	HLA_AII	HLA_BI	HLA_BII	HLA_CI	HLA_CII
VNP1	1101	3201	1801	4403	1203	1601
VNP2	0201	0301	0702	4001	0304	0702
VNP3	2902	3004	3501	4501	0401	0602
VNP4	0201	2402	1501	3906	0303	0702
VNP5	0201	3301	NA	NA	NA	NA
VNP6	0101	0201	4402	5701	0501	0701

NA= not available

Table S3: Results for suggestive associations from genome-wide association analysis of rapid progressors. Reported frequencies and OR are with respect to the A1 (minor) allele. Positions are relative to Human genome build 18. (Separate file in attachment)

SNP	CHR	POS	A1	A2	Case freq	Control freq	OR	P	Gene Symbol	Functional Class
rs2236479	21	45743560	T	C	0,32	0,44	2,44	3,66E-06	COL18A1	intron
rs7765736	6	122622802	A	G	0,33	0,17	2,51	4,02E-06	intergenic	na
rs2406233	4	154254023	T	C	0,45	0,31	2,30	5,43E-06	intergenic	na
rs2085402	6	122563824	G	A	0,35	0,18	2,47	5,50E-06	intergenic	na
rs7763794	6	122470609	C	T	0,33	0,17	2,50	7,04E-06	intergenic	na
rs17595027	15	89591306	T	C	0,28	0,12	2,69	8,94E-06	SV2B	intron
rs9321030	6	125627675	T	C	0,36	0,19	2,44	9,61E-06	intergenic	na
rs477687	5	134495599	T	C	0,34	0,18	2,44	1,37E-05	intergenic	na
rs1474967	6	122649009	G	A	0,32	0,17	2,38	1,87E-05	intergenic	na
rs1346793	2	55823785	G	A	0,31	0,48	0,42	2,02E-05	intergenic	na
rs9375122	6	122724915	T	G	0,32	0,17	2,37	2,12E-05	intergenic	na
rs12978218	19	17663817	G	A	0,42	0,25	2,23	2,23E-05	intergenic	na
rs7966596	12	84729764	C	T	0,48	0,30	2,21	2,75E-05	PAMCI	intron
rs2374688	12	106197332	T	G	0,27	0,45	0,41	3,01E-05	intergenic	na
rs11770182	7	155628329	T	G	0,32	0,17	2,29	3,53E-05	LOC393076	locus-region
rs10840942	12	18097102	G	A	0,32	0,17	2,31	3,87E-05	intergenic	na
rs11883596	2	27238779	G	A	0,21	0,09	2,77	3,88E-05	intergenic	na
rs12522426	5	120235051	T	C	0,23	0,11	2,59	3,95E-05	intergenic	na
rs6064102	20	52717142	T	C	0,23	0,11	2,74	4,01E-05	intergenic	na
rs6547286	2	79946055	G	A	0,30	0,16	2,33	4,26E-05	CTNNA2	intron
rs7310294	12	106214481	T	C	0,27	0,45	0,42	4,34E-05	intergenic	na
rs6472748	8	74197234	A	G	0,39	0,41	2,19	4,39E-05	intergenic	na
rs867870	17	6302512	C	T	0,45	0,29	2,20	4,55E-05	PITPNM3	intron
rs7970960	12	17993577	C	T	0,26	0,12	2,44	4,58E-05	intergenic	na
rs10129026	12	18053778	G	A	0,46	0,29	2,17	4,77E-05	intergenic	na
rs12998797	2	79944934	G	A	0,30	0,16	2,32	4,85E-05	CTNNA2	intron
rs10840939	12	18085845	G	A	0,32	0,17	2,28	4,85E-05	intergenic	na
rs1901515	5	101739148	C	T	0,46	0,37	2,24	4,88E-05	SLCO6A1	intron
rs931503	3	4639754	G	A	0,22	0,10	2,60	4,91E-05	ITPR1	intron
rs17004052	4	80821651	G	A	0,32	0,17	2,29	5,00E-05	intergenic	na
rs9846423	3	115420025	A	G	0,17	0,05	2,89	5,36E-05	intergenic	na
rs661882	10	27808089	T	C	0,44	0,22	2,82	5,91E-05	intergenic	na
rs7773088	6	19460990	T	C	0,35	0,19	2,32	6,06E-05	intergenic	na
rs10148309	14	50271461	A	G	0,27	0,13	2,40	6,25E-05	NIN	intron
rs11117184	12	85000129	G	T	0,45	0,28	2,14	6,27E-05	GNTIVH	mrna-utr
rs12562995	1	4259026	A	G	0,14	0,04	3,39	6,59E-05	intergenic	na
rs1878926	19	41622361	A	G	0,43	0,40	2,14	7,33E-05	intergenic	na
rs10251765	7	129510943	A	C	0,27	0,45	0,44	7,63E-05	KIAA0265	intron
rs9572485	13	70009211	C	T	0,13	0,30	0,35	7,85E-05	intergenic	na
rs4240095	23	32177890	A	G	0,31	0,43	2,89	8,04E-05	DMD	intron
rs530855	13	75235781	T	G	0,34	0,48	2,18	8,12E-05	LMO7	intron
rs7584323	2	79943428	T	G	0,30	0,17	2,29	8,34E-05	CTNNA2	intron
rs4236628	7	129538321	A	G	0,22	0,10	2,65	8,69E-05	KIAA0265	intron
rs10235665	7	103384192	A	C	0,11	0,03	3,86	9,30E-05	RELN	intron
rs9473045	6	47339700	T	C	0,48	0,35	2,05	9,68E-05	TNFRSF21	intron
rs2315654	20	61870085	C	T	0,26	0,13	2,39	9,73E-05	BTBD4	intron
rs914503	6	44242559	G	A	0,44	0,27	2,11	9,80E-05	CAPN11	mrna-utr
rs10503942	8	33300800	A	G	0,11	0,04	3,57	9,80E-05	intergenic	na
rs17641748	5	147380348	T	C	0,24	0,11	2,41	9,99E-05	intergenic	na

Table S3: Differentially expressed genes in $CD8^+$ T cells of rapid progressors

Upregulated in rapid progressors

Symbol	ttest_p.value	eb_p.value	ttest_FDR	eBayes_FDR	logFC	FC Direction
ACSS1	0,000745799	0,000451055	0,051997472	0,037248735	-0,32573435	EC down vs RP
ACTB	0,000360927	0,000170773	0,039304757	0,026976527	-0,5785265	EC down vs RP
ACTB.1	0,001491166	0,000808416	0,067223998	0,049448284	-0,547421	EC down vs RP
ACTG1	0,000842499	0,000537133	0,054599841	0,040421439	-0,2977545	EC down vs RP
AGTRAP.1	0,000738026	0,000427951	0,051868005	0,036521071	-0,3554814	EC down vs RP
ALG3	0,000489621	0,000418447	0,042543622	0,036031675	-0,21512355	EC down vs RP
ALG8	0,000417406	0,000347082	0,040961965	0,033546943	-0,22210985	EC down vs RP
ANKFY1	0,000178002	9,42E-05	0,031829803	0,018776488	-0,3770363	EC down vs RP
ANKRD13A	0,000317411	0,000306719	0,038895006	0,031653156	-0,2021158	EC down vs RP
AP1B1	5,58E-05	3,75E-05	0,01974063	0,014321392	-0,2857779	EC down vs RP
APOBEC3A	0,000886598	0,000448066	0,055567875	0,03724017	-0,6373306	EC down vs RP
ARHGDIB	0,000721322	0,000476252	0,050881122	0,038576376	-0,28081	EC down vs RP
ARL6IP4.1	0,000692111	0,000437721	0,049738341	0,036861136	-0,29984495	EC down vs RP
ARPC2	0,000850923	0,000524161	0,054599841	0,039828356	-0,3170285	EC down vs RP
ATP2A3	0,001057326	0,000691861	0,060702005	0,046634299	-0,28687685	EC down vs RP
ATP5B	3,86E-05	2,69E-05	0,01905309	0,012548005	-0,280712	EC down vs RP
ATP5F1	0,000875245	0,000549567	0,055271961	0,040585361	-0,30634235	EC down vs RP
BAT3	4,15E-06	3,50E-06	0,006603636	0,00445973	-0,27481005	EC down vs RP
BAT3.1	0,000190786	0,000114862	0,031829803	0,021112474	-0,3136423	EC down vs RP
BLVRA	3,46E-05	1,40E-05	0,01905309	0,009892764	-0,5306747	EC down vs RP
C16ORF33	0,000481646	0,000267581	0,042543622	0,0311086	-0,37373395	EC down vs RP
C16ORF57	0,000584361	0,000349406	0,046204577	0,033546943	-0,32781775	EC down vs RP
C1ORF85	0,000257936	0,000176896	0,035503405	0,026976527	-0,27037505	EC down vs RP
C4ORF33	0,000357449	0,000272923	0,039270062	0,03142892	-0,24113515	EC down vs RP
C6ORF173	0,000684069	0,000502421	0,049721124	0,039041791	-0,24506955	EC down vs RP
C7ORF24	0,000964672	0,000635687	0,057627123	0,044675696	-0,28288205	EC down vs RP
C9ORF37	0,000264059	0,000163079	0,035799665	0,026233033	-0,3047343	EC down vs RP
CAMK2G	0,000906901	0,000591692	0,056127584	0,042521761	-0,287112	EC down vs RP
CAPZB	0,000431685	0,000234808	0,041467749	0,029530244	-0,3869778	EC down vs RP
CAT	0,000932381	0,000810609	0,056906327	0,049448284	-0,2035402	EC down vs RP
CD164	0,000434273	0,000612065	0,041507854	0,043821074	-0,1582888	EC down vs RP
CDC20	0,001316382	0,000755307	0,064956498	0,04799421	-0,40110275	EC down vs RP
CDC45L	8,47E-05	4,09E-05	0,025289806	0,01531342	-0,41733425	EC down vs RP
CDCA5	0,000401464	0,000201252	0,040961965	0,027633186	-0,47737245	EC down vs RP
CDCA7	0,001008011	0,000525044	0,058984967	0,039828356	-0,5639525	EC down vs RP
CHI3L2	0,000964485	0,000509988	0,057627123	0,039310208	-0,5087654	EC down vs RP
CHMP4A	0,001321829	0,00082464	0,064956498	0,049448284	-0,3187403	EC down vs RP
CKS1B	1,17E-05	7,84E-06	0,010024496	0,007337296	-0,2987294	EC down vs RP
CNN2.1	0,000439613	0,00023456	0,041539645	0,029530244	-0,4061224	EC down vs RP
CROP	0,000258867	0,000195559	0,035503405	0,027633186	-0,24664615	EC down vs RP
CTNNBL1	2,07E-05	1,20E-05	0,013792979	0,009210352	-0,32836345	EC down vs RP
CUL1	0,000167651	0,000110304	0,031093437	0,020983429	-0,2842563	EC down vs RP

Symbol	ttest_p.value	eb_p.value	ttest_FDR	eBayes_FDR	logFC	FC Direction
DBI.1	0,000114351	6,29E-05	0,028388668	0,018704509	-0,3484869	EC down vs RP
DBNL.1	0,001281994	0,000753651	0,064566224	0,04799421	-0,369475	EC down vs RP
DECR1	0,00140466	0,000852407	0,066426211	0,049873905	-0,3429984	EC down vs RP
DERA	0,000621425	0,000349725	0,04719021	0,033546943	-0,3736233	EC down vs RP
DNMT1	7,09E-07	3,52E-07	0,006244311	0,003094429	-0,37865145	EC down vs RP
DOCK2	0,000337007	0,000252289	0,039270062	0,030331823	-0,2461615	EC down vs RP
DRAP1	0,001206987	0,000721777	0,063213041	0,047152602	-0,3494557	EC down vs RP
E2F2	3,27E-06	1,04E-06	0,006244311	0,003094429	-0,61755845	EC down vs RP
ECGF1	6,76E-05	2,43E-05	0,022280925	0,01209515	-1,301374	EC down vs RP
EIF2AK2	0,000298329	0,000132416	0,037767253	0,023437545	-0,75672205	EC down vs RP
EIF4E2	0,000162983	8,99E-05	0,030847305	0,018776488	-0,35138985	EC down vs RP
ENDOGL1	0,000350614	0,000202218	0,039270062	0,027633186	-0,33946935	EC down vs RP
EPSTI1	5,66E-06	1,55E-06	0,007208832	0,003094429	-1,2796473	EC down vs RP
FEN1.1	0,00079587	0,000492177	0,053542504	0,038869595	-0,31351025	EC down vs RP
FRAG1	0,000246545	0,000146321	0,035503405	0,024973933	-0,3209041	EC down vs RP
GAPDH	0,000600623	0,000339644	0,046610751	0,033467151	-0,36801775	EC down vs RP
GINS2	0,000244419	0,000112023	0,035503405	0,020994511	-0,5738003	EC down vs RP
GLE1	4,91E-05	2,26E-05	0,019098257	0,01209515	-0,43110885	EC down vs RP
GMPPB	0,000454078	0,000364458	0,04193308	0,03432009	-0,2279793	EC down vs RP
GSTK1	0,000182766	9,26E-05	0,031829803	0,018776488	-0,409581	EC down vs RP
HDAC2	0,000122705	7,99E-05	0,028958556	0,018704509	-0,2886612	EC down vs RP
HDAC4	0,000594477	0,000711376	0,046610751	0,047046182	-0,16771675	EC down vs RP
HINT2	0,000212859	0,000114667	0,033628222	0,021112474	-0,37001835	EC down vs RP
IFI44	1,82E-05	5,58E-06	0,013792979	0,006277311	-1,4982939	EC down vs RP
IFI44L	1,21E-05	3,48E-06	0,010024496	0,00445973	-1,91217765	EC down vs RP
IFI6	0,000162474	6,43E-05	0,030847305	0,018704509	-1,3345225	EC down vs RP
IFI6.1	0,000129935	5,05E-05	0,029184819	0,017397279	-1,205443	EC down vs RP
IFITM1	0,001204212	0,000624844	0,063213041	0,04423894	-0,654052	EC down vs RP
IFITM3	0,000186164	7,41E-05	0,031829803	0,018704509	-1,7301828	EC down vs RP
IGJ	0,001415972	0,000755169	0,066426211	0,04799421	-0,59408185	EC down vs RP
IGLL1	7,12E-05	2,53E-05	0,023054902	0,01209515	-1,8020144	EC down vs RP
IL10RB	0,001054376	0,000699054	0,060702005	0,046888101	-0,2805971	EC down vs RP
IRF7	0,000342436	0,000160717	0,039270062	0,026233033	-0,5880602	EC down vs RP
IRF7.2	0,000157476	6,48E-05	0,030847305	0,018704509	-0,82659775	EC down vs RP
IRF9	0,000143936	6,60E-05	0,030645329	0,018704509	-0,49814585	EC down vs RP
KIAA0372	0,00046597	0,000289133	0,042543622	0,031653156	-0,3055883	EC down vs RP
LAP3	4,47E-05	1,76E-05	0,01905309	0,011837615	-0,5967018	EC down vs RP
LCP1	0,000849603	0,000460711	0,054599841	0,037636568	-0,439687	EC down vs RP
LGALS3BP	0,000691769	0,000398868	0,049738341	0,035463961	-0,35760265	EC down vs RP
LOC26010	0,001320839	0,000834103	0,064956498	0,049448284	-0,3103701	EC down vs RP
LOC650518	0,000482038	0,00029371	0,042543622	0,031653156	-0,3146388	EC down vs RP
LOC652493	0,000907273	0,000436875	0,056127584	0,036861136	-1,27199815	EC down vs RP
LOC727761	0,000316879	0,000188326	0,038895006	0,027364442	-0,32242605	EC down vs RP
LOC728635	0,000642826	0,000440366	0,047765427	0,036921252	-0,26692595	EC down vs RP

Symbol	ttest_p.value	eb_p.value	ttest_FDR	eBayes_FDR	logFC	FC Direction
LRRC28	0,00035489	0,000383545	0,039270062	0,035436418	-0,18663005	EC down vs RP
LSMD1	0,000274303	0,000172365	0,036408994	0,026976527	-0,29823475	EC down vs RP
LY6E	6,34E-06	1,78E-06	0,007263096	0,003094429	-1,13083185	EC down vs RP
MAP3K7.1	0,000111154	8,39E-05	0,028330814	0,018704509	-0,25528845	EC down vs RP
MBD2	0,000391556	0,00029549	0,040535562	0,031653156	-0,2429046	EC down vs RP
MCM5	3,12E-06	1,23E-06	0,006244311	0,003094429	-0,45875925	EC down vs RP
MCM7.1	0,000481999	0,000244961	0,042543622	0,029968417	-0,47722365	EC down vs RP
MDH2	0,000146681	8,18E-05	0,030645329	0,018704509	-0,3446275	EC down vs RP
MGC29506	0,000190947	9,39E-05	0,031829803	0,018776488	-0,43855425	EC down vs RP
MGC4677	6,52E-05	6,29E-05	0,021877658	0,018704509	-0,224231	EC down vs RP
MGC4677.1	0,000914067	0,000490603	0,056365484	0,038869595	-0,4667945	EC down vs RP
MIF4GD	0,001222558	0,000718682	0,063755202	0,047152602	-0,3666701	EC down vs RP
MRPL15	9,25E-05	7,39E-05	0,025753613	0,018704509	-0,24686025	EC down vs RP
MRPS11	0,000200167	0,000110867	0,032427124	0,020983429	-0,3523898	EC down vs RP
MRPS18B.1	0,000253675	0,000136797	0,035503405	0,023558599	-0,37407065	EC down vs RP
MT1A	0,000316345	0,000148277	0,038895006	0,025083792	-0,57315815	EC down vs RP
MT1E	0,000803077	0,000403965	0,053542504	0,035490967	-0,6165032	EC down vs RP
MT1H	0,000607957	0,000615631	0,046673511	0,043911936	-0,1862761	EC down vs RP
MT2A	0,00040044	0,000185938	0,040961965	0,027341525	-0,6758624	EC down vs RP
MX1	2,22E-06	5,29E-07	0,006244311	0,003094429	-1,8491931	EC down vs RP
MYL6.1	0,000323661	0,00018093	0,03916	0,027233542	-0,356689	EC down vs RP
MYL6B	0,000943676	0,000523359	0,056906327	0,039828356	-0,4173379	EC down vs RP
NCKAP1L	0,000469827	0,000307678	0,042543622	0,031653156	-0,2833597	EC down vs RP
NME1	0,000162073	7,32E-05	0,030847305	0,018704509	-0,5363188	EC down vs RP
NPL	0,000289493	0,000297485	0,037645868	0,031653156	-0,1955971	EC down vs RP
NRD1	0,001045027	0,00069283	0,060535568	0,046634299	-0,2805615	EC down vs RP
NTHL1	0,000429675	0,000301664	0,041467749	0,031653156	-0,2608454	EC down vs RP
NUDT14	0,000355648	0,000314788	0,039270062	0,031847525	-0,2134187	EC down vs RP
OAS1.2	0,000404186	0,000200589	0,040961965	0,027633186	-0,49502945	EC down vs RP
OAS2.2	0,00042621	0,000190389	0,041357478	0,027364442	-1,05435825	EC down vs RP
OAS3	0,000583798	0,000294132	0,046204577	0,031653156	-0,5241514	EC down vs RP
OGT.1	0,000169163	9,01E-05	0,031093437	0,018776488	-0,37175705	EC down vs RP
PAICS	0,000147487	7,14E-05	0,030645329	0,018704509	-0,4389628	EC down vs RP
PAQR4	0,000146042	0,000118369	0,030645329	0,02134671	-0,2392358	EC down vs RP
PARP12	0,000754509	0,000394046	0,052035836	0,035436418	-0,49119535	EC down vs RP
PARP9.1	0,000345715	0,00016137	0,039270062	0,026233033	-0,60654795	EC down vs RP
PDCD5	4,53E-05	2,41E-05	0,01905309	0,01209515	-0,35557105	EC down vs RP
PFN1.1	0,000644668	0,000339027	0,047765427	0,033467151	-0,4565755	EC down vs RP
PNKD.2	0,000800068	0,000823089	0,053542504	0,049448284	-0,1799713	EC down vs RP
PNPO	7,61E-05	3,48E-05	0,023460039	0,013841254	-0,4568901	EC down vs RP
POLD1	0,000441125	0,000378717	0,041539645	0,03531494	-0,2156703	EC down vs RP
POLR2B	0,000584929	0,000690789	0,046204577	0,046634299	-0,1692966	EC down vs RP
PPIL5	1,08E-05	8,62E-06	0,009812026	0,007337296	-0,27112565	EC down vs RP
PRDX3	0,000614411	0,000353819	0,046980341	0,033649794	-0,3543678	EC down vs RP

Symbol	ttest_p.value	eb_p.value	ttest_FDR	eBayes_FDR	logFC	FC Direction
PRDX3.1	0,000749833	0,000446391	0,051997472	0,03724017	-0,33630715	EC down vs RP
PRKD2	0,000690235	0,00041684	0,049738341	0,036031675	-0,32504255	EC down vs RP
PRR5.1	0,001187032	0,000675544	0,063213041	0,046285694	-0,4029286	EC down vs RP
PSMA5	0,000762191	0,000390782	0,052035836	0,035436418	-0,53697155	EC down vs RP
PSMA6	3,56E-05	2,41E-05	0,01905309	0,01209515	-0,287332	EC down vs RP
PSMB10	0,000419991	0,000205102	0,040961965	0,027633186	-0,532871	EC down vs RP
PSMB2	0,000383189	0,000209213	0,040027528	0,02777297	-0,37880205	EC down vs RP
PSMB8	0,000899396	0,000482718	0,0560028	0,038869595	-0,46448845	EC down vs RP
PSMB8.1	0,000406133	0,000262181	0,040961965	0,0311086	-0,2886151	EC down vs RP
PSME1	0,001185402	0,000685809	0,063213041	0,046634299	-0,3822175	EC down vs RP
PSME2	5,50E-06	1,71E-06	0,007208832	0,003094429	-0,7100372	EC down vs RP
RASGRP3	0,000223439	0,000314877	0,033898924	0,031847525	-0,16878495	EC down vs RP
RBBP8	0,000260017	0,000176301	0,035503405	0,026976527	-0,2735564	EC down vs RP
RBCK1	2,79E-05	1,27E-05	0,017230679	0,009343214	-0,4241178	EC down vs RP
REC8	0,000182068	8,58E-05	0,031829803	0,018704509	-0,4844286	EC down vs RP
REC8.1	0,000127118	7,22E-05	0,029184819	0,018704509	-0,3353477	EC down vs RP
RNASEN	0,000144101	0,000130425	0,030645329	0,023301051	-0,22207315	EC down vs RP
RNF214.1	0,000342942	0,000645912	0,039270062	0,04491487	-0,1423003	EC down vs RP
RPS21	0,000352245	0,000205268	0,039270062	0,027633186	-0,33379415	EC down vs RP
RPS6KA4	0,000417928	0,000246131	0,040961965	0,029968417	-0,3306817	EC down vs RP
SAC3D1	4,01E-05	3,17E-05	0,01905309	0,013758318	-0,2583145	EC down vs RP
SARS2	2,03E-05	8,83E-06	0,013792979	0,007337296	-0,44389815	EC down vs RP
SH3BP1	0,000153051	7,74E-05	0,030797167	0,018704509	-0,40383275	EC down vs RP
SIDT2	0,00019315	8,59E-05	0,031829803	0,018704509	-0,5985707	EC down vs RP
SIRPG.2	0,000818073	0,000507253	0,054111689	0,039257694	-0,3124292	EC down vs RP
SLC46A2	0,000440658	0,000279865	0,041539645	0,031653156	-0,2951995	EC down vs RP
SNTB1	0,000311751	0,000237684	0,038895006	0,029696484	-0,24281535	EC down vs RP
SNX14	0,000942768	0,000713716	0,056906327	0,047046182	-0,23427275	EC down vs RP
SP140.1	0,000507237	0,00030242	0,043287276	0,031653156	-0,3269626	EC down vs RP
SQRDL	9,07E-05	4,96E-05	0,025753613	0,017397279	-0,3486533	EC down vs RP
STAT1.1	0,001089186	0,000561157	0,061600223	0,041099933	-0,63986395	EC down vs RP
STAT2	0,001356533	0,000731429	0,065343817	0,047557841	-0,53707965	EC down vs RP
STIM1	0,001272985	0,000853109	0,064389972	0,049873905	-0,2767308	EC down vs RP
SULT1A1.3	0,000352565	0,000199629	0,039270062	0,027633186	-0,35058865	EC down vs RP
TCF3	0,000408263	0,000356015	0,040961965	0,033690963	-0,21402135	EC down vs RP
TEX264	0,000100092	7,81E-05	0,026539003	0,018704509	-0,25023825	EC down vs RP
TFDP1	4,67E-05	1,96E-05	0,01905309	0,012092679	-0,51076375	EC down vs RP
TFDP1.1	4,39E-05	2,12E-05	0,01905309	0,01209515	-0,40083865	EC down vs RP
TIMELESS	0,0003682	0,000227276	0,039765548	0,0294205	-0,3063595	EC down vs RP
TIMM10	0,000854015	0,000489532	0,054599841	0,038869595	-0,37215745	EC down vs RP
TK1	9,39E-05	5,14E-05	0,025753613	0,017397279	-0,34867075	EC down vs RP
TNFSF13B.1	0,000789936	0,00038835	0,053358334	0,035436418	-0,7290695	EC down vs RP
TOPBP1	0,00022341	0,000175023	0,033898924	0,026976527	-0,24069215	EC down vs RP
TTC13.2	0,000697018	0,000540111	0,049903355	0,040421439	-0,23112945	EC down vs RP

Symbol	ttest_p.value	eb_p.value	ttest_FDR	eBayes_FDR	logFC	FC Direction
TTC17	0,001057427	0,000747125	0,060702005	0,04799421	-0,2549234	EC down vs RP
TUBA1B	0,000622093	0,000326686	0,04719021	0,032695942	-0,4547395	EC down vs RP
TXNDC5.1	0,000113531	5,46E-05	0,028388668	0,018006002	-0,4310458	EC down vs RP
TYMS	7,49E-05	2,90E-05	0,023460039	0,013201311	-0,75613825	EC down vs RP
UBE2C	0,00110703	0,000651606	0,061758299	0,045130785	-0,36002375	EC down vs RP
UBE2L6.1	0,000803866	0,000404698	0,053542504	0,035490967	-0,6134645	EC down vs RP
UBE2L6.2	0,000121702	5,97E-05	0,028958556	0,018704509	-0,4183568	EC down vs RP
UBE2T	0,0003008	0,00039427	0,03782952	0,035436418	-0,1701424	EC down vs RP
UCRC	1,13E-06	8,70E-07	0,006244311	0,003094429	-0,3006752	EC down vs RP
UHRF1	0,00029664	0,000163305	0,037767253	0,026233033	-0,36447075	EC down vs RP
UQCRC1	0,001241167	0,000755715	0,064237571	0,04799421	-0,33521375	EC down vs RP
UQCRH.1	0,000516367	0,000420843	0,043870499	0,0360755	-0,2234105	EC down vs RP
WDR18	3,99E-06	1,48E-06	0,006603636	0,003094429	-0,4957705	EC down vs RP
WDR4	0,00111374	0,000829153	0,061812514	0,049448284	-0,2377425	EC down vs RP
WSB2	0,000185932	0,000223046	0,031829803	0,029203751	-0,18560885	EC down vs RP
XAF1	0,000824039	0,00039485	0,054131698	0,035436418	-1,10081935	EC down vs RP

Downregulated in rapid progressors

Symbol	ttest_p.value	eb_p.value	ttest_FDR	eBayes_FDR	logFC	FC Direction
LOC439992	1,68E-06	9,92E-07	0,006244311	0,003094429	0,3380143	EC up vs RP
LOC650739.1	2,45E-06	1,69E-06	0,006244311	0,003094429	0,30861985	EC up vs RP
ISYNA1	2,84E-06	2,64E-06	0,006244311	0,004197572	0,26821375	EC up vs RP
C21ORF129	2,47E-06	3,50E-06	0,006244311	0,00445973	0,2316598	EC up vs RP
HS.543649	4,58E-06	3,75E-06	0,006730141	0,00447925	0,277471	EC up vs RP
FSHR	1,01E-05	8,78E-06	0,009630411	0,007337296	0,2603664	EC up vs RP
LOC653293.1	9,55E-06	8,34E-06	0,009610117	0,007337296	0,2608117	EC up vs RP
TEKT2	6,68E-06	8,37E-06	0,007263096	0,007337296	0,2283211	EC up vs RP
LOC642250	1,77E-05	1,20E-05	0,013792979	0,009210352	0,29204285	EC up vs RP
C1QTNF1	2,09E-05	1,80E-05	0,013792979	0,011837615	0,2536519	EC up vs RP
LOC645527	1,87E-06	1,93E-05	0,006244311	0,012092679	0,14814035	EC up vs RP
LOC643287	4,13E-05	2,50E-05	0,01905309	0,01209515	0,3128862	EC up vs RP
LOC650140	2,91E-05	2,35E-05	0,017381985	0,01209515	0,25817355	EC up vs RP
ZNF329	4,07E-05	2,32E-05	0,01905309	0,01209515	0,3315527	EC up vs RP
LOC642082	3,54E-05	2,97E-05	0,01905309	0,013201311	0,250604	EC up vs RP
HS.584167	6,84E-06	3,24E-05	0,007263096	0,013758318	0,1563598	EC up vs RP
EMD	6,15E-05	3,32E-05	0,021113977	0,013780629	0,3517038	EC up vs RP
HS.437760	1,98E-05	3,42E-05	0,013792979	0,013841254	0,1899736	EC up vs RP
GFPT2	3,71E-05	3,57E-05	0,01905309	0,013943804	0,2318001	EC up vs RP
HS.563147	5,19E-05	4,53E-05	0,019098257	0,016664932	0,24023675	EC up vs RP
BRF2	4,16E-05	5,19E-05	0,01905309	0,017397279	0,20387475	EC up vs RP
LOC441032	7,79E-05	5,04E-05	0,023646075	0,017397279	0,2926141	EC up vs RP
ABCC6.1	5,25E-05	8,03E-05	0,019098257	0,018704509	0,1842689	EC up vs RP

Symbol	ttest_p.value	eb_p.value	ttest_FDR	eBayes_FDR	logFC	FC Direction
C4ORF39	0,000100302	6,72E-05	0,026539003	0,018704509	0,28240815	EC up vs RP
FBXW11	5,26E-05	5,84E-05	0,019098257	0,018704509	0,2115365	EC up vs RP
HS.160027	4,90E-05	8,13E-05	0,019098257	0,018704509	0,17960545	EC up vs RP
HS.546079	0,000118113	7,90E-05	0,028946853	0,018704509	0,2817418	EC up vs RP
HS.558485	6,19E-05	8,61E-05	0,021113977	0,018704509	0,18900795	EC up vs RP
LOC493754	7,60E-05	8,33E-05	0,023460039	0,018704509	0,2077152	EC up vs RP
LOC651453	8,88E-05	6,57E-05	0,02570848	0,018704509	0,26120245	EC up vs RP
LOC653820	0,000166472	7,55E-05	0,031093437	0,018704509	0,53191045	EC up vs RP
SNURF	0,000128208	7,72E-05	0,029184819	0,018704509	0,31271825	EC up vs RP
TOB1	0,000159395	7,25E-05	0,030847305	0,018704509	0,52071475	EC up vs RP
TTR	0,000101347	7,68E-05	0,026539003	0,018704509	0,25549615	EC up vs RP
C12ORF57	0,000181942	9,43E-05	0,031829803	0,018776488	0,391684	EC up vs RP
HS.528174	9,43E-05	9,21E-05	0,025753613	0,018776488	0,2176947	EC up vs RP
MOSPD1	2,34E-05	9,25E-05	0,014917508	0,018776488	0,14689175	EC up vs RP
HS.130916	0,000131298	9,92E-05	0,029184819	0,019557211	0,25332285	EC up vs RP
XBP1	0,000192731	0,000105589	0,031829803	0,020596399	0,3577776	EC up vs RP
LOC730273	0,000106245	0,000109601	0,027445761	0,020983429	0,2095854	EC up vs RP
HS.123538	4,27E-05	0,000116475	0,01905309	0,021205156	0,15404285	EC up vs RP
MT1E.1	5,30E-05	0,00013487	0,019098257	0,023558599	0,15424615	EC up vs RP
ZCCHC10	0,000150744	0,000135989	0,030788716	0,023558599	0,22194895	EC up vs RP
HS.569204	0,000199088	0,000157827	0,032427124	0,026233033	0,23969515	EC up vs RP
LOC652565	8,77E-05	0,000161503	0,02570848	0,026233033	0,16445055	EC up vs RP
HS.570977	0,000177947	0,000177811	0,031829803	0,026976527	0,20607215	EC up vs RP
UBQLN1.2	0,000259946	0,000174857	0,035503405	0,026976527	0,2758707	EC up vs RP
CGRRF1	0,000271898	0,000182361	0,036346808	0,027234486	0,2765467	EC up vs RP
SPRY4	0,000218245	0,000185089	0,033807012	0,027341525	0,22669805	EC up vs RP
AKAP1.1	0,000130067	0,000189671	0,029184819	0,027364442	0,1744035	EC up vs RP
LHX3.1	0,000187511	0,000204843	0,031829803	0,027633186	0,19500965	EC up vs RP
LOC390998	0,000143668	0,000194528	0,030645329	0,027633186	0,1787683	EC up vs RP
COPZ2	0,000257047	0,000208706	0,035503405	0,02777297	0,23232115	EC up vs RP
HS.374221	0,000251456	0,000217477	0,035503405	0,028670906	0,2216893	EC up vs RP
FXYD2	0,000256554	0,000228468	0,035503405	0,0294205	0,2168194	EC up vs RP
KLHDC8B	0,000325719	0,000229319	0,03916	0,0294205	0,26176285	EC up vs RP
C21ORF125	0,000151399	0,000234446	0,030788716	0,029530244	0,1676761	EC up vs RP
HS.445079	0,000209097	0,000239435	0,033309192	0,029720982	0,18843425	EC up vs RP
SLC35D1	0,000219296	0,00024398	0,033807012	0,029968417	0,1907216	EC up vs RP
DDIT3	0,000523659	0,000252115	0,044098067	0,030331823	0,6314424	EC up vs RP
LOC283202	4,68E-05	0,000256596	0,01905309	0,030656811	0,12637035	EC up vs RP
HS.541249	0,000243491	0,000268514	0,035503405	0,0311086	0,1901	EC up vs RP
TSPYL1	0,00048869	0,000265591	0,042543622	0,0311086	0,3939102	EC up vs RP
ZNF273	0,000208633	0,000264827	0,033309192	0,0311086	0,17863285	EC up vs RP
C20ORF107	0,000347094	0,000302652	0,039270062	0,031653156	0,2161692	EC up vs RP
CCDC16.1	0,000329563	0,000278779	0,039270062	0,031653156	0,2219658	EC up vs RP
HS.254509	0,000325283	0,000306482	0,03916	0,031653156	0,20525475	EC up vs RP

Symbol	ttest_p.value	eb_p.value	ttest_FDR	eBayes_FDR	logFC	FC Direction
HS.37648	0,000483525	0,000304967	0,042543622	0,031653156	0,2985382	EC up vs RP
HS.540764	0,00015652	0,000304233	0,030847305	0,031653156	0,1525625	EC up vs RP
LOC344167	0,000412875	0,000284826	0,040961965	0,031653156	0,26623275	EC up vs RP
LOC646549	0,000267823	0,000307987	0,036054323	0,031653156	0,18424905	EC up vs RP
LOC727815	0,000244315	0,000286452	0,035503405	0,031653156	0,18369665	EC up vs RP
MRVI1	0,00023306	0,000297966	0,035080113	0,031653156	0,176292	EC up vs RP
TMED4	0,00048285	0,000286748	0,042543622	0,031653156	0,3283733	EC up vs RP
TATDN3.1	0,00021794	0,00031321	0,033807012	0,031847525	0,16763565	EC up vs RP
CALML4	0,000453715	0,000324714	0,04193308	0,032669634	0,25509525	EC up vs RP
HS.116301	0,000334939	0,000332124	0,039270062	0,03306711	0,1979218	EC up vs RP
C13ORF27	0,000573125	0,000343621	0,046204577	0,033546943	0,3258539	EC up vs RP
NOL9	0,000412093	0,000350983	0,040961965	0,033546943	0,21793985	EC up vs RP
PFKFB3	0,000576906	0,000349684	0,046204577	0,033546943	0,319753	EC up vs RP
MRFAP1	0,00053629	0,000373837	0,044572697	0,035030743	0,2618495	EC up vs RP
LOC401296	0,000481982	0,000392605	0,042543622	0,035436418	0,22432935	EC up vs RP
LOC730196	0,000370563	0,000390124	0,039795945	0,035436418	0,1890052	EC up vs RP
ANKRD6.1	0,000361877	0,000397337	0,039304757	0,035463961	0,1845596	EC up vs RP
FLJ33387	0,000558608	0,000404741	0,045829817	0,035490967	0,25027275	EC up vs RP
IDH3B.1	0,000382037	0,000415502	0,040027528	0,036031675	0,18478865	EC up vs RP
RPL23AP13	0,000550492	0,000414897	0,045555028	0,036031675	0,24008065	EC up vs RP
HS.571130	0,00062926	0,000434065	0,04754521	0,036861136	0,2646316	EC up vs RP
LOC285588	0,000277789	0,000454015	0,036408994	0,037248735	0,154548	EC up vs RP
LOC642563	0,000581649	0,000452756	0,046204577	0,037248735	0,23189715	EC up vs RP
HS.537591	0,000583622	0,000471783	0,046204577	0,038377058	0,2236485	EC up vs RP
CACNG8	0,000278077	0,000497376	0,036408994	0,038869595	0,14870175	EC up vs RP
DERL2	0,000599084	0,000485398	0,046610751	0,038869595	0,2228709	EC up vs RP
HS.541977	0,000245757	0,000487818	0,035503405	0,038869595	0,1444701	EC up vs RP
LOC643870	0,000827907	0,000497049	0,054199532	0,038869595	0,33311715	EC up vs RP
PLEKHA1.1	0,000644335	0,000498172	0,047765427	0,038869595	0,2324374	EC up vs RP
LOC652211	0,000379895	0,000514574	0,040027528	0,039504424	0,1636633	EC up vs RP
HS.582468	0,000380026	0,000541321	0,040027528	0,040421439	0,15958865	EC up vs RP
LOC653636	0,000413992	0,000535908	0,040961965	0,040421439	0,16618125	EC up vs RP
HS.298416	0,000529733	0,000545622	0,044413931	0,040584075	0,1862206	EC up vs RP
DNAH7	0,000714007	0,000549885	0,050710838	0,040585361	0,23237485	EC up vs RP
DEFB107A	0,00037934	0,00055869	0,040027528	0,041076625	0,15704775	EC up vs RP
LOC648099	0,000656243	0,000569852	0,048064159	0,041577454	0,2084582	EC up vs RP
HS.561149	0,000443602	0,000573303	0,041568082	0,041670165	0,16522065	EC up vs RP
LOC649073	0,000331592	0,000577568	0,039270062	0,041821201	0,14759835	EC up vs RP
KRTAP19.6	0,000293468	0,000583797	0,037767253	0,042112704	0,1415989	EC up vs RP
H19	0,000636072	0,000618034	0,047742151	0,043919464	0,1911688	EC up vs RP
HS.192506	0,000467891	0,000628017	0,042543622	0,044299515	0,16108965	EC up vs RP
HSPA5	0,001088702	0,000638284	0,061600223	0,044693895	0,36298455	EC up vs RP
LOC648223	0,000532069	0,000646139	0,044415007	0,04491487	0,16806275	EC up vs RP
SNTG2	0,000759176	0,000670754	0,052035836	0,046122787	0,20344645	EC up vs RP

Symbol	ttest_p.value	eb_p.value	ttest_FDR	eBayes_FDR	logFC	FC Direction
TSC22D3	0,001273248	0,000668712	0,064389972	0,046122787	0,61878085	EC up vs RP
SNORD68	0,001161547	0,000682816	0,062811864	0,046616835	0,36379255	EC up vs RP
HS.540005	0,000349163	0,000703393	0,039270062	0,047014204	0,1381855	EC up vs RP
LOC729446	0,000716255	0,000705938	0,050710838	0,047019901	0,18739055	EC up vs RP
ADSS.1	0,000558295	0,000708731	0,045829817	0,04704204	0,16312095	EC up vs RP
EIF5.2	0,001249222	0,00072273	0,064366946	0,047152602	0,3860263	EC up vs RP
PRAMEF4	0,000747824	0,000740498	0,051997472	0,04798424	0,1860903	EC up vs RP
LOC649139	0,000122706	0,00074961	0,028958556	0,04799421	0,11022075	EC up vs RP
PPM1B	0,000883281	0,000771353	0,05554213	0,048645541	0,2034543	EC up vs RP
RHBDD1	0,000522238	0,000773606	0,044098067	0,048645541	0,15146105	EC up vs RP
THRA.1	0,000604701	0,000769431	0,046610751	0,048645541	0,1616101	EC up vs RP
ASNSD1	0,001154529	0,000777494	0,062767131	0,048729756	0,2739886	EC up vs RP
TTTY6B	0,000498788	0,000780861	0,042757121	0,048780863	0,1481893	EC up vs RP
LOC642987	0,000486586	0,000783934	0,042543622	0,048813275	0,1465998	EC up vs RP
HS.551128	0,000750748	0,000795601	0,051997472	0,049221465	0,17727515	EC up vs RP
LOC440359	0,001433148	0,000796494	0,066426211	0,049221465	0,4678277	EC up vs RP
TM9SF1.3	0,000636862	0,000798214	0,047742151	0,049221465	0,1621519	EC up vs RP
EFNA4	0,001009002	0,000829142	0,058984967	0,049448284	0,21378065	EC up vs RP
GSTM3	0,000997508	0,000807497	0,058852984	0,049448284	0,2172119	EC up vs RP
HIC1	0,000942066	0,00082837	0,056906327	0,049448284	0,20133115	EC up vs RP
LOC645018	0,001263247	0,000835432	0,064389972	0,049448284	0,28312905	EC up vs RP
MRPS26	0,000767119	0,00083552	0,052142509	0,049448284	0,1736828	EC up vs RP
WFDC10A	0,00044894	0,000818272	0,041863071	0,049448284	0,13998075	EC up vs RP
ZNF652	0,000897907	0,000815701	0,0560028	0,049448284	0,1963658	EC up vs RP
CCM2	0,001021449	0,000853147	0,059362578	0,049873905	0,21019265	EC up vs RP
GNA11	0,001123095	0,000847888	0,061870551	0,049873905	0,2336273	EC up vs RP

CHAPTER 3d. Highly Pathogenic Adapted HIV-1 Strains and Limited Immune Responses Dictate Rapid Disease Progression. *SUPPL MATERIALS.*

Table S5: Heterologous neutralization against a panel of standard virus

		Plasma	94UG103	92BR020	93IN905	MGRM-C-026	92TH021	MGRM-SCB-006	MGRM-D-009	MGRM-AG-007	1196	SF162	JRCSF	NL43	aMLV
RAPID PROGRESSORS (RP)	BASELINE	49	57	59	57	107	104	(-)	(-)	(-)	67	798	32	37	(-)
		58	61	35	44	54	128	23	56	44	25	43	35	(-)	(-)
		59	95	73	87	82	118	(-)	99	(-)	138	1811	(-)	204	(-)
		62	52	49	73	44	92	53	126	42	85	5154	34	214	(-)
		44	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	1695	(-)	182	(-)
		47	(-)	195	138	132	149	(-)	132	(-)	137	17619	120	608	(-)
		52	(-)	(-)	58	(-)	44	(-)	(-)	(-)	(-)	935	(-)	75	(-)
		53	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	257	(-)	(-)	(-)
		75	(-)	(-)	(-)	(-)	36	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
		65	(-)	(-)	101	116	156	85	155	(-)	120	4119	(-)	210	(-)
		76	46	25	25	38	120	27	(-)	28	(-)	70	(-)	(-)	(-)
		48	45	47	64	52	69	(-)	35	(-)	(-)	1436	48	669	(-)
	POST	45	90	121	163	154	136	(-)	90	111	135	5150	106	517	(-)
		47	(-)	106	124	(-)	(-)	(-)	96	(-)	(-)	13898	(-)	448	(-)
		49	33	39	46	54	56	28	26	29	52	394	31	23	(-)
		53	(-)	(-)	95	(-)	(-)	(-)	(-)	(-)	(-)	398	(-)	175	(-)
		44	(-)	80	78	66	(-)	(-)	(-)	(-)	74	6017	(-)	137	(-)
		47	(-)	66	55	55	46	(-)	64	(-)	59	10606	44	408	(-)
		52	(-)	(-)	(-)	(-)	79	(-)	(-)	(-)	(-)	811	(-)	(-)	(-)
		58	87	71	112	103	179	81	171	76	171	2253	84	805	(-)
ELITE/LONG TERM NON-PROGRESSORS (EP)	BASELINE	CM9	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
		CM53	(-)	186	66	63	52	(-)	69	(-)	98	1267	74	305	(-)
		CM2	273	506	617	288	170	98	326	426	355	33764	152	2048	(-)
		CM41	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	678	(-)	56	(-)
		CM10	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	801	(-)	(-)	(-)
		CM11	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	439	(-)	52	(-)
		CM39	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	482	(-)	112	(-)
		CM12	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	92	(-)	(-)	(-)
		CM43	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	252	(-)	(-)	(-)
		CM44	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
		CM48	41	39	53	90	57	26	39	(-)	35	218	(-)	81	(-)
		CM19	37	40	59	83	49	(-)	39	28	64	528	(-)	349	(-)
		CM54	(-)	(-)	(-)	77	42	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
		CM49	(-)	(-)	(-)	69	31	(-)	(-)	30	(-)	(-)	(-)	(-)	(-)
		CM21	(-)	(-)	(-)	92	(-)	(-)	(-)	(-)	(-)	142	(-)	61	(-)
		CM50	193	139	194	(-)	194	(-)	136	(-)	124	7504	(-)	972	(-)
		CM23	126	107	255	170	104	(-)	93	62	127	5045	68	330	(-)
		CM51	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
		CM52	76	(-)	72	143	107	(-)	67	(-)	62	403	(-)	(-)	(-)
		CM25	105	(-)	124	107	85	(-)	107	(-)	60	1540	(-)	331	(-)
		CM59	48	56	128	64	113	(-)	97	(-)	95	6775	(-)	661	(-)
		CM26	(-)	(-)	(-)	(-)	84	(-)	(-)	(-)	21	73	(-)	(-)	(-)
		CM61	71	126	92	69	86	(-)	102	(-)	94	2192	77	120	(-)
		CM29	77	105	72	(-)	88	(-)	(-)	(-)	100	1076	(-)	100	(-)
		CM35	46	75	75	54	45	(-)	227	(-)	58	7622	(-)	923	(-)
		CM64	83	(-)	(-)	111	(-)	(-)	(-)	(-)	(-)	81	(-)	(-)	(-)
		CM37	(-)	(-)	96	(-)	(-)	(-)	(-)	(-)	(-)	1046	(-)	372	(-)
		CM66	73	122	235	198	(-)	(-)	74	(-)	191	5412	79	1107	(-)
		CM67	(-)	(-)	121	122	142	(-)	116	(-)	(-)	738	(-)	139	(-)
		CM68	(-)	(-)	153	(-)	(-)	(-)	162	(-)	(-)	811	(-)	244	(-)
		CM69	85	(-)	209	251	241	(-)	76	(-)	113	1308	(-)	208	(-)
		CM1	47	194	(-)	172	81	(-)	53	(-)	188	53571	54	115	(-)
	POST	CM17	(-)	119	251	(-)	40	53	46	N/A	58	18895	39	128	(-)
		CM9	33	78	38	49	55	(-)	34	23	75	2260	37	283	(-)
		CM53	44	192	76	67	77	(-)	48	(-)	140	1943	59	361	(-)
		CM66	102	143	208	448	(-)	(-)	60	(-)	184	5234	232	1835	(-)
		CM68	30	51	43	42	23	(-)	127	24	74	2395	(-)	250	(-)
		CM1	63	582	96	89	97	(-)	62	(-)	236	83576	87	221	(-)
		CM11	60	101	54	(-)	(-)	(-)	(-)	(-)	115	3346	81	113	(-)
		CM39	35	44	59	(-)	38	(-)	69	83	56	3066	39	646	(-)
		CM12	34	(-)	42	(-)	(-)	(-)	113	(-)	54	2036	(-)	396	(-)
		CM41	(-)	85	63	(-)	(-)	(-)	70	(-)	75	1390	(-)	226	(-)
		CM43	(-)	89	101	86	(-)	(-)	86	(-)	(-)	1077	(-)	334	(-)
		CM44	(-)	82	87	(-)	59	(-)	79	N/A	61	25440	(-)	181	(-)
		CM45	47	56	69	46	54	(-)	67	(-)	93	2965	(-)	295	(-)
		CM48	92	120	306	128	92	53	249	66	227	4529	67	730	(-)
		CM19	88	149	99	107	93	32	145	45	355	14726	53	2882	(-)
		CM54	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)	353	(-)	119	(-)
		CM21	(-)	(-)	179	124	(-)	(-)	157	(-)	(-)	1207	(-)	420	(-)
		CM23	143	102	244	275	132	45	157	96	128	5499	109	316	(-)
		CM51	61	86	91	81	79	(-)	89	66	117	2560	(-)	331	(-)
		CM52	54	79	64	53	71	(-)	54	36	108	2347	(-)	320	(-)
		CM25	73	76	188	106	89	(-)	211	(-)	125	8312	112	1452	(-)
		CM59	73	93	393	104	178	(-)	269	(-)	153	23261	(-)	1153	(-)
		CM26	263	112	189	124	143	355	226	(-)	183	9950	131	5592	(-)
		CM35	53	80	94	(-)	(-)	(-)	61	(-)	109	14327	(-)	1209	(-)
		CM36	77	(-)	97	(-)	(-)	(-)	(-)	(-)	(-)	1795	(-)	89	(-)
		CM37	(-)	(-)	210	264	(-)	(-)	(-)	(-)	101	2002	(-)	298	(-)
		CM67	(-)	(-)	131	(-)	(-)	(-)	197	(-)	144	9931	(-)	470	(-)
		CM69	77	73	260	188	263	71	94	46	130	1630	72	171	(-)

IC50 (1/dil'n)	<20	20-100	100-250	250-500	>500
Virus group	Sensitive	Moderate	Resistant		

Table S6: HLA Class I phenotype frequency and allelic distribution in RP and SP study groups

HLA-A* alleles	% RP (n=74)	% RP (n=74)	p-value	q-value
A*02:01	59.5	39.6	0.041	0.543
A*03:01	23.0	18.8	0.654	0.938
A*24:02	21.6	18.8	0.819	0.956
A*01:01	18.9	18.8	1.000	1.000
A*68:01	9.50	4.20	0.480	0.938
A*11:01	6.80	18.8	0.077	0.543
A*31:01	6.80	2.10	0.401	0.938
A*33:01	6.80	4.20	0.702	0.938
A*29:02	6.80	8.30	0.737	0.938
A*68:02	4.10	4.20	1.000	1.000
A*02:05	4.10	6.30	0.678	0.938
A*30:02	2.70	4.20	0.645	0.938
A*32:01	2.70	6.30	0.380	0.938
A*26:01	1.40	8.30	0.077	0.543
HLA-B* alleles	**% RP (n=74)**	**% RP (n=74)**	**p-value**	**q-value**
B*07:02	21.6	8.30	0.078	0.344
B*44:02	16.2	8.30	0.276	0.558
B*08:01	14.9	4.20	0.075	0.344
B*51:01	14.9	12.5	0.794	0.919
B*44:03	13.5	20.8	0.322	0.558
B*40:01	12.2	6.30	0.361	0.558
B*35:01	10.8	8.30	0.762	0.919
B*18:01	9.50	16.7	0.267	0.558
B*35:03	8.10	4.20	0.478	0.657
B*38:01	6.80	0.00	0.155	0.488
B*58:01	5.40	0.00	0.153	0.488
B*14:02	5.40	8.30	0.710	0.919
B*35:02	5.40	4.20	1.000	1.000
B*40:02	4.10	0.00	0.278	0.558
B*50:01	4.10	4.20	1.000	1.000
B*49:01	2.70	6.30	0.380	0.558
B*13:02	2.70	2.10	1.000	1.000
B*52:01	1.40	6.30	0.298	0.558
B*27:05	1.40	8.30	0.077	0.344
B*14:01	0.00	6.30	0.058	0.344
B*57:01	0.00	8.30	0.022	0.344

*Only alleles with a phenotypic frequency in the overall population >3% are represented in the table

Chapter 8

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Contribution of Immunological and Virological Factors to Extremely Severe Primary HIV Type 1 Infection

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Background. During acute human immunodeficiency virus (HIV) infection, high viral loads and the induction of host immune responses typically coincide with the onset of clinical symptoms. However, clinically severe presentations during acute HIV type 1 (HIV-1) infection, including AIDS-defining symptoms, are unusual.

Methods. Virus isolates were tested for clade, drug susceptibility, coreceptor use, and growth rate in 2 case reports of sexual transmission of HIV-1 infection. Human leukocyte antigen (HLA) genotype was determined, and HIV-1–specific cytotoxic T lymphocyte responses to an overlapping peptide set spanning the entire HIV clade A and clade B proteome were assayed.

Results. The viruses isolated in the 2 unrelated case reports of severe primary HIV-1 infection showed R5/X4 dual-mixed tropism, belonged to clade B and CRF02-AG, and were highly replicative in peripheral blood mononuclear cell culture. Impaired humoral responses were paralleled by a profound absence of HIV-1–specific cytotoxic T lymphocyte responses to the entire viral proteome in the 2 case reports. In 1 case report for which the virus source was available, there was a remarkable HLA similarity between the 2 patients involved in the transmission event, because 3 of 4 HLA-A and HLA-B alleles had matched HLA supertype for both patients.

Conclusions. The data suggest that concurrence of viral and host factors contributes to the clinical severity of primary HIV-1 infection and that patients infected with highly replicative, dual-tropic viruses are more prone to develop AIDS-defining symptoms during acute infection if they are unable to mount humoral and cellular HIV-1–specific immune responses. The presence of concordant HLA supertypes might facilitate the preferential transmission of HLA-adapted viral variants, further accelerating disease progression.

Symptoms of acute retroviral syndrome typically coincide with high-level viremia and the induction of the host's initial adaptive immune response [1, 2]. However, clinically severe presentations during acute HIV-1 infection, including AIDS-defining symptoms, are considered to occur infrequently [3]. Furthermore, epidemiological studies have revealed that, in the absence of treatment, in <0.5% of HIV-1–infected individuals, the infection progresses to AIDS within 1 year after primary infection [4]. A complex interplay between multiple viral and host factors is most likely to be involved in accelerating disease progression. Among these myriad factors, CXCR4 tropism has been associated with high virulence [5]. Moreover, HLA class I concordance between individuals and the inability to elicit specific cytotoxic T lymphocyte (CTL) responses have been suggested to increase the rate of transmission of HIV-1 infection and disease progression [6, 7].

In our study, we investigated the immunological and virological factors that contributed to the development of AIDS-defining pathogenesis in 2 independent case reports of unusually severe, acute, sexually transmitted HIV infection. The clinical and diagnostic outcomes are shown in figure 1.

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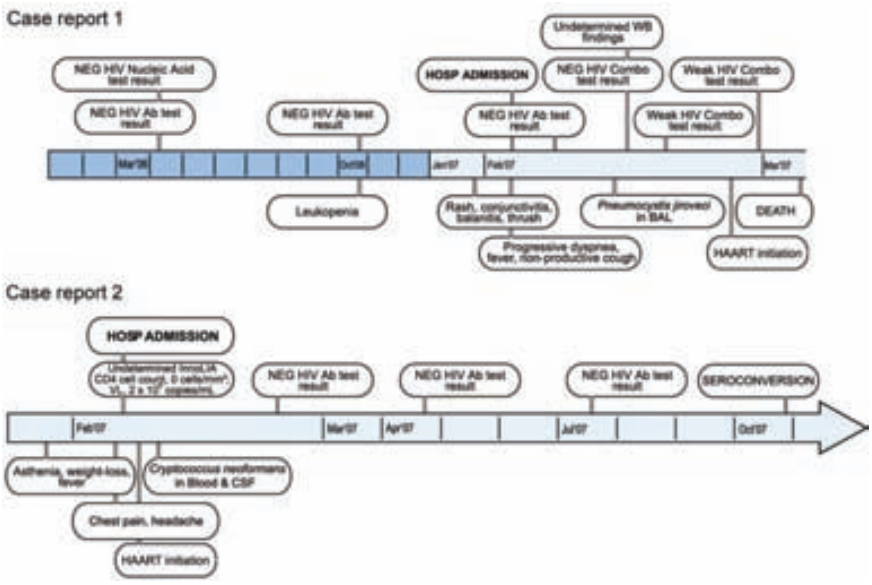

Figure 1. Description and outline of clinical and diagnostic outcomes in the 2 case reports of sexual transmission of severe primary HIV-1 infection in this study. Ab, antibody; HOSP, hospital; NEG, negative; WB, Western blot.

PATIENTS, MATERIALS, AND METHODS

The study participants provided written informed consent to participate in this study, which was approved by the institutional review boards of the hospitals where the participants received medical care. HIV-1 was isolated from the patients' PBMCs, and the viral stocks were titrated in TZM-bl cells [8]. Coreceptor use of primary HIV-1 isolates was assessed by infection of U87.CD4 cells expressing either CCR5 or CXCR4 [9]. Syncytia induction was determined in vitro in MT-2 cells. CCR5 was genotyped in genomic DNA extracted from cryopreserved PBMCs to detect the Δ32 deletion. The growth rate of the viral isolates was determined by infecting phytohemagglutinin-stimulated donor PBMCs [10]. To assess the presence of drug resistance–associated mutations, we sequenced the HIV protease region (codons 1–99) and reverse-transcriptase region (codons 40–247) from a plasma sample obtained before the initiation of antiretroviral therapy.

To determine whether the index patient in case report 2 harbored the same virus as the suspected source patient, viral RNA was extracted from plasma. The *pol* (protease and first 235 codons of the reverse transcriptase) and *env* (C2 to V5 regions) genes were sequenced [10, 11]. In addition, a total of 46 molecular clones encompassing the *env* gene were used to estimate diversity in the plasma viral RNA for the source and index patients [11]. Sequence alignments were obtained using Sequencher, version 4.6 (Gene Codes), and ClustalW and were manually edited in the regions of variable length. Genetic distances and evolutionary rates were computed using a Kimura 2–parameter model. Neighbor-joining phylogenetic trees of each patient's *pol* and *env* sequences were constructed using MEGA3. The reliability of phylogram clustering was assessed by bootstrapping analyses. Coreceptor use was inferred from *env* clonal sequences with use of phenotype prediction tools [12].

HLA class I and class II genotypes were identified by high-resolution sequencing in an approved clinical laboratory. HLA class I supertype assignment was based on functional classification for the many different 4-digit, high-resolution HLA alleles that overlap in their peptide-binding specificities [13].

Cellular immunity to HIV and Epstein-Barr virus (EBV) was assessed by IFN-γ enzyme-linked immunospot assays. T cell responses to an overlapping peptide set spanning the entire HIV clade A and clade B protein sequence were detected [14]. In addition, optimal epitopes known to be presented by the patients' HLA class I alleles were either included in their clade-specific consensus version or based on sequence variants identified in the index or source patient. To assess general immune reactivity, 3 peptide pools containing a previously described set of EBV-derived optimal CTL epitopes were also tested [15]. Positive responses were determined on the basis of specific cutoffs that are defined elsewhere [16].

CASE REPORTS

Case report 1. A 26-year-old Spanish man with a history of recurrent conjunctivitis and mild bronchial asthma who reported having had only 1 heterosexual partner during the pre-

vious 2 years was admitted to a hospital in Ferrol, Spain, for progressive dyspnea, tachypnea, tachycardia, fever, and nonproductive cough. Four months before hospital admission, the patient had received a diagnosis of leukopenia but had negative results of HIV serological examination. Four weeks before hospital admission, he developed maculopapular rash, conjunctivitis, balanitis, and oral thrush. At hospital admission, laboratory testing revealed leukocytosis, bilateral alveolar-interstitial lung infiltrates, and severe hypoxia. Two days later, he fulfilled the criteria for acute respiratory distress syndrome and required orotracheal intubation, mechanical ventilation, and broad-spectrum antibiotic therapy. An EIA was weakly positive for HIV-1 antibodies, and the Western blot results were undetermined. *Pneumocystis jiroveci* was detected in a bronchoalveolar lavage specimen, and *Candida albicans* was isolated from oropharyngeal exudates. The patient's plasma HIV-1 RNA level was 31,600 copies/mL, and his $CD4^+$ T cell count was 108 cells/mm^3. Antiretroviral therapy with tenofovir, emtricitabine, lopinavir-ritonavir, and enfuvirtide was initiated. The acute respiratory distress syndrome was refractory to treatment, and the patient died of multiorgan failure 3 weeks after hospital admission.

Case report 2. Case report 2 involved an index patient and the presumed source patient. The index patient was a 15-year-old girl from Ecuador who was admitted to a hospital in Badalona, Spain, with fever, severe headache, fatigue, malaise, severe odynophagia, and a 4-day history of continuous retrosternal chest pain. She had a 1-month history of asthenia, a 2-kg weight loss, and fever. Her previous medical history was unremarkable. The index patient reported having had only 1 regular sexual partner during the previous 2 years. At hospital admission, she was febrile and had enlarged bilateral cervical lymph nodes, oral thrush, and soft, nonswollen hepatomegaly (1.5 cm). *C. albicans* was subsequently isolated from oropharyngeal and vaginal exudates. *Haemophilus influenzae* (biotype 2) was isolated from a sputum sample. Serological testing was reactive for IgG against cytomegalovirus and *Toxoplasma gondii.* An EIA for HIV-1 antibodies was weakly reactive, and a line immunoassay for detection of IgG antibodies against HIV-1/2 disclosed only 1 band for gp41 in 2 independent samples obtained 5 days apart. The index patient's HIV-1 RNA level was 2×10^6 copies/mL, and her $CD4^+$ T cell count was 0 cells/mm^3. The patient initiated therapy with tenofovir, emtricitabine, and lopinavir-ritonavir. Three days later, she developed cognitive deficits. Cranial MRI revealed ischemic and hemorrhagic supratentorial and infratentorial lesions in the cortical-subcortical interface and basal ganglia. *Cryptococcus neoformans* was isolated from blood and CSF cultures. The patient had a favorable clinical course with appropriate treatment. Two months later, her HIV RNA level was undetectable, and her $CD4^+$ T cell count was 51 cells/mm^3 ($CD4^+$ T cell percentage, 3%). Despite repetitive antibody testing, complete seroconversion did not occur until 9 months after initial presentation.

The index patient's partner, presumably the source patient, was a 23-year-old man who was also from Ecuador. He confirmed having had several sexual partners, but he had never been tested for HIV-1 infection. EIA and line immunoassay were reactive for HIV-1. His plasma HIV-1 RNA level was 40,000 copies/mL, and his $CD4^+$ T cell count was 32 cells/mm^3 (4%). The source patient initiated therapy with tenofovir, emtricitabine, and lopinavir-ritonavir. Nine months later, his HIV RNA level was undetectable, and his $CD4^+$ T cell count was 130 cells/mm^3.

RESULTS

Case report 1. Laboratory assessment of the patient indicated a change in HIV-1 antibody reactivity near the time of presentation. The results of 3 previous assays (HIV-1 antibody–, nucleic acid–, and antigen-based assays) performed within 9 months before presentation were negative. Antibody and antigen tests and Western blots became partially reactive, and plasma HIV-1 RNA was present at the time of presentation, suggesting HIV-1 primary infection (figure 1 and table 1).

The replication-competent virus isolated from PBMCs was able to infect and replicate in both CCR5 and CXCR4-U87.CD4 cells, as concluded from the p24 antigen production and the formation of syncytia in the cell cultures (figures 2*A* and 2*B*). The patient did not have a Δ32 genotype in the CCR5 chemokine receptor gene that might have explained an early selection of CXCR4-tropic viruses [17]. The production of p24 antigen in growth kinetics cultures of donor PBMCs was similar to the production seen with the laboratory-adapted viral strain $HIV\text{-}1_{NL4\text{–}3}$ (figure 2*C*). Phylogenetic analyses with boot-scanning methods for the genetic subtyping of *pol* indicated the presence of a subtype B virus. The HIV-1 genotype had no drug resistance–associated mutations. The results of HLA typing are shown in table 1.

Case report 2. Clinical symptoms and analytical results for the index patient were consistent with a diagnosis of advanced HIV-1 infection and AIDS. However, the patient denied HIV risks other than sexual contact with her partner during the previous 2 years. The patient's mother tested negative for HIV-1 infection, thus excluding potential vertical transmission. Moreover, the viruses isolated from the source and index patients were similar both phenotypically and genotypically (figure 3 and table 1). Laboratory assessment of the index patient's original sample provided clear reactivity data on the presence of HIV-1 antigens, but despite high levels of immunoglobulins, antibody-based tests showed partial reactivity, indicating a lack of HIV-1–specific antibodies (figure 1 and table 1).

Bootstrap analysis of *pol* and *env* sequences from the index and source patients revealed values of ⩾99% in 1000 replicates

Table 1. Laboratory assessment of the patients involved in 2 case reports of sexual transmission of severe HIV-1 infection.

Variable	Case report 1: index patient	Case report 2	
		Index patient	Source patient
Plasma HIV-1 RNA level, copies/mL	32×10^4	2×10^6	4×10^4
$CD4^+$ T cell count, cells/mm^3	108	0	33
$CD4^+$ T cell percentage	27	0	4
$CD8^+$ T cell count, cells/mm^3	Not performed	203	481
$CD8^+$ T cell percentage	Not performed	59	59
Result of standard Ab HIV test	Negative	Weak[a]	Positive
HIV Western blot findings	Result undetermined	Result undetermined	Positive
Result of nucleic acid and/or viral load test	Positive	Positive	Positive
Plasma p24 level, pg/mL	6.3	72.6	12.5
Viral subtype	B	AG	AG
Drug resistance genotype			
Protease	K20M and M36I	L- 10V, I13V, G16E, M36I, H69K, and L89I	L- 10V, I13V, G16E, K20I, M36I, H69K, and L89I
Reverse transcriptase	None	None	None
Coreceptor use[b]	R5/X4	R5/X4	R5/X4
CCR5 Δ32 genotype	WT/WT	WT/WT	WT/WT
HLA alleles			
Class I[c]	A*0201 (A2), A*1101 (A3) B*3503 (B7), B*4001 (B44) Cw*0304, Cw*0401	A*0201 (A2), A*0301 (A3) B*0702 (B7), B*1801 (B44) Cw*0702, Cw*1203	A*6802 (A2), A*6801 (A3) B*5101 (B7), B*5301 (B7) Cw*0401, Cw*1502
Class II	DRB1*0701, DRB1*1302 DQB1*0202, DQB1*0604	DRB1*0405, DRB1*1201 DQB1*0301, DQB1*0302	DRB1*0101, DRB1*0701 DQB1*0202, DQB1*0501

NOTE. Ab, antibody; WT, wild type.

[a] Different HIV antibody tests provided nonreactive or weakly reactive results.

[b] Viruses from all of the patients were syncytia-inducing viruses.

[c] Supertypes are shown in parentheses (A*68 and B*53 [increased susceptibility] and B*51 [increased protection]).

(figure 3*A* and 3*B*), indicating that sequence clustering was unlikely to have occurred by chance. The genetic distance between the *pol* sequences from the index and source patients was <0.1%, whereas the mean genetic diversity between randomly selected sequences from local, epidemiologically unrelated HIV-infected individuals with the CRF02-AG subtype was 3.0%. The high degree of similarity between viral sequences indicates a likely viral transmission from one patient to the other. Clonal analysis of *env* sequences indicated that all sequences from the index patient were closely related (mean diversity, 1.8%), and the source patient's sequences had a mean diversity of 2.4% (figure 3*C* and 3*D*).

After 5 days of culture, the viruses isolated from the source and index patients were able to infect and replicate in both CCR5 and CXCR4-U87.CD4 cells, as indicated by the p24 antigen production and the formation of syncytia in the cell cultures (figure 3*E* and 3*F*). Neither of the individuals showed a Δ32 genotype in the CCR5 chemokine receptor gene. Phenotypic inference of the V3 amino acid sequence in multiple clones from each individual suggests that all clones from the index patient could use CXCR4 (R5X4 tropism) for viral entry, whereas the source patient had clones that could use only CCR5 and clones that could use CXCR4 (R5X4 tropism) (figure 3*C*). The production of p24 antigen in growth kinetics cultures in PBMCs was equal in the 2 viral isolates and comparable to that in the laboratory-adapted viral strain HIV-1_{NL4-3} (figure 3*G*).

Genetic subtyping of the *gag, pol,* and *env* genes in the patients' viruses indicated that both patients had the AG circulating recombinant form 02 (CRF02-AG). Drug-resistance genotyping revealed no drug resistance–associated mutations in the reverse transcriptase. Several polymorphisms were detected in the protease gene; this might have been associated with possible tipranavir resistance in non–subtype B viruses (table 1).

Cellular immune responses. The patient in case report 1 showed a single weak response against 1 overlapping peptide that was not subsequently observed in the reconfirmation test and only a borderline response to 1 EBV-peptide pool. This atypical lack of EBV-specific CTL responses suggests a widespread impairment of the ability to mount adequate CTL responses [16].

The index and source patients in case report 2 expressed HLA class I alleles that were highly related. In fact, 3 of the 4 HLA-A and -B alleles had matched HLA supertype for both 2 patients (table 1). Because the transmission of escape mutants

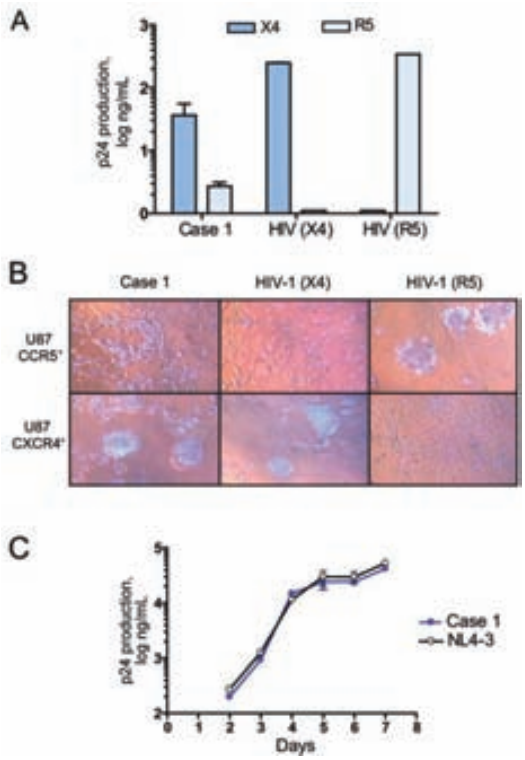

Figure 2. Virological data for case report 1. Viral coreceptor use based on p24 production *(A)* and syncytia formation *(B)* in U87.CD4 cells expressing either CXCR4 or CCR5. Control viral strains HIV-1_{NL4-3} (CXCR4-tropic, syncytia inducer) and HIV-1_{NFN-SX} (CCR5-tropic, nonsyncytia inducer) were included in both assays. *C,* Viral replication growth rates in phytohemagglutinin-stimulated primary donor PBMCs infected with the patient's viral isolate. The laboratory-adapted HIV-1_{NL4-3} reference strain was grown in parallel. One of 2 representative experiments with PBMCs from 2 different donors is shown.

arising in the source patient may have prevented the induction of an effective T cell response in the index patient, the cellular immune response to the entire viral proteome was assessed in both patients at 1 and 9 months after the transmission event. The analyses included 2 comprehensive sets of overlapping peptides spanning HIV clade A and clade B consensus sequences, as well as autologous peptide variants (from the index and source patients) of optimally defined epitopes presented by the patients' HLA types (table 2). The source patient showed weak responses to 3 different regions of the virus (table 3), which represented an overall weak response rate, compared with a median of 17 responses among >300 previously tested patients with chronic HIV infection (authors' unpublished data) [14]. The index patient showed an even weaker HIV-specific T cell response to only a single peptide, which was detected before the patient received treatment but was subsequently lost 9 months after infection. Importantly, the index patient was able to mount a T cell response to a peptide pool containing EBV-derived CTL epitopes, which indicated that the absence of HIV-specific T cells was not attributable to poor cell viability or a general immune incompetence in this individual.

Although some responses to autologous sequence variants that were not tested may exist, the data are in line with a remarkable absence of HIV-specific T cell immunity in both the patient in case report 1 and the index patient in case report 2. This absence of immunity may be related to the extraordinarily fast disease progression in these individuals.

DISCUSSION

The interplay between the viral and host factors influencing accelerated disease progression is complex and poorly understood. The 2 temporarily coincident case reports described here suggest immediate progression to AIDS from primary HIV-1 infection after sexual transmission. In both cases, the diagnosis of primary HIV-1 infection was supported by nucleic acid– and antigen-based screening tests, with an evolving antibody pattern. The patient in case report 1 tested negative for HIV several times before presentation, and subsequent HIV-1 Western blot was only partially reactive. The index patient in case report 2 lacked previous negative test results but presented with a very high plasma HIV RNA level, which is consistent with acute HIV infection [18]. In addition, HIV-1–specific antibody tests were only partially reactive and did not become positive until 9 months after the initiation of antiretroviral treatment. Although detection of HIV-specific antibodies after the onset of symptoms of primary infection can take 5–15 days [19], complete seroconversion may occasionally be delayed until 12 months after identification of infection by antigen testing, when virological control has been achieved with effective antiretroviral therapy [19, 20]. The fact that serum levels of IgG, IgM, and IgA (in the index patient in case report 2) were within the reference range or higher suggests polyclonal B cell activation [21]. Moreover, positive IgG responses to cytomegalovirus, *T. gondii,* and hepatitis A virus indicate the ability of antibodies to maintain an appropriate response against microorganisms that cause persistent latent infection. Although plasma viral load had greatly decreased with treatment, $CD4^+$ T cell recovery increased slowly from total absence at presentation, which could have delayed HIV seroconversion.

The diversity of the viral population in HIV-1 *env* increases in parallel with divergence at a rate of 1% per year for a few years after seroconversion, before reaching a peak and then leveling off or decreasing [22]. Nevertheless, the rates of diversity are higher among patients who have a sharp decrease in their CD4 T cell count [23]. In case report 2, the mean HIV-1 diversity was lower in the index patient than it was in the source patient, indicating that viral evolution took longer in the latter. Although viral diversity tends to decrease in the later

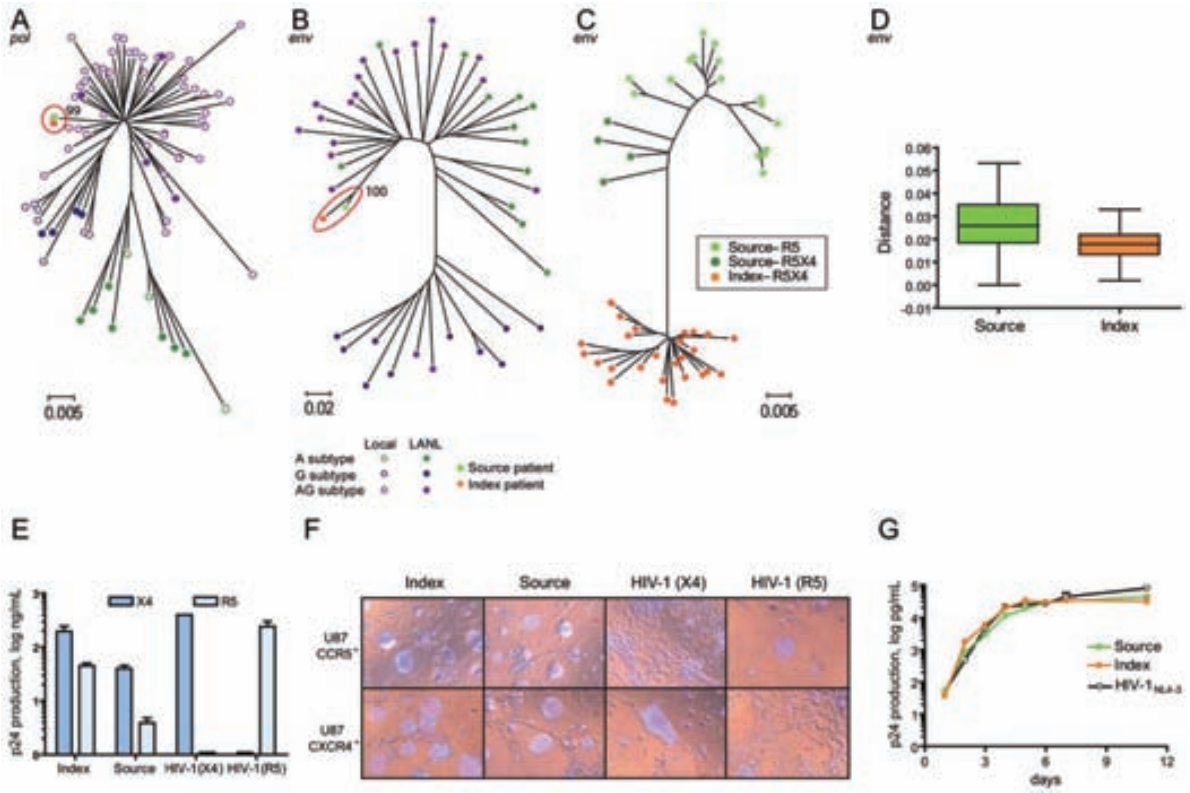

Figure 3. Virological data for case report 2, including data on samples from the source patient and index patient. Neighbor-joining phylograms of *pol (A)* and *env (B)* sequences derived from viral RNA in plasma are shown. The scale for the genetic distance is based on the Kimura 2-parameter method. *C,* Phylogenetic analysis of the *env* C2V3 clonal sequences derived from the index and source patients' viral isolates, with different color patterns to represent the virtual tropism of each clone. *D,* Intrapatient diversity. Viral coreceptor use based on p24 production *(E)* and syncytia formation *(F)* in U87.CD4 cells expressing either CXCR4 or CCR5 are shown. Control viral strains HIV-1_{NL4-3} (CXCR4-tropic, syncytia inducer) and HIV-1_{NFN-SX} (CCR5-tropic, nonsyncytia inducer) were included in both assays. *G,* Viral replication growth rates in phytohemagglutinin-stimulated primary donor PBMCs infected with the patients' viral isolates. The laboratory-adapted HIV-1_{NL4-3} reference strain was grown in parallel. One of 3 representative experiments involving different PBMC donors is shown.

stages of infection, most of the genetic distances would remain >2% [22], thus supporting the direction of transmission in this pair and the theory of a very early presentation after viral transmission to the index patient.

The development of acute retroviral syndrome typically coincides with high-level viremia and the host's initial immune response. However, these 2 case reports reveal primary HIV-1 infection with unusually severe clinical symptoms. Other reports have described severe presentations during primary HIV-1 infection, including acute myopericarditis, renal failure, acute liver failure, and opportunistic infections [24–27], but viral and host factors have not been addressed in detail.

In both case reports, the virus isolated from the patients' PBMCs was able to use CCR5 and/or CXCR4 as entry coreceptors and to replicate very efficiently in phytohemagglutinin-stimulated donor PBMCs. These data indicate that both viral isolates were either dual-tropic viruses or a mixed population of CCR5-tropic and CXCR4-tropic viruses with high replication capacity. This observation would suggest that the transmitted virus had the ability to deplete $CCR5^+$ and $CXCR4^+/CD4^+$ T lymphocytes, which may help to explain the total loss of the $CD4^+$ T cell population and rapid clinical progression observed in the index patient at the time of transmission. Infection with dual-tropic HIV-1 variants in injection drug users has been associated with an immediate and rapid decrease in total T cell count and progression to AIDS within 4 years after the estimated time of infection [28]. Furthermore, patients who experienced seroconversion of $CCR5^{\Delta 32/\Delta 32}$ and showed the uncommon pattern of early syncytia-inducing virus and rapid decrease in $CD4^+$ T cell count had a uniformly high viral load and dual-tropic coreceptor use [29]. A link between the detection of syncytia-inducing variants and a rapid decrease in $CD4^+$ T cell count in vivo has already been established [30].

Despite the fact that the characterized viral subtype CRF02-

Table 2. Optimal epitopes showing sequence diversity between viruses from the source and index patients on case report 2.

Epitope	Protein	HXB2 position	Reference	Sequence
A02 AL9	Vpr	59–67	con B	AIIRILQQL
			source/index	AIIRILQQL
			ConAG	AIIRILQQL
A02 FK10	Gag	70–79	con B	FLGKIWPS**Y**K
			source/index	FLGKIWPS**H**K
			ConAG	FLGKIWPS**Y**K
A02 GT9	Vpr	41–49	con B	GLGQHIY**E**T
			source	GLGQHIY**N**T
			index	GLGQHIY**D**T
			ConAG	GLGQHIY**E**T
A02 SL9	Gag	77–85	con B	SL**Y**NT**V**ATL
			source/index	SL**F**NT**I**ATL
			ConAG	SL**F**NT**I**ATL
A02 RI9	Vpr	62–70	con B	RILQQLLF**I**
			source/index	RILQQLLF**T**
			ConAG	RILQQLLF**V**
A02 RI10	Env	311–320	con B	**R**GPG**RA**F**VTI**
			source/index	**I**GPG**QT**F**YTI**
			ConAG	**I**GPG**QT**F**YAT**
A02 YV9	Pol	127–135	con B	YTAFTIPS**V**
			source/index	YTAFTIPS**L**
			ConAG	YTAFTIPS**V**
A03 AK9	Pol	158–166	con B	AIFQ**S**SMTK
			source/index	AIFQ**A**SMTK
			ConAG	AIFQ**A**SMTK
A03 RK9	Gag	20–28	con B	RLRPGGKK**K**
			source/index	RLRPGGKK**Q**
			ConAG	RLRPGGKK**K**
A03 RY10	Gag	20–29	con B	RLRPGGKK**K**Y
			source/index	RLRPGGKK**Q**Y
			ConAG	RLRPGGKK**K**Y
A03 TN10	Vpr	19–28	con B	TLELLEELK**N**
			source/index	TLELLEELK**H**
			ConAG	TLELLEELK**H**
A68 DL9	Pol	30–38	con B	DTVLE**EWN**L
			source/index	DTVLE**EIN**L
			ConAG	DTVLE**IWL**L
A68 IV9	Pol	3–11	con B	ITLWQRP**L**V
			source/index	ITLWQRP**V**V
			ConAG	ITLWQRP**L**V
A*6801 DR11	Vpr	52–62	con B	DTW**A**GV**E**AIIR
			source	DTW**E**GV**M**AIIR
			index	DTW**E**GV**V**AIIR
			ConAG	DTW**E**GV**E**AIIR
A*6802 EV10	Vpr	48–57	con B	**E**TYGDTW**A**GV
			source	**N**TYGDTW**E**GV
			index	**D**TYGDTW**E**GV
			ConAG	**E**TYGDTW**E**GV
B07 RI10	Env	298–307	con B	RP**N**NNTRK**SI**
			source	RP**S**NNTRK**NI**
			index	RP**N**NNTRK**GV**
			ConAG	RP**N**NNTRK**SV**
B07 TL9	Gag	48–56	con B	TPQDLN**T**ML
			source/index	TPQDLN**M**ML
			ConAG	TPQDLN**T**ML

(continued)

Table 2. ***(Continued.)***

Epitope	Protein	HXB2 position	Reference	Sequence
B18 FK10	Gag	161–170	con B	FRDYVDRF**Y**K
			source/index	FRDYVDRF**F**K
			ConAG	FRDYVDRF**F**K
B51 LI9	Env	416–424	con B	L**P**CRIKQII
			source/index	L**Q**CRIKQII
			ConAG	L**P**CRIKQII
B51 TI8	Pol	128–135	con B	TAFTIPS**I**
			source/index	TAFTIPS**L**
			ConAG	TAFTIPS**I**
B53 QW9	Gag	176–184	con B	QA**S**QEVK**N**W
			source	QA**T**QEVK**H**W
			index	QA**T**QEVK**N**W
			ConAG	QA**T**QEVK**N**W
B53 TL9	Gag	48–56	con B	TP**Y**D**I**N**Q**ML
			source/index	TP**Q**D**L**N**T**ML
			ConAG	TP**Y**D**I**N**Q**ML
X-GL12	Vpr	9–20	con B	GPQREP**H**NEWTL
			source/index	GPQREP**F**NEWTL
			ConAG	GPQREP**F**NEWTL

NOTE. Changes in the sequences are represented by boldface type.

AG in case report 2 is rather unusual in our area (1.1% of *pol* sequences tested for antiretroviral resistance during 1999–2007), it is still the second most common non-B subtype. CRF02-AG is the predominant and most rapidly spreading HIV strain in West Africa and western Central Africa [31, 32], which raises concerns about its superior replication fitness and/or transmission efficiency. In fact, primary HIV-1 CRF02-AG isolates from Cameroon exhibited higher ex vivo replicative fitness than did subtype A and G viruses from the same geographic region [33, 34]. These observations are consistent with the high replication rate that we observed in primary phytohemagglutinin-stimulated PBMCs, although we compared these rates with the rate of replication of the laboratory-adapted subtype-B HIV-1_{NL4-3} strain.

In our case reports, concurrent host factors may have also contributed to higher susceptibility to HIV-1 infection or disease progression. For example, specific HLA haplotypes have been proposed as an important risk factor in this context [35]. Among these, HLA-B*35, which is in high linkage disequilibrium with HLA-Cw*04, has been consistently associated with rapid progression to AIDS [36–38]. Specifically, the allele HLA-B*3503, present in the patient in case report 1, has been reported to increase the risk of progression to AIDS by 2.7-fold (95% CI, 1.7–4.3; $P < .001$) [39]. In case report 2, the source patient expressed the HLA-A*68, HLA-B*53, and HLA-Cw04 alleles, which have been associated with rapid disease progression [35]. Although, none of the alleles in the index patient have been associated with accelerated disease progression, the donor and recipient expressed 3 of 4 HLA-A and HLA-B alleles that were in the same HLA supertypes (i.e., clusters of functionally related, 4-digit, high-resolution HLA class I alleles) [13]. This may have facilitated the transmission of viruses with CTL escape mutations, thus diminishing the number of epitopes recognized in the newly infected individual [6, 7, 40, 41]. Remarkably, optimal epitope variants representing autologous sequence diversity did not elicit a response, which suggests effective CTL escape (table 2). This hypothesis would fit with the fact that the index patient in case report 2 showed almost complete absence of HIV-1–specific CTL responses; this is rather unusual during primary HIV-1 infection. In a previous study, only 1 of 5 patients who presented with primary HIV-1 infection showed absence of precursor CTL specific for cells expressing viral proteins [1]. Another study involving acute and early HIV-infected patients reported a slightly higher breadth and magnitude of HIV-1–specific CTL responses [42]. However, that study used a less comprehensive pool of overlapping peptides, and contrary to our case reports, in which there was a persistent absence of responses at month 9, CTL responses increased after 6–12 months of treatment. Although we could not identify the source patient in case report 1, the lack of HIV-1–specific CTL responses in the index patient might allow us to speculate a potential HLA class I concordance at transmission. Nonetheless, the inability to elicit HIV-1–specific CTL responses at the time of primary infection was paralleled in these 3 patients with AIDS-defining pathogenesis and severe clinical presentation. Although HIV-1–specific CTL responses have been considered to be a crucial factor in HIV disease progression, we had limited experimental and clinical evidence of the detrimental effect that the inability to elicit these responses might have in symptomatic primary HIV-1 infection [1, 43]. Moreover, the coincident inability in these 2 case reports for the patients to mount an effective adaptive immune re-

Table 3. Cellular immune responses in patients involved in 2 case reports of sexual transmission of severe primary HIV-1 infection.

Patient, peptide	Protein	HXB2 position	Sequence	SFC per 1×10^6 PBMCs
Case report 1 (index patient): OLP 83 con B	Nef	118–135	**tQGYFPDWQNY**TPGPGIRY	47
Case report 2				
Source patient				
OLP 42 con A	Gag	172–189	LRAE**QATQEVKGW**MTETL	41
OLP 42 con B			LRAE**QASQEVKNW**MTETL	72
B53 QW9 con B	Gag	176–184	**QASQEVKNW**	97
B53 QW9 index/con A			**QATQEVKNW**	97
OLP 84 con A	Nef	126–143	NYTPGPGIRYPLCFGWCF	10
OLP 84 con B			NYTPGPGIRYPLTFGWCF	103
OLP 223 con A	Pol	583–600	QLEKDPIAGAETFYVDGA	52
OLP 223 con B			QLEKEPIVGAETFYVDGA	21
OLP 224 con A&B			GAETFYVDGAANRETKL	72
Index patient				
OLP 296 con A&B	Env	52–69	**LFCASDAKAY**DTEVHNVW	18
A24 LY10 con B&A/index	Env	52–61	**LFCASDAKAY**	24

NOTE. Sequence changes between peptides are underlined, and optimal peptides are shown in boldface type. con, Consensus; OLP, overlapping peptide; SFC, spot-forming cells.

sponse against HIV-1, albeit not to other pathogens, might be a consequence of a potential defect in the innate immunity. Clearly, further studies involving these and other patients with accelerated disease progression will be needed to address these factors.

In conclusion, we describe 2 case reports of sexual transmission of highly replicative, dual-tropic HIV-1 of subtypes B and CRF02-AG that resulted in an aggressive clinical progression to severe symptomatic AIDS in young patients. Adaptive cellular and humoral immune responses in the host might have simultaneously failed to control the virus.

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Research article

Comparative transcriptomics of extreme phenotypes of human HIV-1 infection and SIV infection in sooty mangabey and rhesus macaque

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High levels of HIV-1 replication during the chronic phase of infection usually correlate with rapid progression to severe immunodeficiency. However, a minority of highly viremic individuals remains asymptomatic and maintains high CD4$^+$ T cell counts. This tolerant profile is poorly understood and reminiscent of the widely studied nonprogressive disease model of SIV infection in natural hosts. Here, we identify transcriptome differences between rapid progressors (RPs) and viremic nonprogressors (VNPs) and highlight several genes relevant for the understanding of HIV-1–induced immunosuppression. RPs were characterized by a specific transcriptome profile of CD4$^+$ and CD8$^+$ T cells similar to that observed in pathogenic SIV-infected rhesus macaques. In contrast, VNPs exhibited lower expression of interferon-stimulated genes and shared a common gene regulation profile with nonpathogenic SIV-infected sooty mangabeys. A short list of genes associated with VNP, including *CASP1*, *CD38*, *LAG3*, *TNFSF13B*, *SOCS1*, and *EEF1D*, showed significant correlation with time to disease progression when evaluated in an independent set of CD4$^+$ T cell expression data. This work characterizes 2 minimally studied clinical patterns of progression to AIDS, whose analysis may inform our understanding of HIV pathogenesis.

Introduction

HIV infection leads to severe immunodeficiency in most infected subjects, in an average of 10 years; however, there are marked departures from this estimate. Attention has been directed at understanding the determinants of nonprogressive disease, as exemplified by the clinical course of long-term nonprogressors and of elite controllers (1–3). The other extreme of the spectrum of disease — rapid progression — has been the subject of much less research. Rapid progressors (RPs) can be defined by a number of criteria — generally including progressive immunosuppression soon after seroconversion and, in many cases, high levels of viremia (4, 5). Limited data suggest that the concurrence of viral and host factors contributes to the severity of early disease (6). There are, however, few such individuals in clinical cohorts — the main limitations for prospective recruitment are the need to identify patients with a known date of infection (seroconverters), and the short window of clinical observation before antiretroviral treatment is initiated. These constrain the availability of relevant biological material for study.

There are also very rare individuals that can tolerate very high viral loads, comparable to those of RPs, while maintaining stable CD4$^+$ T cell counts for many years in the absence of treatment. Choudhary et al. (7) described 3 HIV-infected individuals with long-term asymptomatic disease who maintained stable CD4$^+$ T cell counts and low levels of immune activation, despite viral replication in the range of 10^4 to 10^5 HIV-1 RNA copies per ml of plasma. This profile of tolerance of viral replication is reminiscent of the pattern of SIV infection in the natural host. The importance of such model for the understanding of HIV/AIDS pathogenesis has been underscored by

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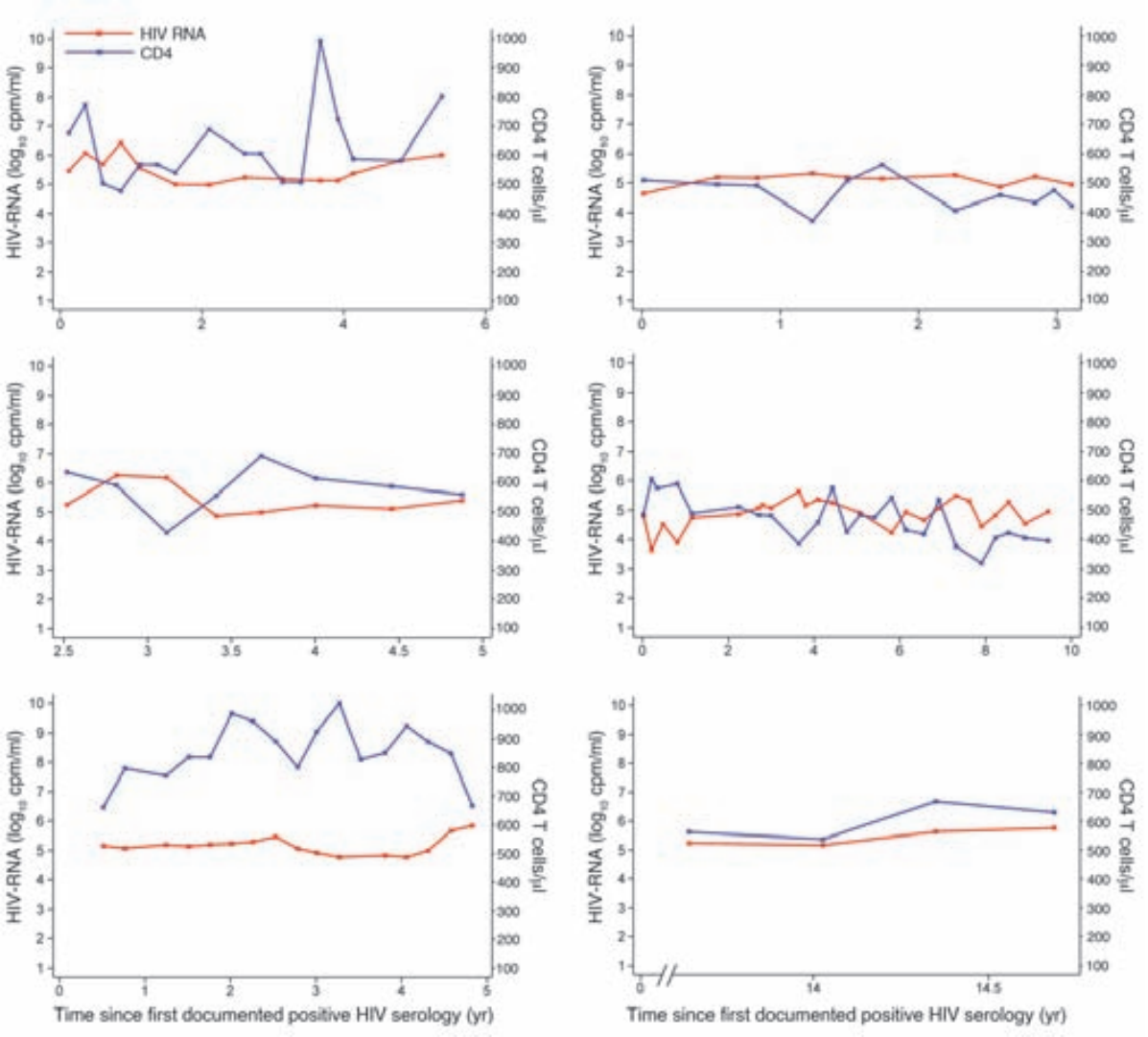

Figure 1
Individual viral loads and CD4+ T cell profiles of VNPs. Viremia is shown in red, and CD4+ T cell count is shown in blue.

studies in sooty mangabeys and in African green monkeys (8–12). Sooty mangabeys have nonprogressive disease despite chronic virus replication that is characterized by low levels of immune activation, while pathogenic SIV infection of rhesus macaques is associated with chronic immune activation. The consequences of immune activation include increased cell turnover, the skewing of lymphocytes toward more activated and differentiated subpopulations, and the induction of cellular exhaustion, senescence, and low renewal potential (reviewed in ref. 13).

The first goal of the present study was to explore a set of standard criteria to identify HIV-infected individuals presenting those 2 distinct clinical patterns: rapid progression and the contrasting setting of nonprogressive disease, despite prolonged and very high levels of viremia (extreme viremic nonprogressors [VNPs]). We then used immunogenetic, genomic, and transcriptomic tools and biomarkers to identify differences between those extreme groups as well as exploring genomic patterns previously defined in comparative studies of SIV infection in the pathogenic and the nonpathogenic models of rhesus macaques and sooty mangabeys, respectively (8–10). The study revealed characteristic biomarker and transcriptome patterns and highlighted several genes of relevance for the understanding of pathogenesis of HIV-1–induced immunosuppression.

Results

Clinical and immunogenetic profiles. We identified 6 individuals that fulfilled strict clinical criteria of VNPs and had material available for analysis; plots of the infection course for each VNP individual are shown in Figure 1. We further identified 66 individuals who fulfilled the criteria of rapid progression and had materials available for study; the collective plot is shown in Figure 2. Notably, at

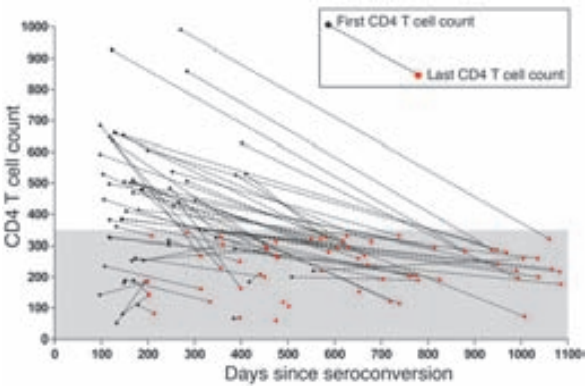

Figure 2
Evolution of T cell count in individuals with a profile of rapid progression. The first CD4+ T cell count determination (black symbols) and the last CD4+ T cell count determination (red symbols) (connected by dashed lines) in individuals, defined by the progression to fewer than 350 CD4+ T cells (denoted by the gray area) in fewer than 3 years after seroconversion. Only values beyond the 3-month window after seroconversion are considered.

the time of analysis, VNPs had higher levels of viral replication (set point HIV RNA, 5.4 $\log_{10}$ cp/ml; interquartile range [IQR], 5.1–5.5 $\log_{10}$ cp/ml]) compared with those of RPs (set point HIV RNA, 4.7 $\log_{10}$ cp/ml [IQR, 4.3–5.2 $\log_{10}$ cp/ml]). Transcriptome analysis also included 9 elite/viremic controllers (ECs) and 5 chronic progressors, as previously defined (5). Patient characteristics are detailed in Supplemental Table 1 (supplemental material available online with this article; doi:10.1172/JCI45235DS1).

The HLA and KIR alleles were determined in all individuals, compared across clinical groups and compared to the allele frequencies of 1,609 participants of the SHCS (Supplemental Figure 1). Protective alleles were underrepresented, and risk alleles were more common in RPs compared with the general population. In contrast to HLA alleles, there was no depletion of protective KIR alleles or KIR/HLA combinations in RPs (Supplemental Figure 2). HLA alleles of VNPs are shown in Supplemental Table 2. To determine whether any common variants of very large effect could be implicated in mediating rapid progression, the study was completed with a genome-wide association across an approximately 500,000-loci study that included 66 RPs and 757 participants of the SHCS. No SNPs reached genome-wide significance (Supplemental Figure 3 and Supplemental Table 3), likely due to the limited power to detect anything other than very large effect sizes. A previous genome-wide association study of rapid progression (4) identified 8 SNPs that passed the study-wide false discovery rate (FDR) cutoff of 25%. These failed confirmation in our study (Supplemental Table 4).

Transcriptome analysis in CD4+ T cells. To investigate differences at the transcriptome level between RPs and VNPs, we performed microarray analysis on purified CD4+ cells from 27 RPs, 5 VNPs, 5 chronic progressors, and 9 ECs (Supplemental Table 1B). RPs, with and without transcriptome analysis, were similar with regard to CD4+ T cell counts and HIV viral load. The median CD4+ T cell counts at baseline were 440 cells/μl (IQR, 350–506 cells/μl) and 382 cells/μl (IQR, 315–497 cells/μl) for those with and without transcriptome analysis, respectively; the median baseline HIV viral loads were 4.8 cp/ml (IQR, 4.1–5.5 cp/ml) and 4.9 cp/ml (IQR, 4.3–5.1 cp/ml). During follow-up, the median CD4+ T cell counts were 263 cells/μl (IQR, 197–313 cells/μl) and 223 cells/μl (IQR, 186–299 cells/μl), and median HIV viral loads were 4.8 cp/ml (IQR, 4.3–5.4 cp/ml) and 5.0 cp/ml (IQR, 4.4–5.2 cp/ml) ($P > 0.4$ for all comparisons). Thirteen (20%) individuals had an AIDS-defining event within 3 years of seroconversion.

Principal component analysis identified 4 outliers that were excluded from further analysis. Various parameters were assessed as covariates (clinical center, gender, age, CD4+ T cell viability and laboratory date, and microarray chip batch); we retained chip batch as a statistically significant covariate. To contrast specific patient profiles, we applied a Bayesian approach to the analysis of gene expression (14). Analysis of RPs versus ECs identified 14 differentially expressed genes at a FDR-adjusted *P* value of less than 0.05. Interferon-stimulated genes (ISGs) are well known to be upregulated in patients with progressive HIV disease. Consistent with this knowledge, 6 ISGs, *IFI44* (and its ligand *IFI44L*), *MX1*, *EIF2AK2*, *IFI6*, *LY6E*, *TRIM22*, were upregulated in RPs. Other upregulated genes included *SYNCRIP* that encodes a nuclear ribonucleoprotein (hnRNP-Q) associated with the *APOB* mRNA editosome complex that may modulate the posttranscriptional C to U RNA-editing *PRIC285* that encodes a helicase acting as a transcriptional coactivator for a number of nuclear receptors, *EPSTI1* and *MRPS18B*. Genes downregulated in RPs included *TRK1*, which encodes a kinase, and *FOXJ2*, a transcriptional activator. Next, we specifically searched genes uniquely associated with the VNP profile by contrasting this profile with that of RPs or chronic progressors. This analysis failed to identify FDR-adjusted differentially expressed genes.

Transcriptome analysis in CD8+ T cells. We also performed microarray analysis on purified CD8+ T cells derived from the same PBMC samples used for CD4+ T cell transcriptome analysis. Expression analysis was successfully completed for 25 RPs and 5 VNPs as well as 5 chronic progressors and 8 elite and viremic controllers (Supplemental Table 1B). No outliers were identified, and all samples progressed to further analysis. As above, we retained microarray chip batch as covariate in all definitive analyses. Using the same sensitive Bayesian approach as for the CD4+ T cell analysis (14), contrasting of RPs and ECs yielded 317 differentially expressed genes at a FDR-adjusted *P* value less than or equal to 0.05 (Supplemental Table 5). Among the 180 genes upregulated in RPs, prominent groups of genes included multiple members of the proteasome and interferon-induced immunoproteasome, ISGs, and cell cycle, cell division, and metabolic genes indicating cell proliferation (Supplemental Figure 4). No apparent mechanisms were deduced from the collective analysis of 137 genes downregulated in RPs by using EMBL Search Tool for the Retrieval of Interacting Genes/Proteins (STRING), Ingenuity Pathway Analysis 7.0 (IPA), and KEGG pathway analysis (see Methods). As for the CD4+ T cells, we specifically searched genes uniquely associated with the VNP profile by contrasting this profile with that of RPs or chronic progressors. Given power limitations, this analysis failed to identify FDR-adjusted differentially expressed genes. Thus, we proceeded to the analysis of specific pathways and of the genes identified in primate studies of nonpathogenic SIV infection (9, 10).

Analysis of genes of the interferon response. Recent publications (8–12) highlight a distinctive downregulation of the interferon response after SIV infection of natural host species, such as sooty mangabeys and African green monkeys. In contrast, SIV infection of the pathogenic models of rhesus or pig-tailed macaque is character-

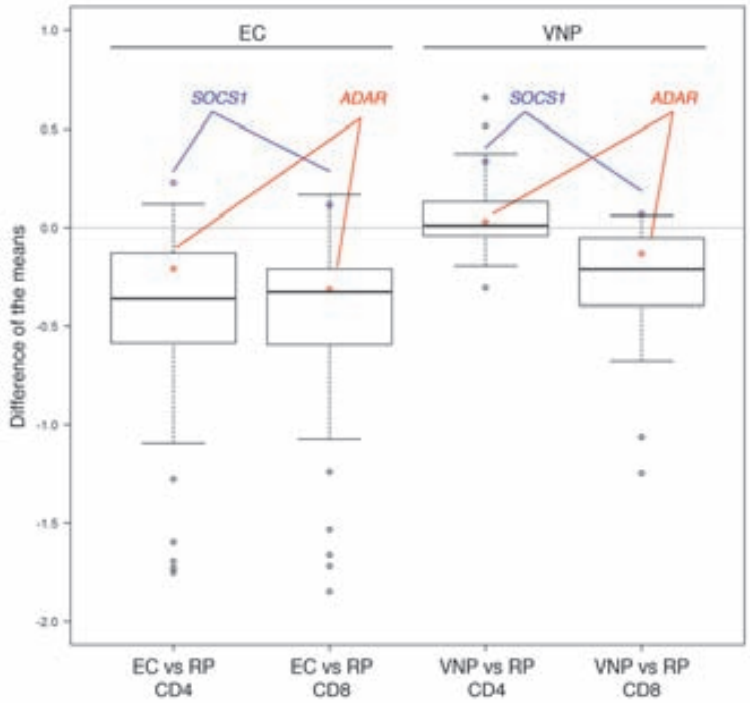

Figure 3
Analysis of differential expression of ISGs. Consistent with the primate natural infection model, a representative set of ISGs (n = 29) had lower expression levels in CD8+ T cells from VNPs than in those from RPs. The box-and-whisker plot indicates that the differences are more pronounced for the comparisons of ECs and RPs. The horizontal bars indicate the median values, the boxes indicate the 25th to 75th percentiles, and whiskers indicate extremes. Each dot represents the difference in expression value for a given gene across groups. The profiles of the inhibitor of interferon response, *SOCS1*, and of *ADAR* are highlighted (blue and red, respectively).

ized by persistence of deregulated interferon responses. Consistent with the primate model of natural infection, we observed a lower level of expression of ISGs (see Methods for the specific ISGs) in $CD8^+$ T cells of individuals with a VNP profile in comparison with that of individuals with a RP profile (Figure 3) (difference of the means, median –0.21 [IQR, –0.05 to –0.40]; paired t test, $P = 0.014$). However, these differences were not observed in $CD4^+$ T cells, (difference of the means, 0.01 [IQR, 0.13 to –0.04]; $P = 0.59$). As expected, more profound differences in expression of ISGs were found in the comparison between ECs and RPs (median –0.36 [IQR, –0.13 to –0.59], $P = 2.5 \times 10^{-5}$ in $CD4^+$ T cells, and median –0.33 [IQR, –0.21 to –0.59], $P = 3.8 \times 10^{-6}$ in $CD8^+$ T cells) (Figure 3). The expression of *SOCS1*, involved in a negative feedback loop in the regulation of signal transduction through the JAK/STAT5 pathway, was higher in $CD4^+$ T and $CD8^+$ T cells of VNPs and ECs compared with that of RPs; the differences were statistically significant for the comparison of ECs and RPs in $CD4^+$ T cells ($P = 0.02$) (Figure 3). This trend was not observed for a second regulator, *ADAR*.

Gene set enrichment analysis of human VNPs and SIV-infected sooty mangabeys. To examine whether the phenotype maintained by VNPs and natural host species was due to a shared, underlying molecular mechanism, we used gene set enrichment analysis (GSEA) of the human transcriptome data sets with gene sets derived from the analysis of sooty mangabeys and rhesus macaques (9). GSEA tests the relative position of a collection of genes ("query gene set") within an independent, ranked data set ("reference gene set"). Because GSEA relies on an additive signal of multiple genes within a data set, it is less dependent on arbitrary cutoffs, such as fold change of specific P values, making its ability to detect an underlying process within transcriptome data potentially more sensitive than a "single-gene" approach using traditional statistics. The use of rank data rather than absolute intensity measurements in GSEA also affords greater flexibility to make comparisons between diverse gene-expression data (i.e., between tissues, species, or array platforms) (15).

As presented in Table 1 and in Supplemental Figure 5, the query set of ISGs identified as differentially expressed in the rhesus macaque was associated with enrichment in human RPs, although the P values were only consistent with a statistical trend. The $CD8^+$ T cell expression data was particularly enriched for the ISGs; the data set comprised 15,879 nonredundant genes, and the lowest-ranked

Table 1
Analysis of gene sets of the primate model

	VNP vs. RP CD4 pre-ranked data set		VNP vs. RP CD8 pre-ranked data set	
Gene set	**ES**	***P* value**	**ES**	***P* value**
ISGs (RM)	–0.70	0.156	–0.85	0.089
IA (RM)	–0.69	0.141	–0.83	0.075
SM > RM chronic phase	0.98	0.010	–0.30	0.786
Random	0.28	0.910	–0.44	0.667

Enrichment of genes upregulated in sooty mangabeys (SMs) or rhesus macaques (RMs) after SIV infection was analyzed by GSEA in expression data sets derived from contrasting human HIV-infected RPs with VNPs. Positive enrichment scores (ES) indicate enrichment in the VNP phenotype, and negative scores indicate enrichment in the RP phenotype. "SM > RM" denotes genes that were upregulated in sooty mangabeys to a higher degree than in rhesus macaques. IA, immune activation genes.

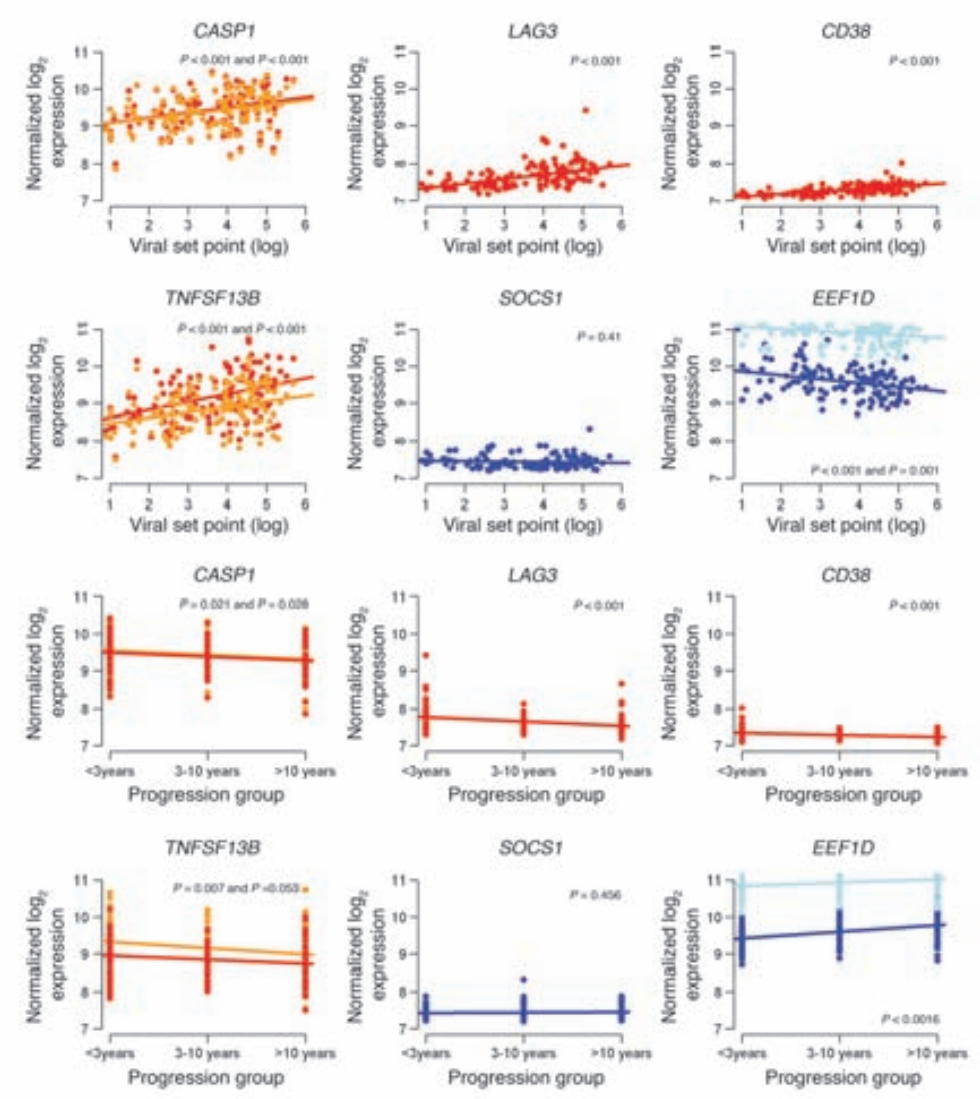

Figure 4
Analysis of the candidate VNP signature in an independent CD4+ T cell expression data set. The signature associated with the VNP profile upon transcriptome analysis in humans and nonhumans was tested in an independent validation set of 153 individuals, contributing CD4+ T cell expression data across all levels of viral set point after seroconversion. Correlations with individual gene expression levels and viral set point after seroconversion are shown in the 6 top panels. Correlations with disease progression, as indicated by time to CD4+ T cell count depletion to fewer than 350 cells/μl, are shown in the 6 bottom panels. Multiple probes for 1 gene are shown in different colors: orange/red is used for genes differentially upregulated in RPs, and blue/light blue is used for genes differentially upregulated in the VNPs. Where there are 2 *P* values, the first value represents the red/blue lines, and the second value represents the orange/light blue lines. Each dot represents an individual. The regression lines from the linear models are shown.

ISG was at position 10,562, well above the phenotype threshold at position 7,556, below which genes demonstrated higher expression in VNPs, and 12 out of 20 queried ISGs were higher than position 14,500 (Supplemental Figure 5). Genes found to be correlated with immune activation in rhesus macaques were also enriched in the RP phenotype in humans in both CD4 and CD8+ T cell data (Table 1 and Supplemental Figure 5). The enrichment of immune activation genes in RPs would indicate that VNPs have reduced cellular activation/proliferation relative to RPs. Taken together, these data suggest that VNPs, at least at the transcriptional level, are able to reduce the chronic immune activation seen in pathogenic HIV/SIV infection and that this attenuation largely overlaps with comparisons between sooty mangabeys and rhesus macaques. Because the human VNP and RP samples were obtained from the postacute phase of infection, we reasoned that genes found to be differentially expressed between sooty mangabeys and rhesus macaques during

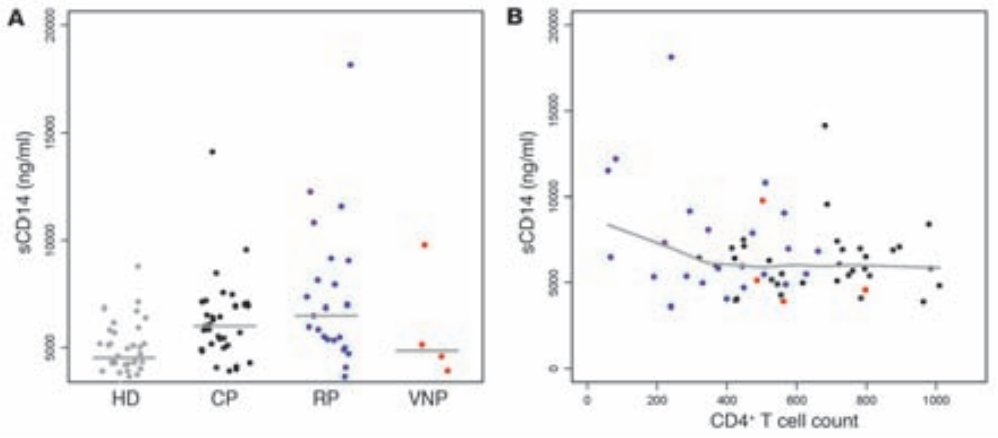

Figure 5
Analysis of sCD14 plasma levels. (**A**) sCD14 levels were measured during the 3-year period after seroconversion and/or transcriptome analysis in RPs (n = 24) and VNPs (n = 4). Chronic progressors (CP; n = 39) and healthy donors (HD; n = 38) contributed reference data. The gray line represents the median values. (**B**) RP sCD14 levels were higher at lower CD4+ T cell counts. The gray line represents the LOWESS curve fitted to the sample population. Each dot represents an individual

chronic infection may be enriched in the VNP phenotype. When we performed GSEA using genes found to be significantly higher in sooty mangabeys than rhesus macaques during chronic infection against the human data sets, we found that there was no significant enrichment in either phenotype in CD8+ T cells, but that there was significant enrichment in the VNP phenotype of CD4+ T cells (Table 1). The enrichment was largely driven by a single gene, *SV2A*, that ranked extremely high in the VNP phenotype. Taken together, these results suggest that sooty mangabeys and VNPs share some similarities in expression during chronic SIV/HIV infection; however, these similarities were not statistically significant.

Detailed analysis of genes identified in nonpathogenic primate models of natural infection. We extended the above analysis to examine in detail a list of genes reported by Bosinger et al. (9). We used a heuristic approach to inform this list (see Methods) by assessing (a) the consistency and direction of the association (downregulation or upregulation) between the primate model and the human expression profile, (b) the general correlation between CD4+ T cell and CD8+ T cell observations, and (c) the statistical support for the different associations in this subanalysis. Six genes fulfilled the criteria; genes *CASP1*, *CD38*, *LAG3*, and *TNFSF13B* presented lower expression levels in VNPs and in the nonpathogenic animal model, and *SOCS1* and *EEF1D* presented greater expression levels in VNPs and in the nonpathogenic animal model of infection. The short list of genes was constituted into a signature to be evaluated in an independent set of data. For this, we used the large data set of CD4+ T cell expression (16) to assess the association of the signature genes with viral load and with progression of immunosuppression (as defined by time to fewer than 350 CD4+ T cells/μl). In unadjusted regression, the following genes showed statistically significant association with time to progression to fewer than 350 CD4+ T cells/μl: *CASP1*, *LAG3*, *CD38*, *TNFSF13B*, and *EEF1D* (Figure 4). A multigene model explained 19.5% of the variance in disease progression (P = 0.0003). Inclusion of viral load in the model improved the proportion of variance explained to 26% ($P = 4.8 \times 10^{-7}$). However, there was significant colinearity with viral load and, after its inclusion in the model, only *EEF1D* remained as an independent variable (P = 0.013).

Association of soluble CD14 levels with clinical groups. To further assess whether the differences between RPs and VNPs reflected differences in mechanisms of pathogenesis, we assessed plasma levels of soluble CD14 (sCD14), which is produced by monocytes on becoming activated by LPS. Thus, plasma sCD14 levels reflect the host response to translocated bacterial products and are a significant independent predictor of mortality in HIV infection (17, 18). We analyzed samples from 24 RPs and 4 VNPs collected within 3 years after seroconversion. To contextualize these data, we measured plasma sCD14 levels in healthy volunteers and from chronic progressors. sCD14 levels were significantly higher in the plasma samples from RPs than in samples from chronic progressors, healthy donors, and for 3 out of 4 VNP samples analyzed (median 6,235 ng/ml [IQR, 5,069–8,808 ng/ml], median 6,065 ng/ml [IQR, 4,973–7,043 ng/ml], median 4,516 ng/ml [IQR, 3,972–5,304 ng/ml], and median 4,852 ng/ml [IQR, 4,069–8,612 ng/ml]); the differences between RPs versus healthy controls and chronic progressors versus healthy controls were significant (P < 0.0001) (Figure 5A). Additional plasma samples of the fourth VNP were consistently elevated. There was a trend toward increasing levels of sCD14 for individuals sampled at the time of advanced immunosuppression, with CD4+ T cell counts of below 350 cells/μl (Figure 5B).

Discussion

The current study defines 2 presentations of HIV infection that share a similar level of high viral replication but differ in the degree of immunological damage and in the pattern of clinical evolution, i.e., RPs and VNPs. The proportion of individuals with rapidly progressive disease was estimated in the SHCS (19). In this nationwide and representative cohort, 7.9% of HIV-infected individuals with a known seroconversion date fulfilled the criteria of RPs. Severity of the disease, rapid initiation of treatment, and the need for precise knowledge of the seroconversion window hampered recruitment of

RPs into clinical cohorts and research protocols in the past. VNPs constitute a group of individuals that sustain prolonged periods of high viral load, in the range of 100,000 copies/ml, while maintaining stable $CD4^+$ T cell counts. VNPs represent a very uncommon pattern of disease progression; prevalence estimates in the SHCS indicate that only 0.1% of HIV-infected individuals would fulfill the strict definition of VNPs used in the current work. However, the selected individuals likely represent the extreme of the distribution of VNPs, and relaxed criteria compared with those used in the present study will lead to different estimates of frequency.

The various genomic analyses in this study associate rapid progression with an enrichment for HLA alleles linked to adverse prognosis and a depletion of protective alleles. This pattern validates the phenotypic set of criteria elaborated to define rapid progression. In contrast, we found no association of the RP cohort with KIR alleles or KIR/HLA combinations previously related to disease progression or viremia (20). The genome-wide association study was conducted to exclude a major impact of common variants and to assess the candidates from a previous study of similar power (4) that could not be validated here. The transcriptome profile did confirm the deregulation of the ISGs in $CD8^+$ T cells in RPs, as previously documented for $CD4^+$ T cells (16, 21, 22) and in lymphatic tissue (23). It also identified a characteristic pattern of upregulation in $CD8^+$ T cells of RPs for genes involved in cell proliferation and cell division as well as in the immunoproteasome. RPs shared a number of features with the chronic SIV infection of rhesus macaques, in particular the prominent expression of a ISG and of immune activation markers. The absence of persistent immune activation during chronic SIV infection is a key characteristic of natural host species, such as the sooty mangabeys (24), and the presence of proliferation/activation markers on $CD4^+$ and $CD8^+$ T cells is an accurate predictor of disease in HIV-infected individuals (25). The immune activation gene set assessed in the present study was originally identified as being correlated with $CD8^+$ T cells expressing the activation marker Ki67 in SIV-infected rhesus macaques but was not expressed in SIV-infected sooty mangabeys (9).

More remarkable were the observations in VNPs. While the study did not have the power to allow a discovery that was not a priori, it permitted the assessment of a number of characteristics that have been previously described in SIV-infected sooty mangabeys. Individuals with the VNP profile display a limited deregulation of the ISG when compared with RPs, particularly in $CD8^+$ T cells. It should be stressed that these differences were present despite greater levels of viremia among VNPs than in RPs. In addition, to assess whether VNPs demonstrated lower immune activation and/or chronic interferon responses relative to RPs, we ranked the $CD4^+$ and $CD8^+$ expression data sets according to the significance value determined by the Bayesian analysis and used GSEA to test the relative position of ISGs/immune activation genes and genes differentially expressed in SIV-infected sooty mangabeys and rhesus macaques. This analysis supported the notion that the human profile of VNPs shares common features, at the transcriptome level, with the nonpathogenic model of SIV infection in the natural host. Reduced ISG expression is a consistent feature of natural host infection and not due to temporal fluctuation (10). Although the observation of reduced ISGs in VNPs in the current study is cross-sectional, it was consistent in showing ISG reduction relative to RPs. How differences in transcription levels of the ISGs translates into protein and the mechanisms of regulation should be the focus of future research (26).

We investigated in detail a set of genes identified through a comparative analysis of human and nonhuman primate transcriptome data; *CASP1*, *CD38*, *LAG3*, and *TNFSF13B* were upregulated in rhesus macaque and in human RPs; *SOCS1* and *EEF1D* were upregulated in sooty mangabeys and in human VNPs. The shared expression pattern between VNPs and sooty mangabeys supports their role in lentiviral pathogenesis. Caspase-1 precursor (CASP1) is a well-known intermediate of the inflammatory processes and apoptosis. The lymphocyte differentiation antigen CD38 is associated with immune exhaustion during immune activation and with adverse prognosis (27–29). LAG3 negatively regulates the expansion of activated T cells, and T cell homeostasis and is required for maximal regulatory T cell function (30) and has been demonstrated to associate with immune dysfunction/exhaustion of $CD8^+$ T cells in LCMV infection (31). Tumor necrosis factor ligand superfamily member 13B (TNFSF13B) is a receptor involved in the stimulation of B and T cell function and the regulation of humoral immunity. Suppressor of cytokine signaling (SOCS1) is involved in a negative feedback loop in the regulation of cytokine signal transduction signaled through the JAK/STAT5 pathway. Although *SOCS1* was downregulated in RPs compared with ECs and VNPs, its expression levels did not exhibit a significant association with viral set point or disease progression in the validation data set of $CD4^+$ T cell transcription data (16).

We completed the study by the analysis of a biomarker of compromised intestinal mucosal barrier, the monocyte-expressed LPS receptor sCD14 (18). Our data show higher plasma levels among RPs, in particular during advanced immunosuppression, than for other clinical progression groups. Although only 4 VNPs could be tested, 3 presented low sCD14 plasma levels, a pattern fitting other observations of lesser immunopathogenesis in these individuals. The transcriptome and biomarker data thus complement the work of Choudhary et al. (7) on VNPs that presented less extreme viral loads. They identified a lower percentage of activated $HLA\text{-}DR^+CD38^+CD4^+$ and $CD8^+$ T cells and lower levels of proliferating Ki67-expressing $CD4^+$ and $CD8^+$ T cells in VNPs compared with those of progressors. In contrast, viral isolates from VNPs and progressors replicated to similar levels and shared the capacity to deplete $CD4^+$ thymocytes or $CD4^+$ T cells in secondary lymphoid tissue and were equally cytopathic.

Future studies should extend analyses to plasmacytoid dendritic cells, as they are key activators of the immune system in HIV and SIV infection. Assessment of this cell population is limited by the low percentage of these cells in fresh blood, in particular, in the infected individual (32). The study has limited power due to the rarity of the study phenotypes and inherent limitations in recruitment. However, this work highlights the importance of 2 poorly understood clinical patterns of disease progression that have been minimally studied in the past and provides working definitions that should help identifying additional individuals to allow greater power in future genomic and functional studies. In addition, this report of a strong phenotypic similarity between nonpathogenic SIV infection of sooty mangabeys and a subset of HIV-infected individuals emphasizes the importance of studying natural SIV infection as a model to better understand HIV/AIDS pathogenesis.

Methods

Ethics statement. All participating centers provided local institutional review board approval for genetic analysis, and each participant provided informed consent for genetic testing. The Institutional Review Boards are

Comission d'Ethique de la Recherche Clinique, Faculté de Médecine, Université de Lausanne, Lausanne, Switzerland, and Comitè Etic d'Investigació Clínica, Hospital Germans Trias i Pujol, Badalona, Spain.

Patients and definition of clinical profiles. Study participants were followed in the SHCS (www.shcs.ch) or at the HIVACAT. The selection criteria for RPs included a HIV seroconversion window of less than 1 year with documented negative and positive serology and either of the following possibilities: (a) more than 2 $CD4^+$ T cell counts below 350 cells/μl within 3 years of seroconversion and no subsequent rise of $CD4^+$ T cells above 350/μl in the absence of combination antiretroviral therapy or (b) beginning antiretroviral therapy within 3 years of seroconversion and a $CD4^+$ T cell count within 1 month of starting antiretroviral therapy of less than 350/μl. $CD4^+$ T cell values in the first 6 months after seroconversion were excluded to avoid the $CD4^+$ T cell nadir during acute HIV infection.

The selection criteria of VNPs included more than 3 years of follow-up, median HIV viremia from more than 3 measurements of more than 100,000 cp/ml, HIV viremia consistently above 10,000 cp/ml, a $CD4^+$ T cell count above 350/μl, and no HIV treatment during follow-up.

Study candidates were identified by a standardized database search. Subsequently, the individual $CD4^+$ T cell profiles of all candidates were visually inspected before final inclusion. In addition to individuals fulfilling the definition of RP or of VNP, the study included 9 ECs as reference group.

Immunogenetic and genome-wide association analyses. High-resolution genotyping of *HLA-A*, *HLA-B*, *HLA-Cw*, and *DRB1* alleles was performed by sequence-based typing methods. *KIR* gene typing was performed by a sequence-specific oligonucleotide probe using the Luminex microbead technology. For genome-wide association analysis, participants were genotyped using Illumina BeadChips Human660W-Quad. For quality control purposes, SNPs were removed based on their absence (locus absence >5%), minor allele frequency (>1%), and Hardy-Weinberg Equilibrium deviation ($P < 1 \times 10^{-6}$). Participants were filtered based on call rate, gender check (heterozygosity testing), cryptic relatedness, and population structure (33).

Cell isolation, RNA extraction, and transcriptome profiling. For transcriptome analysis, we included all RPs ($n = 27$) for whom viable cells were available from the time of seroconversion (>6 months to 3 years from acute infection) and before initiation of antiretroviral treatment. Samples from all VNPs were included. The CD4 and viral load values at the time of transcriptome analysis are presented in Supplemental Table 1. $CD4^+$ and $CD8^+$ T cells were positively selected from frozen PBMCs (median time of cryopreservation was 1,485 [IQR, 821–2,558] days) using magnetically labeled $CD4^+$ or $CD8^+$ microbeads and subsequent column purification according to the manufacturer's protocol (Miltenyi Biotec). The median $CD4^+$ T cell purity, verified by flow cytometry, was 96.8% (range, 93.9%–98.9%), whereas the median $CD8^+$ T cell purity was 88.8% (range, 84.8%–92.1%). $CD4^+$ and $CD8^+$ T cell viability was assessed by the trypan blue dye exclusion method using the Vi-CELL (Beckman Coulter). Total RNA was extracted from purified $CD4^+$ and $CD8^+$ T cells using the mirVana miRNA Isolation Kit (Ambion) according to the manufacturer's protocol for total RNA extraction. The amount of RNA was estimated by spectrophotometry using the Nanodrop 1000 (Thermo Fisher). RNA quality was determined by the Agilent RNA 6000 Pico Kit on an Agilent 2100 Bioanalyzer. Samples were collected between 1993 and 2008 and investigated in 2009. The median of $CD4^+$ T cell viability for samples that were successfully analyzed was 79% (IQR, 64%–87%). The median of $CD8^+$ T cell viability for samples that were successfully analyzed was 82% (IQR, 74%–87%). Viability was minimally dependent on time of cryopreservation and more dependent on collection center. These covariates were assessed in the analyses. Target preparation was performed starting from 200 ng total RNA using the Illumina TotalPrep-96 RNA Amplification kit (Ambion). cDNA and cRNA were purified using the MagMAX Express Magnetic Particle Processor (Applied Biosystems). cRNA quality was assessed by capillary electrophoresis on the Agilent 2100 Bioanalyzer. Hybridization on HumanHT-12 v3 Expression BeadChips (Illumina) was carried out according to the manufacturer's instructions.

Transcriptome data analysis. Bead summary data were the output from Illumina's BeadStudio software without background correction, as this has previously been shown to have detrimental effects (34). Genes declared as nonexpressed ($P > 0.01$) were excluded from analysis. Data preprocessing, including quantile normalization and $\log_2$ transformation was completed in the Partek Genomics Suite package (Partek Inc.). Outliers were identified based on principal component analysis using 3 standard deviations as the cutoff for inclusion. For the differential expression analysis, we applied an empirical Bayes analysis approach, as implemented in the "limma" package of the R programming language, to model the variation profiles of all genes and used that information as prior knowledge to better estimate the variance of each gene expression (14).

The selected analysis included the following genes: *APOBEC3H*, *BST2*, *EIF2AK2*, *IFI27*, *IFI35*, *IFI44*, *IFIH1*, *IFITM1*, *IFITM3*, *IRF1*, *IRF9*, *ISG15*, *JAK1*, *JAK2*, *MX1*, *MX2*, *OAS3*, *STAT2*, *TAP*, *TRIM22*, *TYK2*, *ZBP1*, *APOBEC3F*, *APOBEC3G*, *IFI6*, *IFIT1*, *IFIT3*, *OAS1*, *OAS2*, *OASL*, *PSMB8*, *PTPN2*, *RNASEL*, *STAT1*, and *TRIM5* as previously described (16).

Signature analysis and validation. Because of the rarity of individuals with a VNP profile, we used a heuristic approach to assessing possible genetic markers associated with the clinical profile. This approach included the analysis of a preliminary signature, including genes identified as possibly associated with the VNP profile upon transcriptome analysis because of concordant signals in both $CD4^+$ and $CD8^+$ T cells as well as genes identified as potentially relevant in studies of SIV infection in the natural host: sooty mangabey and African green monkey. The signature was tested in an independent validation set of 153 individuals from a previous transcriptome analysis (16).

Pathway and network analyses. STRING (http://string.embl.de/) was used to identify known and predicted interactions (derived from 4 sources: genomic context, high-throughput experiments, coexpression, and previous knowledge). IPA (http://www.ingenuity.com/) and KEGG (http://www.genome.jp/kegg/pathway.html) were used for the analysis of pathway enrichment.

GSEA and gene set selection. The GSEA algorithm uses a Kolgorimov-Smirnov statistic to determine the significance of distribution of a set of genes within a larger, ranked data set (35). To evaluate the enrichment of SIV-inducible genes in the rhesus macaques and sooty mangabeys and in our human data set, we performed GSEA as follows: transcriptome data from VNPs and RPs were ranked according to their calculated Bayesian statistic; genes in which the mean was greater in VNPs were classified as positive, and genes with a greater mean in RPs were classified as negative. The data were ranked by the inverse Bayesian *P* value, resulting in a data set in which the most significant genes, overexpressed in VNPs, were listed at the top, and the most significant genes, overexpressed in RPs, were listing at the bottom. We next defined discrete query gene sets (Supplemental Table 6) from a large microarray data set, detailing longitudinal SIV infection in rhesus macaques, which develop disease, and sooty mangabeys, a nonpathogenic, natural host species, described previously (9). The ISG set comprised genes known to be regulated by type I interferon that were found to be differentially expressed in SIVmac239-infected rhesus macaques after 180 days of infection. The immune activation gene set was defined by multiple criteria: significant correlation of expression with lymphocyte activation assessed by circulating levels of $Ki67^+CD8^+$ T cells in SIVmac239-infected macaques (FDR = 0.0106), significant induction of expression assessed by ANOVA (FDR = 0.0075), a minimum of 2-fold upregulation in macaques at 1 or more time points, and expression in sooty mangabeys not exceeding 1.5× at any interval. To determine whether

gene expression maintained chronically in VNPs shared similarity with that of sooty mangabeys, we defined the sooty mangabey chronic query gene set as follows: robust multiarray average $\log_{10}$ intensity values from baseline samples were subtracted from chronic time points for individual animals of both species, and 2-sample *t* test was performed on the subsequent fold-change data; genes with a higher average fold change in sooty mangabeys relative to that in rhesus macaques were ranked according to *P* value, with the top 50 most significantly overexpressed genes selected for gene set inclusion. GSEA was performed using the desktop module available from the Broad Institute (www.broadinstitute.org/gsea/). GSEA was performed on the pre-ranked human data sets using 1,000 permutations, median collapse of duplicates, and random seeding.

Analysis of sCD14 levels. sCD14 levels were quantified in plasma samples using a commercially available ELISA assay (Diaclone). Plasma samples were diluted (1:50 or 1:100) and tested in duplicate. Plasma aliquots were collected either in EDTA (n = 55) or BD Vacutainer CPT Cell Preparation Tube with Sodium Citrate (CPT) tubes (n = 12). The CPT tubes contained a nonnegligible amount of molar sodium citrate solution (1 ml for the tubes, 8 ml draw capacity) and polysaccharide/sodium diatrizoate solution (FICOLL Hypaque solution; 2 ml for the tubes, 8 ml draw capacity), therefore samples collected with these tubes were considered to be diluted 1.44 times, and values were corrected accordingly.

Statistics. Comparisons of clinical and demographic characteristics used Fisher's exact tests for dichotomous variables and the Wilcoxon rank-sum test for continuous variables (STATA SE, release 11; StataCorp LP). In genome-wide association studies, association between genotype and phenotype (rapid progression) was tested using logistic regression, including top population principal components as covariates to correct for stratification. Genome-wide significance was assessed, using a cutoff of $P < 5 \times 10^{-8}$ to correct for multiple tests. In transcriptome analysis, we used a FDR method (36) to control for multiple testing. Probes selected for further analysis had an FDR-adjusted *P* value of less than 0.05. Statistical analyses dedicated to GSEA are detailed in the relevant section (see *GSEA and gene set selection*). Multiple regression analyses and graphical representations were performed by using the statistics package R (www.r-project.org).

Microarray data accession number. Microarray results have been deposited in the Gene Expression Omnibus database; the accession number is GSE28128.

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In–Depth Characterization of Viral Isolates from Plasma and Cells Compared with Plasma Circulating Quasispecies in Early HIV-1 Infection

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Abstract

Background: The use of *in vitro* models to unravel the phenotypic characteristics of circulating viral variants is key to understanding HIV-1 pathogenesis but limited by the availability of primary viral isolates from biological samples. However, overall *in vivo* genetic variability of HIV-1 within a subject may not be reflected in the viable viral population obtained after isolation. Although several studies have tried to determine whether viral populations expanded *in vitro* are representative of *in vivo* findings, the answer remains unclear due to the reduced number of clonal sequences analyzed or samples compared. In order to overcome previous experimental limitations, here we applied Deep Pyrosequencing (DPS) technology in combination with phenotypic experiments to analyze and compare with unprecedented detail the composition of viral isolates and *in vivo* quasispecies.

Methodology/Principal Findings: We amplified by DPS HIV-1 genomic regions covering *gag*, protease, integrase and *env*-V3 to characterize paired isolates from plasma and peripheral blood mononuclear cells and compare them with total plasma viral RNA in four recently HIV-1 infected subjects. Our study demonstrated the presence of unique haplotypes scattered between sample types with conservation of major variants. In addition, no differences in intra- and inter-population encoded protein variability were found between the different types of isolates or when these were compared to plasma viral RNA within subjects. Additionally, *in vitro* experiments demonstrated phenotypic similarities in terms of replicative capacity and co-receptor usage between viral isolates and plasma viral RNA.

Conclusion: This study is the first in-depth comparison and characterization of viral isolates from different sources and plasma circulating quasispecies using DPS in recently HIV-1 infected subjects. Our data supports the use of primary isolates regardless of their plasma or cellular origin to define genetic variability and biological traits of circulating HIV-1 quasispecies.

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Introduction

Human immunodeficiency virus (HIV-1) exhibits a high degree of genetic diversity particularly difficult to characterize due to the complexity of the RNA viral populations. This complexity is associated with factors such as the lack of proof-reading activity of HIV-1 polymerase, the high rate of generation of viral particles, and the recombination and hypermutagenesis process favored by host cellular proteins [1,2,3,4,5,6,7]. Consequently, the HIV-1 population is composed of a swarm of genetically related variants, known as viral quasispecies, which grant the virus with the ability to quickly adapt to various selective pressures. Examples of the rapid adaptive machinery of HIV-1 are the selection of mutations enabling escape from the humoral and cellular host immune responses [8,9,10,11] and the selection of mutations generating resistance to currently available antiretroviral drugs [12]. Therefore, to define the composition of HIV-1 quasispecies and identify virus diversity or variability within a single infected subject or at the population level it is essential to understand the pathogenesis of HIV-1 and design optimal antiretroviral treatments and vaccines.

Some studies associated pathogen diversity with poor prognosis [13,14,15], and increased diversity of HIV-1 has been related to

disease progression [16,17]. As a result, the maintenance of virus population structures in primary isolates is a key feature for the accurate study of specific viral biological traits, such as fitness and co-receptor usage, which are central to completing our understanding of the HIV-1 pathogenesis. The recent development of a new generation of massively parallel sequencing technologies has enabled us to carry out comprehensive studies of the genotypic characteristics of viral populations, genetically comparing thousands of sequences and increasing our chances of identifying minority variants. Deep Pyrosequencing (DPS) technology has made possible to describe the complexity of viral dynamics during immune escape, to quantify the presence of minority drug resistance variants, and to define virus co-receptor use for the management of CCR5 antagonists [18,19,20,21,22].

This study aims to investigate with the use of DPS technologies whether viral isolates from biological samples preserves the variability of circulating viruses and the phenotypic features found *in vivo*. For that reason, we compared paired HIV-1 isolates obtained from plasma and cells with total plasma viral RNA in four recently HIV-1-infected subjects. We combined multiple-amplicon DPS covering *gag*, protease, integrase, and *env*-V3 with *in vitro* replicative capacity and virus co-receptor use assays in order to address the genetic and phenotypic associations between HIV-1 isolates and viral quasispecies.

Results

Efficiency of HIV-1 recovery correlates with sample viral load for both plasma-derived and cell-derived viral isolates

In order to compare the efficiency of the methods used to obtain primary HIV-1 isolates from plasma or peripheral blood mononuclear cells (PBMCs), we analyzed a total 94 samples from different subjects at unique time-points, with the exception of the four included in the study; 56 plasma samples and 38 PBMCs samples with viral loads ranging from 10 to $>10^6$ copies/ml. Of those, we recovered a total of 63 primary isolates (34 from plasma samples and 29 from PBMCs). After stratification of samples by viral load, we observed an increase in the efficiency of virus recovery concomitant with the increase in viral load for both plasma and PBMCs HIV-1 isolation methods, Fig. 1. Furthermore, the categorization of viral load ranges into linear values demonstrated the existence of a direct correlation between sample viral load range and efficiency of virus recovery (Plasma: $r = 0.94$, $p<0.016$; PBMCs: $r = 0.94$ $p<0.016$ [Spearman correlation test]). Therefore, overall efficiency of the HIV-1 isolation methods used was similar and correlated to sample viral load.

Phylogenetic analysis of multiple-amplicon DPS reveals clusters of interspersed variants between cell virus isolates, plasma virus isolates, and plasma viral RNA

Four naïve, recent HIV-1-infected subjects were enrolled in the study. A summary of their clinical and epidemiological characteristics is shown in Table 1. Three sample types from a unique blood sample were obtained per subject, as represented in Fig. 2, for comparative purposes: 1.Total plasma viral RNA (RNA); 2. Plasma virus isolates (VP) after HIV-1 capture from plasma and virus *in vitro* expansion and; 3. Cell virus isolates (VC) obtained from PBMCs co-culture and virus *in vitro* expansion.

VP and VC primary isolates were expanded *in vitro* for a period of 2 to 3 weeks and 3 to 4 weeks respectively. Afterwards, virus were harvested for further genotypic (DPS) and phenotypic characterization (Tropism and Replicative Capacity) Fig. 2.

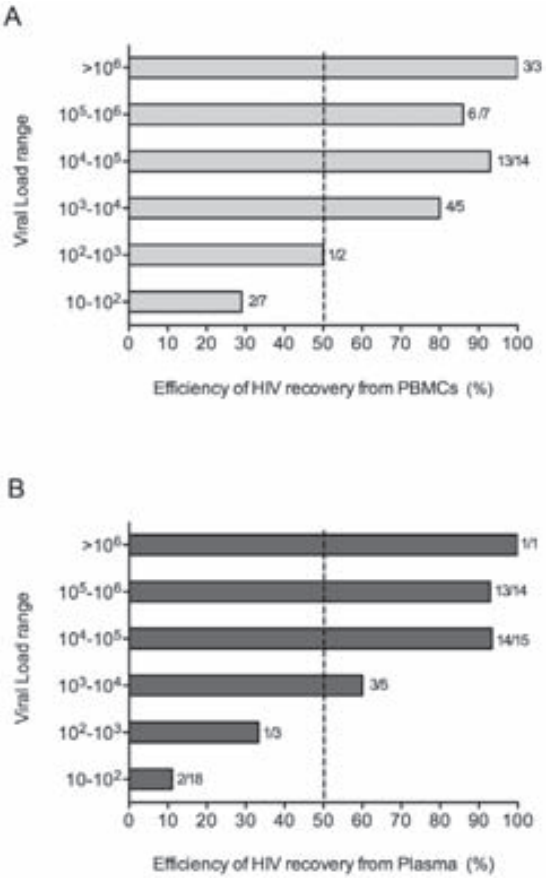

Figure 1. Comparison of the efficiencies of HIV-1 isolation methods from PBMCs or plasma samples. To determine the efficiency of HIV-1 isolation from PBMCs and plasma, we compared virus recovery from 56 plasma samples and 38 PBMCs samples with viral load ranging from 10 to $>10^6$ copies/ml. (**A**) Efficiency of HIV-1 recovery from PBMCs in percentages per viral load range. (**B**) Efficiency of HIV-1 recovery from plasma samples in percentages per viral load range. Bars represent mean values. Numbers next to the bars indicate (number of positive samples/total number of samples tested).
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Multiple-amplicon DPS was carried out in the three samples types RNA, VP, and VC, thus covering the *gag*, protease, integrase, and *env*-V3 regions with an average number of reads per nucleotide of 4039, 4193, 3629, and 4488, respectively. Data extracted using DPS were corrected for sequencing errors, filtered to a final number of unique reads, and merged into haplotypes (unique sequences represented in $\geq 1\%$), a resume of the sequences obtained after the various filtering steps is represented in Table 2. Final haplotypes were used to build phylogenetic trees based on the best-inferred model for conserved regions *gag*, protease and integrase as well as variable regions *env*-V3 of the HIV-1 proteome. As shown in Fig. 3, the phylogenetic trees for *gag*, protease, and integrase did not show segregation of clusters between VC, VP, and RNA variants, with low genetic distances between sample types and preservation of major variants after *in vitro* culture. A similar tree topology was observed for the variable *env*-V3 loop region, with clear interspersion of major variants from VC, VP,

Table 1. Epidemiological and clinical data of study subjects.

Subject	Sex	Virus[a] Subtype	Time after seroconversion (months)	Viral Load at sample collection (HIV-1 RNA copies/ml)	CD4 T-cell count at sample collection (cells/μl)	Nadir CD4 T-cell count (cells/μl)
P20	Male	B	5.5	69,000	190	144
P21	Male	B	13.4	320,000	181	181
P22	Male	BF	4.2	27,000	576	242
P23	Female	B	1.3	170,000	627	197

[a]Virus subtype was determined based on sequences from *gag*, pol, and *env*-V3 using the REGA HIV-1 Subtyping tool. BF denotes the recombinant BF HIV-1 form.
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and RNA. A tendency toward clustering of VP was found in the case of P21 for *gag*, P23 for integrase, and P22 for *env*-V3. However, this pattern was not consistent for other genes within the same subjects. In summary, VC, VP, and total plasma viral RNA populations were structured in closely related quasispecies represented by interspersed variants with a low genetic distance between them.

Low intra- and inter-population variability for VC, VP, and RNA variants among HIV-1 proteins

To define in detail VC, VP, and total viral RNA populations, we calculated intra- and inter-population variability, defined as the tendency for individual genomes to vary from one to another in a population. For that purpose, we simulated a viral population by considering the sequences obtained in the DPS run as a sample of the real population. We measured pairwise intra- and inter-population variability according to sample type for each HIV-1 protein and subject. We found low intra-population variability, with values close to zero for VC, VP, and RNA populations in all subjects and genetic regions (Table 3). Additionally, inter-population analyses comparing RNA with VC, RNA with VP, and VC with VP (Table 3) demonstrated a similar pattern of low variability. These results indicated that VC, VP, and plasma viral RNA populations were composed of HIV-1 variants with low intra- and inter-population variability.

VC and VP isolates display similar *in vitro* replicative capacity in primary cultures

In certain cases, heterogeneity in the distribution of quasipecies during *in vitro* passage of HIV-1 modified virus fitness in the absence of changes in the consensus sequences [23]. In order to test whether minor genetic changes described in our populations (synonymous changes, differences in the number of unique variants) affected the phenotypic properties of VC and VP

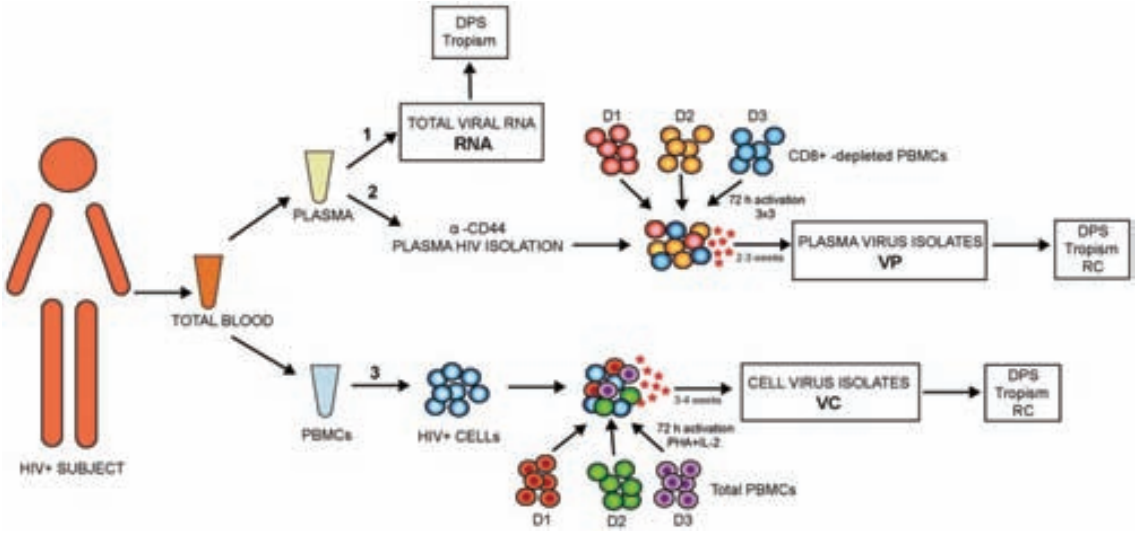

Figure 2. Schematic representation HIV-1 sample types analyzed for comparative purposes per study subject. Total blood was separated into plasma and PBMCs for the following: **1.** Total viral RNA extraction (RNA) used for DPS and virus tropism. **2.** Plasma virus isolation (VP) used for DPS, virus tropism and Replicative Capacity (RC): VP isolates were obtained by mixing plasma extracted anti-CD44 HIV-1 particles with a pool of $CD8^+$-depleted PBMCs from three seronegative-donors (D1, D2, and D3) and culture during 2 to 3 weeks for virus *in vitro* expansion. **3.** Cell virus isolation (VC) used for DPS, virus tropism and RC. VC isolates were obtained by co-culture of HIV+ cells with a pool of total PBMCs from three seronegative-donors (D1, D2, and D3) and culture during 3 to 4 weeks for virus *in vitro* expansion. Colored red, orange and dark blue circles represent cells from CD8+ depleted seronegative donors D1, D2 and D3 respectively. Colored red, green and purple circles represent cells from seronegative donors D1, D2 and D3 respectively. Light blue circles represent HIV+ cells. Red stars indicate virus production.
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Table 2. Number of sequences obtained for each subject and sample type by DPS.

Subject	Protein	Sample	Total Reads[a]	Valid Reads[b]	Unique Haplotypes[c]
P20		RNA	2,979	786	4
	Gag	VC	4,812	197	17
		VP	4,842	1,383	3
		RNA	3,180	2,321	2
	PR	VC	2,374	1,800	10
		VP	2,541	1,726	5
		RNA	6,898	4,271	3
	IN	VC	2,970	2,102	4
		VP	1,640	1,266	5
		RNA	1,823	1,535	3
	Env-V3	VC	4,776	2,951	3
		VP	3,101	2,701	3
P21		RNA	2,425	1,213	2
	Gag	VC	3,850	89	12
		VP	3,875	2,541	4
		RNA	6,503	5,115	2
	PR	VC	5,385	4,315	5
		VP	3,443	2,806	2
		RNA	3,931	2,702	4
	IN	VC	212	120	6
		VP	1,484	899	9
		RNA	3,073	2,511	2
	Env-V3	VC	5,723	3,594	2
		VP	4,265	3,545	1
P22		RNA	2,960	2,370	3
	Gag	VC	5,284	2,238	1
		VP	3,910	3,146	3
		RNA	2,582	2,138	3
	PR	VC	7,605	3,063	3
		VP	1,812	1,443	2
		RNA	8,334	4,527	6
	IN	VC	6,593	4,143	8
		VP	1,301	1,070	5
		RNA	2,698	2,412	1
	Env-V3	VC	10,669	3,476	2
		VP	5,140	4,335	4
P23		RNA	5,707	4,255	4
	Gag	VC	4,585	2,802	7
		VP	3,244	2,748	3
		RNA	4,189	3,640	2
	PR	VC	2,694	2,282	5
		VP	8,019	6,565	2
		RNA	5,752	4,746	4
	IN	VC	2,906	2,387	2
		VP	1,527	1,289	4
	Env-V3	RNA	2,417	2,083	1
		VC	5,534	3,298	4
		VP	4,639	4,180	1

Table 2. Cont.

[a]Total reads is the total coverage of sequences obtained after direct DPS.
[b]Valid reads are those sequences obtained after cleaning the total reads by selecting unique sequences with >70% homology to HXB2 and manual correction of homopolymer tacks.
[c]Unique haplotypes are defined by similar sequences represented as a proportion ≥1% from the total unique reads. DPS, deep pyrosequencing; PR, protease; IN, integrase; RNA, plasma viral RNA; VC, cell virus isolates; VP, plasma virus isolates.
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isolates, we measured replicative capacity for VC and VP isolates in primary cells. After infection, viral growth was monitored by p24 production for one week and the log-transformed data on the exponential growth phase used to calculate the virus growth rate (slope of the linear regression) for each type of isolate. As shown in Fig. 4, VC and VP pairs display similar replication kinetics with no differences in replicative capacity per pair in any of the study subjects. Thus, in spite of minor genetic differences in quasispecies composition, our data revealed no differences in replicative capacity between VC and VP isolates for each subject.

Phenotypic determination and genotypic prediction of co-receptor usage in VC isolates, VP isolates, and plasma RNA

HIV-1 co-receptor use is a key determinant of viral pathogenesis; the presence of CXCR4 using strains has been related to disease progression, and detection of minor CXCR4 variants has a clear clinical interest in the management of CCR5-antagonists [21,24,25,26]. Therefore, in order to understand the relationship between VC, VP and plasma RNA, we compared virus co-receptor use by means of U87 cells in VC and VP isolates and by means of ESTA in plasma RNA. Furthermore, for genotypic prediction of virus co-receptor, we used the PSSM and g2p algorithms in *env*-V3 loop from the most frequent haplotypes, with cut-off values of -4.75 and ≤ 3.5, respectively. These results are summarized in Table 4. Phenotypic data show a concordance of 100% between U87 and ESTA results. Moreover, genotypic prediction of co-receptor usage with PSSM was 75% (9/12) concordant with g2p. In spite of minor discrepancies between the methods used, plasma RNA, VC, and VP isolates exhibited good matching in terms of virus co-receptor per study subject and sample type. Additionally, a more detailed prediction of co-receptor use was made in VC, VP, and RNA by inference of g2p and PSSM scores in the unique *env*-V3 sequences extracted from DPS. We observed a cluster of combined variants from VC, VP, and RNA with low intra-patient deviation and preferential R5 use, with the exception of p20 Fig. S1. In the case of P20, g2p and PSMM scores from DPS sequences suggest the presence of a homogeneous population of X4R5 dual tropic virus when compared to previously defined R5+X4R5 or X4R5 HIV-1 isolates Fig. S1 [27]. Therefore, inference of phenotypic and genotypic tropism in VC and VP pairs and plasma viral RNA demonstrated concurrence in virus co-receptor usage among sample types for each study subject.

Discussion

Primary viral isolates play a key role in our understanding of the HIV-1 pathogenesis and are a common approach for various *in vitro* studies such as antibody neutralization, drug testing, or virus co-receptor use assays. Furthermore, the relevance of using

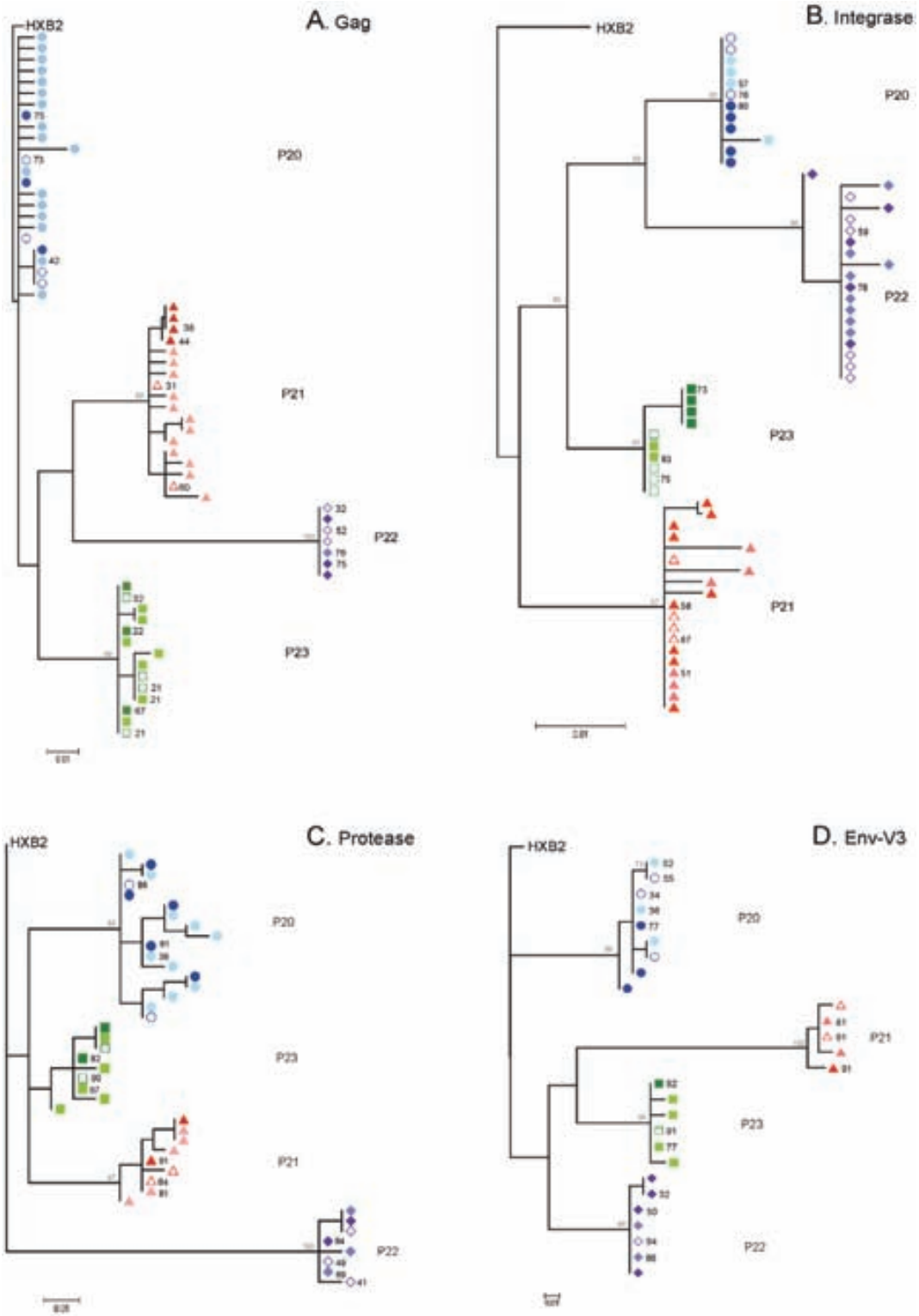
A. Gag
HXB2
P20
P21
P22
P23
B. Integrase
HXB2
P20
P22
P23
P21
C. Protease
HXB2
P20
P23
P21
P22
D. Env-V3
HXB2
P20
P21
P23
P22

Figure 3. Phylogenetic trees for HIV-1 *gag*, protease, integrase and *env*-V3 sequences extracted from VC, VP, and RNA using DPS. Symbols represent unique haplotypes extracted from DPS for subjects P20 ○, P21 △, P22 ◇, and P23 □, according to sample type: VC (light symbols), VP (dark symbols), and RNA (empty symbols). Numbers next to symbols indicate haplotype frequencies obtained from the total reads; only values above 20% are indicated in the figure. Node numbers indicate bootstrap values over 75%. (**A**) *gag* maximum-likelihood phylogenetic tree based on the TrN model. (**B**) Integrase maximum-likelihood phylogenetic tree based on the HKY model. (**C**) Protease maximum-likelihood phylogenetic tree based on the HKY+ G ($\alpha = 0.565$) model. (**D**) *env*-V3 maximum-likelihood phylogenetic tree based on the TrN model.
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primary isolates for an accurate description of virus phenotype has been highlighted by differences in replicative capacity found between recombinant viruses and full isolates [28,29].

Previous studies in the HIV-1 field have determined whether viral populations from primary isolates were representative of *in vivo* findings with contradictory results. Some of them report a decrease in HIV-1 gp120 diversity in isolates [30], while others support the maintenance of major variants in blood after co-culture with PBMCs [31]. Additionally, most of these studies are limited by the number of samples analyzed, the number of clonal sequences obtained, and their focus on comparing proviral DNA to primary isolates recovered from co-cultured PBMCs. In order to overcome previous experimental limitations, we carried out multiple-amplicon DPS to genetically compare thousands of sequences in four regions of the HIV-1 genome and clearly define phylogenetic relationships between primary isolates obtained from VP, VC after *in vitro* HIV-1 expansion and plasma circulating quasispecies (RNA) *in vivo*. Our results demonstrate a structured population of interspersed major VC, VP, and RNA variants with fluctuations in low frequency unique sequences in most of the HIV-1 genes studied (*gag*, protease, integrase, and *env*-V3) and among subjects but with no significant differences in the total numbers of unique haplotypes (data not shown). The presence of major variants in similar frequencies for VC and VP primary isolates, when compared to *in vivo* RNA, demonstrates the maintenance of high frequency variants after *in vitro* expansion in both VC and VP isolates. Furthermore, the low intra- and inter-population variability, with values close to zero, reflects homogeneous populations both within HIV-1 proteins or sample types. Nevertheless, relative homogeneous viral populations have been reported in both proviral HIV-1 DNA and plasma HIV-1 RNA during early infection [16,32]. As a consequence, the low level of genetic variability found among primary viral isolates and total RNA, could be related to the short time after seroconversion in our samples, where homogeneous viral populations will be present before diversification at later stages of disease [16]. On the other hand, recent studies on founder virus evolution support early variation in the HIV-1 genome after transmission and accumulation of changes over the first year after infection [33]. In this context, our results suggest an adequate representation of RNA circulating quasispecies after HIV-1 *in vitro* expansion. However, these results should be viewed with caution until they are confirmed in chronically infected samples.

Table 3. Comparison of intra- and inter-population variability for each subject, HIV-1 protein, and sample type (RNA, VC, or VP).

		Π^a_{intra}			Π_{inter}		
Subject	**Protein**	**Π_{int} RNA**	**Π_{int} VC**	**Π_{int} VP**	**RNA vs VC**	**RNA vs VP**	**VC vs VP**
P20	Gag	0.0011	0.0110	0.0008	0.0010	<0.0001	0.0009
	PR	0.0005	0.0075	0.0041	0.0022	0.0033	0.0002
	IN	0.0005	0.0013	0.0004	<0.0001	<0.0001	<0.0001
	Env-V3	0.0051	0.0050	0.0018	<0.0001	0.0037	0.0035
P21	Gag	0.0240	0.0300	<0.0001	0.0037	0.0023	0.0040
	PR	0.0006	0.0010	<0.0001	<0.0001	<0.0001	<0.0001
	IN	0.0009	0.0025	0.0014	<0.0001	<0.0001	<0.0001
	Env-V3	0.0002	0.0006	<0.0001	<0.0001	0.0194	0.0194
P22	Gag	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001
	PR	0.0033	0.0018	0.0009	0.0013	0.0012	<0.0001
	IN	0.0009	0.0016	0.0005	<0.0001	<0.0001	0.0002
	Env-V3	<0.0001	<0.0001	0.0437	<0.0001	0.0033	0.0033
P23	Gag	0.0023	0.0036	<0.0001	<0.0001	0.0007	0.0012
	PR	0.0002	0.0029	0.0010	<0.0001	<0.0001	<0.0001
	IN	0.0004	0.0002	0.0006	<0.0001	0.0042	0.0042
	Env-V3	<0.0001	0.0012	<0.0001	<0.0001	<0.0001	<0.0001

[a]Average number of nucleotide differences per site between sequences. PR, protease; IN, integrase; RNA, plasma viral RNA; VC, cell virus isolates; VP, plasma virus isolates.
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RNA virus populations are composed of a swarm of closely related genotypes or quasispecies in which viral evolution operates as a unit and adaptation is the result of cooperative interactions between multiple genomes [15]. Various studies have demonstrated how minor genetic differences in composition and quasispecies heterogeneity can modulate HIV-1 fitness in the absence of changes in population sequence [23,34]. Additionally, genetic similarities in studied regions cannot be extrapolated to the whole viral genome. Therefore, similarities in virus genotype might not take the form of similarities in virus phenotype. In this context, our data revealed no differences in terms of virus replicative capacity in paired VC and VP isolates, regardless of minor differences in genotypic composition of the viral quasispecies studied. However, our approach is limited by the short-term *in vitro* culture of the replicative capacity experiments and presence of antiretroviral drugs, neutralizing antibodies, cytotoxic T lymphocytes, or other selective pressures may induce unpredictable fluctuations in closely related viral populations, which are not capture in this study.

Together with replicative capacity, HIV-1 co-receptor use is an essential trait when defining HIV-1 pathogenesis. The presence of CXCR4-using HIV-1 variants is associated with disease progression [24,25,26], and detection of minor CXCR4 HIV-1 populations has become a key marker for the management of CCR5 antagonists [21,35]. A previous study showed high concordance of co-receptor usage in paired plasma and PBMCs samples during primary infection [36]. In agreement with this observation, we found concordance in co-receptor use between VC isolates, VP isolates, and plasma RNA as measured both by ESTA and U87. Comparable results were obtained by genotypic inference of virus co-receptor use in DPS *env*-V3 sequences with g2p and PSSM. Regardless of small differences in the methods applied intra-subject, co-receptor use was very homogeneous.

Many studies have described the use of DPS in combination with genotypic algorithms in the *env*-V3 variable region as a key tool when detecting minor CXCR4 populations for the management of CCR5 antagonists. We used the same approach to compare VC isolates, VP isolates, and circulating plasma quasispecies.

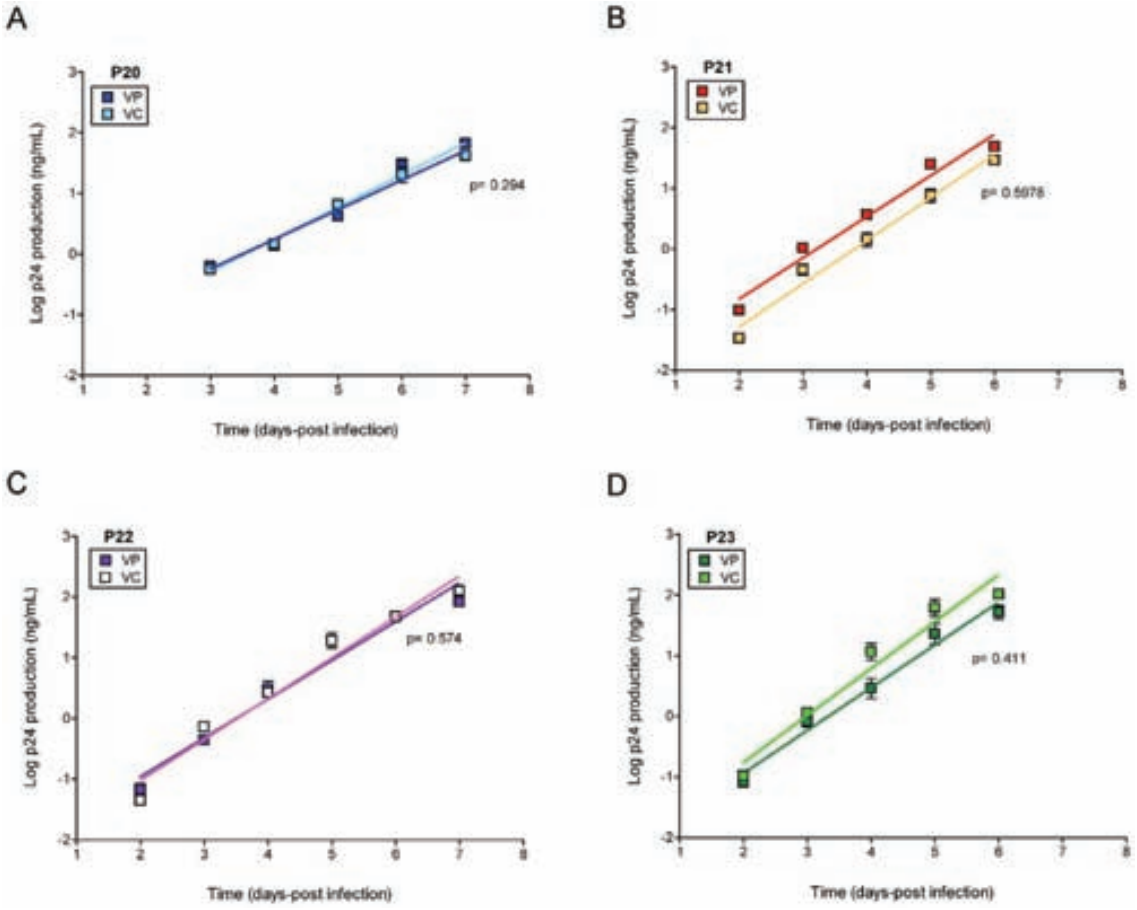

Figure 4. Replicative capacity of VC and VP HIV-1 isolates in primary cells. PBMCs stimulated from seronegative donors were infected in triplicate with each viral variant, and virus growth was monitored by p24 production over one week. Slopes were compared for VC and VP pairs, and $p<0.001$ was considered significant. Squares represent mean values and bars represent the standard error of the mean. Light squares correspond to VC and dark squares correspond to VP. (**A**) P20, (**B**) P21, (**C**) P22, and (**D**) P23.
doi:10.1371/journal.pone.0032714.g004

We found clusters of mixed sequences from VC, VP, and RNA sequences with homogeneous populations and preferential R5 use. These data contrast with those of previous studies, where DPS revealed the presence of more heterogeneous populations in proviral quasispecies [35], but argue in favor of homogeneous replication-competent populations obtained after *in vitro* expansion in VC primary isolates, regardless of the heterogeneity in proviral DNA.

In summary, our study provides the first direct comparison of viral isolates with plasma circulating quasipecies using DPS in recently HIV-1 infected subjects. Our data demonstrated that VC and VP share genotypic characteristics with HIV-1 quasispecies and maintain the presence of major variants after virus *in vitro* expansion. In spite of minor genetic differences, phenotypic data reveal similarities in paired VP and VC isolates with regard to replicative capacity and co-receptor use. Our data support the potential use of VP or VC primary isolates as a reliable tool to characterize the circulating quasispecies. Nevertheless, further comparisons will help to clarify whether our findings also apply to later stages of the disease.

Methods

Study subjects and Ethics Statement

The study sample comprised four treatment-naïve HIV-1-infected subjects. Epidemiological and clinical data are summarized in Table 1. Virus subtype was assigned based on *gag*, pol, and *env*-V3 sequences using the REGA HIV-1 Subtyping tool. The study was approved by the institutional review board of Hospital Germans Trias i Pujol, and all four subjects gave their written informed consent to participate.

Cell lines

The following reagents were obtained through the NIH AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: TZM-bl from Dr. John C. Kappes, Dr. Xiaoyun Wu and Tranzyme Inc; U87CXCR4 and U87CCR5 from Dr. HongKui Deng and Dr. Dan R. Littman as previously described [37,38].

Plasma virus isolation

Viral isolates were obtained from plasma samples using anti-CD44 beads following the manufacturer's protocol (Miltenyi

Table 4. Phenotypic and genotypic prediction of co-receptor usage from total plasma RNA, VC, and VP primary isolates.

Sample	Phenotype		Genotype	
	U87	ESTA[a]	PSSM[b]	g2p[b]
P20 RNA	-	X4/R5	R5	Non-R5
VC	X4/R5	-	R5	Non-R5
VP	X4/R5	-	R5	Non-R5
P21 RNA	-	R5	R5	R5
VC	R5	-	R5	R5
VP	R5	-	R5	R5
P22 RNA	-	R5	R5	R5
VC	R5	-	R5	R5
VP	R5	-	R5	R5
P23 RNA	-	R5	R5	R5
VC	R5	-	R5	R5
VP	R5	-	R5	R5

[a]ESTA, Enhance Sensitivity Trofile Assay. This assay has a detection limit of 0.3% for non-R5 variants.
[b]Cut-off values to define non-R5 using sequences were −4.75 for PSSM and ≤3.5 for g2p, respectively. Non-R5 sequences include X4 and X4/R5 dual tropic virus. RNA, plasma viral RNA, VC, cell virus isolates; VP, plasma virus isolates. – Indicates non-determined.
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Biotec, Germany) with minor modifications as previously described [29]. Briefly, before virus extraction, PBMCs from three HIV-1-seronegative donors were isolated and $CD8^+$ T cells depleted using the RosetteSep human CD8+ depletion cocktail (Stemcell Technologies, France). Pooled $CD8^+$-depleted PBMCs were then stimulated under three different conditions ('3×3' method, Miltenyi Biotech). After 72 hours, cells were mixed to a final concentration of 10^6 cells/ml in R10 supplemented with IL-2 (100 U/ml) (Roche, Spain), and 200 µl of the extracted virus was added to the culture. Cultures were fed weekly with 10^6 cells/ml fresh 3×3-stimulated cells. Viral growth was monitored weekly using p24 enzyme-linked immunosorbent assay (ELISA) (Innogenetics, Spain). Virus isolates were harvested when the p24 concentration in the supernatant reached at least 100 ng/ml and then stored at −80°C.

Cell virus isolation

Viral isolates from cryopreserved cells were obtained by co-culture of PBMCs from each HIV-1-infected subject with a pool of PBMCs from three HIV-1-seronegative subjects that had been previously stimulated with phytohemagglutinin (PHA) (3 µg/ml) and IL-2 (10 U/ml) for 72 hours. Viral growth was monitored weekly by p24 ELISA and cultures were fed weekly with fresh cells. Viral stocks were harvested and stored at −80°C.

PCR amplification and amplicon preparation

Total viral RNA was extracted (QIAamp Viral RNA Mini Kit™, QIAGEN, CA) from plasma (2 ml), plasma viral isolates (1 ml), and cell viral isolates (1 ml) in order to carry out PCR amplification. *gag*, pol, and *env*-V3 were amplified using one-step reverse transcriptase polymerase chain reaction (RT-PCR) (SuperScript® III One-Step RT-PCR System with Platinum® Taq High Fidelity, Invitrogen, Carlsbad, CA, USA) based on a primer set containing 5′-GCA GAA TGG GAT AGA TTG CAT CCA-3′ (1,417→1,440, HXB2) and 5′-CCT TGT TAT GTC CTG CTT GAT ATT CAC-3′ (5,438←5,464, HXB2), and 5′-TAG AGC CCT GGA AGC ATC CAG GAA G-3′ (5853→5877, HXB2) and 5′-TTG CTA CTT GTG ATT GCT CCA TGT-3′ (8,913←8,936, HXB2) for *gag*, pol, and *env*-V3, respectively. Amplification conditions were as follows: 30 minutes at 52°C during reverse transcription, 2 minutes at 94°C, 30 seconds at 94°C, 30 seconds at 55°C, and 4 minutes at 68°C for 25 cycles. A final polymerization step of 5 minutes at 68°C was applied. The enzyme used for the RT-PCR was the Super-Script III one-step PCR (Invitrogen, USA). Amplicons for QDS were generated using carried 454 adaptor A and subject-specific multiple identifiers; pyrosequencing was unidirectional. The conditions for the enzyme were 5 minutes at 94°C, 30 seconds at 52°C, and 1 minute at 68°C for 25 cycles. A final polymerization step of 5 minutes at 68°C was applied. The enzyme used was Platinum High Fidelity (Invitrogen, USA). The specific primer set was composed of the forward primers 5′-CAG GAT TTA AAC ACC ATG CTA AA-3′ (1,333→1,355 HXB2), 5′-AAT TTG CCA GGA AGA TGG-3′ (2,361→2,378 HXB2), 5′-TTA AGG CCG CCT GTT G-3′ (4,606→4,621 HXB2), and 5′-TGG CAG TCT AGC AGA AGA AG-3′ (7,010→7,029 HXB2), and the reverse primers 5′-TAT CCA TCT TTT ATA GAT TTC TCC-3′ (1,564←1,587 HXB2), 5′-CAA TAG GAC TAA TGG GAA AA-3′ (2,546←2,565 HXB2), 5′-TTT TGT AAT TTG TTT TTG TAA TTC-3′ (4,863←4,886 HXB2), and 5′-CTG GGT CCC CTC CTG AGG-3′ (7,315←7,332 HXB2) for *gag*, protease, integrase, and *env*-V3, respectively. All PCR reactions were performed in triplicate to reduce amplification bias and the founder effects. Triplicate amplifications were pooled before the purification procedure. Reactions were purified using the Agencourt AMPure Kit (Beckman Coulter, Germany) to eliminate the primer-dimers produced. The number of molecules was quantified by fluorometry using the Quant-iT PicoGreen dsDNA assay kit (Invitrogen, USA). When concentrations were below 5 ng/ml, amplicon quality was assessed by spectrometry using BioAnalyzer (Agilent Technologies, USA). Quantitative multiple amplicon DPS was performed in a 454 Genome Sequencer FLX (454 Life Sciences/Roche, USA) using FLX chemistry. A pNL4.3 clone was sequenced to assess the likelihood of errors during DPS. Discrepancies between data obtained by DPS and Sanger sequencing of pNL4.3 clone were attributed to the process.

Multiple amplicon DPS data clean-up and phylogenetic analysis

Data were cleaned in order to increase the quality of the sequences for down-stream analysis after multiple-amplicon DPS. The first step was to retrieve those sequences with a similarity >70%, when compared with HXB2 from the sequencing run. We then manually corrected the homopolymer tracks, since these are the most common sequencing errors produced by the technique. Sequences with stop codons within the open reading frame of the protein were removed from the analysis, and sequences containing gaps were maintained and included in the analysis, rather than being removed using a conservative bias towards an unknown nucleotide at this position. Identical sequences were collapsed into a single unique sequence or haplotype. Haplotypes with less than 1% presence in the population were removed from the analysis. A summary of the number of reads after the various filtering steps and the final number of haplotypes is represented in Table 2. Phylogenetic trees were built on the nucleotide alignment for the total unique reads collapsed into unique haplotypes. The best phylogenetic model was inferred using jModeltest v0.1.1 [39] for each HIV-1 protein in all subjects. Phylogenetic trees were constructed taking into account the inferred model in PhyML over

1000 bootstrap replicates (www.HIV-1.lanl.gov) were: for *gag* (TrN), protease (HKY+G), integrase (HKY) and *env*-V3 (TrN). Newick trees were exported and edited with MEGA4 [40].

Population variability per HIV-1 protein and sample type

To study and reproduce the variability according to sample type and among HIV-1 proteins, we simulated a viral population taking into account the sequences obtained in the sequencing run as a sample of the real population. The percentage of each sequence, based on the sequencing run, was used to create a population of 100 sequences where each haplotype was represented as many times as indicated by the percentage of the sequence in the sequencing run. This population of 100 sequences was used to infer variability among populations in the same patient and among HIV-1 proteins. We measured pairwise intra- and inter-population variability using the best model found by jModeltest v0.1.1, as implemented in MEGA4.

Replicative capacity experiments

Viral isolates obtained from plasma and cells were titrated in the TZM-bl immortalized cell line. Replicative capacity experiments were carried out using PBMCs from three seronegative individuals; previous infection PBMCs were stimulated for 72 hours with PHA (3 μg/ml) and IL-2 (10 U/ml). Stimulated PBMCs were then infected in triplicate with an equal multiplicity of infection of each viral variant at 37°C for 2 hours. Pellets were washed twice with phosphate-buffered saline (PBS) and cultured at 37°C and 5% CO_2 in R20 supplemented with IL-2 (20 U/ml) (Roche, Spain) [41]. Viral growth was measured by p24 ELISA in supernatants over 10 days (Perkin Elmer, Spain). Replicative capacity was calculated by fitting a linear model to the $\log_{10}$-transformed data of p24 production and comparing the slopes as previously described [42].

Determination of virus co-receptor use

Viral tropism from VP and VC was measured in U87 immortalized cell lines expressing CCR5 or CXCR4, as previously described [27,38]. Briefly, 5,000 cells were plated on a 96-well plate and infected with 2 ng of p24 for each viral variant overnight. The next day, virus was washed 3 times with 200 μl of PBS and fresh media added to a final volume of 200 μl. Five days after infection, virus growth was identified microscopically by observation of syncytium formation, and the results were corroborated by p24. Furthermore, virus tropism was assessed in plasma samples at similar time-points using the Enhance Sensitivity Trofile Assay (ESTA, with a detection limit of 0.3% for non-R5 variants). In addition, two algorithms were used to infer virus co-receptor use based on *env*-V3 loop sequences from DPS: PSSM (http://indramullins.microbiol.washington.edu/webpssm) and geno2pheno (g2p) (http://www.geno2pheno.org/) with a false positive rate of 10%. Cut-off values to define non-R5 using sequences were -4.75 for PSSM and ≤ 3.5 for g2p [21].

Supporting Information

Figure S1 Genotipic predicition of co-receptor use in DPS sequences from VC, VP and total plasma RNA. Unique sequences obtained from the DPS of the *env*-V3 loop region were used to run PSSM and g2p algorithms to infer virus co-receptor use per sample type VC (ligth symbols), VP (dark symbols) and RNA (empty symbols) and subject. For comparative purposes *env*-V3 loop sequences from virus with dual mix (R5+R5X4) and dual (R5X4) co-receptor use were included. (**A**) Prediction of co-receptor use based on g2p algorithm with a false positive rate of 10%. Dashed line represents cut-off values (3.5) to infer R5 and non-R5 use. (**B**) Prediction of co-receptor use based on PSSM scores. Dashed line represents cut-off value (-4.75) to infer R5 and non-R5 use.
(TIF)

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Author Contributions

Conceived and designed the experiments: JD FMC JMP JGP. Performed the experiments: JD IE MP CP FMC JGP. Analyzed the data: JD FMC JGP. Wrote the paper: JD FMC JMP JGP BC RP.

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Chapter 9

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Chapter 10

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