MINISTÉRIO DA SAÚDE
FUNDAÇÃO OSWALDO CRUZ
INSTITUTO OSWALDO CRUZ

Doutorado pelo Programa de Pós-Graduação em Biologia Celular e Molecular

CARACTERIZAÇÃO DOS MECANISMOS DE CONTROLE E PERSISTÊNCIA VIRAL EM UMA COORTE DE INDIVÍDUOS INFECTADOS PELO HIV-1 COM SUPRESSÃO NATURAL DA VIREMIA E DA EVOLUÇÃO PARA A AIDS

SUWELLEN SARDINHA DIAS DE AZEVEDO

Rio de Janeiro
Março de 2019

Ministério da Saúde

FIOCRUZ
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Caracterização dos mecanismos de controle e persistência viral em uma coorte de indivíduos infectados pelo HIV-1 com supressão natural da viremia e da evolução para a aids.

Tese apresentada ao Instituto Oswaldo Cruz como parte dos requisitos para obtenção do título de Doutor em Ciências.

Orientador (es): Prof. Dr. Gonzalo Jose Bello Bentancor
Prof. Dr. Thiago Moreno L. Souza

RIO DE JANEIRO
Março de 2019

Sardinha Dias de Azevedo, Suwellen .

Caracterização dos mecanismos de controle e persistência viral em uma coorte de indivíduos infectados pelo HIV-1 com supressão natural da viremia e da evolução para a aids. / Suwellen Sardinha Dias de Azevedo. - Rio de Janeiro, 2019.
xvii, 181 f.; il.

Tese (Doutorado) – Instituto Oswaldo Cruz, Pós-Graduação em Biologia Celular e Molecular, 2019.

Orientador: Gonzalo Jose Bello Bentancor.
Co-orientador: Thiago Moreno Lopes e Souza.

Bibliografia: Inclui Bibliografias.

1. HIV-1. 2. Controladores do HIV-1. I. Título.

Elaborada pelo Sistema de Geração Automática de Ficha Catalográfica da Biblioteca de Manguinhos/ICICT com os dados fornecidos pelo(a) autor(a).

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Aprovada em: 29/03/2019

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Rio de Janeiro, 29 de março de 2019.

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Declaramos, para fins curriculares, que **Suwellen Sardinha Dias de Azevedo**, sob orientação do Dr. Gonzalo José Bello Bentancor e Dr. Thiago Moreno Lopes e Souza, foi aprovada em 29/03/2019, em sua defesa de tese de doutorado intitulada: **"Caracterização dos mecanismos de controle e persistência viral em uma coorte de indivíduos infectados pelo HIV-1 com supressão natural da viremia e evolução para a aids"**, área de concentração: Biologia Celular e Molecular. A banca examinadora foi constituída pelos Professores: Dr. Fernando Augusto Bozza - IOC/FIOCRUZ (presidente), Dr. André Felipe Andrade dos Santos - UFRJ/RJ e Dr. Aguinaldo Roberto Pinto - UFSC/SC e como suplentes: Drª. Milene Dias Miranda - IOC/FIOCRUZ e Dr. Tiago Graf - UFRJ/RJ.

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Documento registrado sob nº DCE-10015/18 no livro nº II, folha 58v em 29/03/2019.

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Dedico este trabalho a todos os indivíduos HIV+ que participaram desse projeto. Que nossas contribuições no entendimento da infecção pelo HIV possam ser revertidas em benefícios a todos que estão nessa luta.

AGRADECIMENTOS

Ao Dr. Gonzalo Bello pela orientação, comprometimento e cuidado singular na minha formação. Agradeço pelas discussões científicas, pelos ensinamentos fundamentais para o meu progresso acadêmico. O seu apoio, incentivo e confiança foram os pilares para que eu pudesse superar o medo de encarar diversos desafios dessa tese. Agradeço também ao meu amigo Gonza, que vibrou comigo as conquistas, e me apoiou nos momentos difíceis.

Ao Dr. Thiago Moreno pela orientação rica em discussões científicas, além dos conselhos pragmáticos e descontraídos, tornando muitos momentos mais leves. Estendo meus agradecimentos também a toda sua equipe de profissionais e alunos que estavam sempre dispostos a me ajudar.

À Dra. Fernanda Côrtes e ao Dr. Edson Delatorre pelo suporte científico, e ensinamentos para a condução de grande parte dos experimentos realizados nessa tese.

À equipe do Laboratório de Aids e Imunologia Molecular (LabAIDS) pela infraestrutura para o desenvolvimento dos experimentos dessa tese e pelas discussões científicas fundamentais para o meu avanço acadêmico.

As médicas do Laboratório de Pesquisa Clínica em DST-AIDS, responsáveis pelo recrutamento e acompanhamento dos pacientes aqui estudados, como também a todos os profissionais que fizeram a coleta do material biológico desses pacientes e deram suporte na obtenção das informações clínicas relevantes para a condução desse estudo. Agradeço também ao Dr. Marcelo Ribeiro-Alves, por todo suporte e pelas análises estatísticas dos experimentos conduzidos nessa tese.

Aos pacientes que voluntariamente durante anos doam seu material biológico e nos confiam esse material para estudo, sem o qual essa tese não seria possível.

À Dra Ana Carolina do Laboratório de Genética Molecular de Microrganismos (LGMM) pelo espaço disponibilizado no seu laboratório para que eu pudesse realizar grande parte das análises desse projeto e também a Dra. Érika e a Fernanda Freitas pelo suporte a equipamentos do LGMM que foram utilizados durante diversos experimentos dessa tese.

À Dra. Aline Moreira, Beatriz Muller e Marília Mello da Plataforma de Sequenciamento de Ácidos Nucléicos de Nova Geração e ao Dr. Otacílio e Angélica da Plataforma de PCR em Tempo Real do Instituto Oswaldo Cruz por todo suporte na condução de diversos experimentos dessa tese. Agradeço pelo zelo e atenção em todos os momentos. Estendo também, meus agradecimentos, a Plataforma de Sequenciamento de Nova Geração do Programa de Pós-Graduação em Genética/Departamento de Genética, IB/UFRJ e sua equipe.

À Leila Botelho e Marcele Bral do Laboratório de Laboratório Tecnologia e Diagnóstico – Biomanguinhos/FIOCRUZ que nos disponibilizou o uso do equipamento Luminex para as análises dos marcadores plasmáticos.

Ao Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) e Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ) pela bolsa de doutorado e taxa especial complementar - Nota 10 concedidas.

A Deus, meus pais e familiares por todo o apoio, incentivo para enfrentar os desafios. Durante o desenvolvimento dessa tese, perdi dois pilares da minha família, meu avô paterno (Nélio) e minha avó materna (Ondina), nos quais agradeço especialmente pelo carinho e por entenderem os momentos que não pude estar presente.

Ao meu marido, Edson Delatorre pela cumplicidade, incentivo em superar os desafios e também por ser meu amparo nos momentos difíceis.

Aos meus queridos amigos Daiana, Guille, Lili, Paula, Zezé, Duduca e Léo que foram grandes incentivadores, conselheiros e companheiros responsáveis por momentos inesquecíveis. Estendo meus agradecimentos aos meus amigos do 5º andar do Pavilhão 26, Rafael, Verônica, Sheila, Rosângela e Jacy, por toda alegria e descontração no dia a dia.

“Você nunca sabe que resultados virão da
sua ação. Mas se você não fizer nada,
não existirão resultados.”
Mahatma Gandhi

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RESUMO

TESE DE DOUTORADO EM BIOLOGIA CELULAR E MOLECULAR

Suwellen Sardinha Dias de Azevedo

Controladores do HIV (HIVc), são um grupo raro e singular de indivíduos infectados pelo HIV que controlam a replicação viral, mantendo a viremia em níveis baixos (<2000 cópias de HIV/ml, do inglês, *viremic controllers* – VC) ou indetectáveis (do inglês, *elite controllers* – EC), na ausência tratamento antirretroviral. Sugere-se que um conjunto de fatores genéticos e adquiridos do hospedeiro, bem como características virais específicas, contribuem para a restrição viral. No entanto, os mecanismos subjacentes ao controle natural da infecção pelo HIV-1 são complexos e multifatoriais, uma vez que a presença ou ausência de um fator isoladamente não é suficiente para desenvolver o perfil HIVc. Nosso objetivo neste estudo foi a caracterização de mecanismos responsáveis pelo controle e persistência viral em uma coorte de indivíduos infectados pelo HIV com diferentes níveis de supressão natural da viremia e evolução para a aids. Inicialmente, observamos que a diversidade das quasispécies provirais do HIV-1 associada ao PBMC (do inglês, *peripheral blood mononuclear cell*) em EC é significativamente menor do que em VC, mas não significativamente diferente entre subgrupos de EC exibindo carga viral indetectável persistente (do inglês*, persistent elite controllers* – PEC) ou episódios ocasionais de viremia transitória (do inglês*, ebbing elite controllers* – EEC). Alguns PEC e EEC exibiram populações provirais altamente homogêneas com grandes grupos de sequências idênticas (EC_{LD}), enquanto outros exibiram populações provirais relativamente diversas com uma elevada proporção de sequências únicas (EC_{HD}). Apesar das diferenças na diversidade genética proviral, os subgrupos EC_{LD} e EC_{HD} apresentaram níveis comparáveis de células T $CD8^+$ ativadas e biomarcadores inflamatórios plasmáticos (IP-10, IL-18, RANTES, PDGF-AA e CTACK) associados a predição da progressão virológica e/ou imunológica. Encontramos também dois indivíduos (um EC e um VC) com superinfecção intersubtipo pelo HIV-1. Através da caracterização longitudinal do reservatório proviral, descobrimos que ambos mantiveram o controle virológico sem evidência de evolução viral ou progressão imunológica por pelo menos 2 anos após o evento de superinfecção. Essas evidências sugerem que os fatores do hospedeiro provavelmente estariam envolvidos no controle virológico alcançado em pelo menos alguns indivíduos dessa coorte. Portanto, investigamos se fatores de restrição do hospedeiro (FR), proteínas da resposta imune inata, teriam um papel no controle natural da infecção pelo HIV-1 observado nesses indivíduos. Encontramos um aumento significativo do RNAm de p21 e MCPIP1 em PBMC de HIVc em comparação com indivíduos infectados pelo HIV em supressão pela terapia e não infectados pelo HIV, e a expressão desses dois FR foi significativamente correlacionada positivamente. Confirmamos a superexpressão de p21 em HIVc e mostramos pela primeira vez a superexpressão de MCPIP1 associada à supressão natural da replicação do HIV-1 *in vivo*. Juntos, esses resultados indicam que não há relação simples entre supressão viral sistêmica e a diversidade proviral intra-hospedeiro em HIVc cronicamente infectados e que os FR do hospedeiro, como p21 e MCPIP1, podem ter um papel importante no controle do HIV-1 em HIVc. A compreensão das vias regulatórias e o efeito desses FR da resposta inata criam novas perspectivas para prevenir a replicação do HIV-1 e a progressão da doença em indivíduos infectados pelo HIV-1.

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CHARACTERIZATION OF MECHANISMS OF VIRAL CONTROL AND PERSISTENCE IN A COHORT OF HIV-1-INFECTED INDIVIDUALS WITH NATURAL SUPPRESSION OF VIREMIA AND EVOLUTION FOR AIDS

ABSTRACT

PHD THESIS IN CELL AND MOLECULAR BIOLOGY

Suwellen Sardinha Dias de Azevedo

HIV controllers (HIVc), are a unique and rare group of HIV+ individuals who control viral replication, maintaining the viremia at low (<2000 HIV copies/ml, viremic controllers - VC) or undetectable (elite controllers - EC) levels in the absence of antiretroviral treatment. A set of host genetic and acquired factors, as well as specific viral characteristics, were suggested to contribute to the viral containment. However, the mechanisms underlying the natural control of HIV-1 infection are complex and multifactorial, as the presence or absence of one factor alone is not sufficient to achieve the HIVc profile. In this study, we aimed the characterization of mechanisms responsible for the viral control and persistence in a cohort of HIV+ individuals with different levels of natural suppression of viremia and evolution to AIDS. First, we found that HIV-1 diversity of the PBMC-associated proviral quasispecies in EC is significantly lower than in VC, but not significantly different between EC subgroups exhibiting persistent (PEC) undetectable viral load or occasional episodes of transient (EEC) viremia. Some PEC and EEC displayed highly homogenous proviral populations with large clusters of identical sequences (EC_{LD}), while others exhibited relatively diverse proviral populations with a high proportion of unique sequences (EC_{HD}). Despite the differences in proviral genetic diversity, the EC_{LD} and EC_{HD} subgroups displayed comparable levels of activated $CD8^+$ T cells and plasmatic inflammatory biomarkers (IP-10, IL-18, RANTES, PDGF-AA, and CTACK) associated with virologic and/or immunologic progression prediction. We also found two individuals (one EC and one VC) that undergone intersubtype HIV-1 superinfection. Through longitudinal characterization of the proviral reservoir, we found that both individuals maintained virologic control with no evidence of viral evolution or immunologic progression for at least 2 years after the superinfection event. These evidences suggested that host factors would probably be involved in the virologic control achieved in at least some individuals of this cohort. Thus, we investigated if host restriction factors (RF), proteins of the immune response, had a role in the natural control of HIV-1 infection observed in these individuals. We found a significant upregulation of p21 and MCPIP1 mRNA in PBMC from HIVc compared with both ART-suppressed and HIV-negative subjects, and the expression of these two RF was significantly positively correlated. We confirmed the overexpression of p21 in HIVc and showed for the first time the overexpression of MCPIP1 associated with natural suppression of HIV-1 replication *in vivo*. Together, these results indicate that there is no simple relationship between systemic viral suppression and intra-host proviral diversity in chronically infected HIVc and that host RF such as p21 and MCPIP1 may have a major role on HIV-1 control in HIVc. The understanding of the regulatory pathways and the effect of these RF of innate response creates novel perspectives to prevent HIV-1 replication and disease progression in HIV^+ individuals.

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ÍNDICE DE FIGURAS

LISTA DE SIGLAS E ABREVIATURAS

ADCC	citotoxicidade mediada por células dependentes de anticorpos
aids	síndrome da imunodeficiência adquirida
AP-1	do inglês, *activator protein 1*
APOBEC	Apolipoproteína B mediante RNAm semelhante ao polipeptídeo catalítico
ART	do inglês, *antiretroviral therapy*
bNAb	anticorpos amplamente neutralizantes
C/EBP	do inglês, *CCAAT/enhancer-binding protein*
CDK	cinases dependente de ciclinas
cDNA	DNA complementar
CO	coinfecção
CPI	complexo pré-integração
CRF	do inglês, *circulating recombinant forms*
DC	do inglês, *dendritic cells*
DNA	ácido desoxirribonucleico
EUA	Estados Unidos da América
FR	fatores de restrição
GALT	mucosa gastrointestinal
GI	gastrointestinal
HIV	vírus da imunodeficiência humana
HLA	do inglês, *Human leukocyte antigen*
HR	do inglês, *heptad repeat*
HSH	homens que fazem sexo com homens
HTLV-III	vírus T-linfotrópico humano do tipo III
ICTV	do inglês, *International Committee on Taxonomy of Viruses*
IF	do inglês, *fusion inhibitors*
IFITM	proteínas transmembranas induzidas por interferon
IFN-α	interferon do tipo 1
IN	integrase
IIN	do inglês, *integrase inhibitors*
ISG	do inglês, *interferon-stimulated genes*
kb	kilobase
KIR	do inglês, *killer immunoglobulin-like receptor*

LAV	vírus associado à linfadenopatia
LEDGF/p75	do inglês, *lens epithelium-derived growth fator*
LTNP	do inglês, *long-term nonprogressors*
LTR	do inglês, *long terminal repeats*
MCPIP1	proteína induzida por proteína quimiotática de monócito 1
MHC	do inglês, *major histocompatibility complex*
Mx	proteína de resistência à Myxovírus
NFAT	do inglês, *nuclear factor of activated T cells*
NF-κB	do inglês, *nuclear factor-κB*
NK	do inglês, *natural killer cells*
nm	nanômetro
NRTI	do inglês, *nucleoside/nucleotide reverse transcriptase inhibitors*
NNRTI	do inglês, non- *nucleoside reverse transcriptase inhibitors*
PAMP	padrões moleculares associados ao patógeno
PBMC	do inglês, *peripheral blood mononuclear cell*
PEP	do inglês, *post-exposure prophylaxis*
PR	protease
PrEP	do inglês, *pre-exposure prophylaxis*
PRR	do inglês*, pattern recognition receptors*
PT	progressores típicos
P-TEFb	do inglês, *positive transcription elongation factor b*
RNA	ácido ribonucléico
RP	do inglês, *Rapid Progressors*
RRE	do inglês, *rev responsive element*
RTC	do inglês*, reverse transcription complex*
SAMHD1	proteína 1 contendo o domínio HD e SAM
SI	superinfecção
SIV	vírus da imunodeficiência símia
Sp-1	do inglês, *specific factor 1*
TAR	do inglês, *tat responsive element*
TNFα	do inglês, *tumor necrosis factor*
TR	transcriptase reversa
UDI	usuários de drogas injetáveis
UNAIDS	do inglês, *Joint United Nations Programme on HIV/AIDS*
URF	do inglês, *unique recombinant forms*

1 INTRODUÇÃO

1.1 Descoberta da Síndrome da Imunodeficiência Adquirida (aids) e identificação do Vírus da Imunodeficiência Humana (HIV) como seu agente etiológico

No início da década de 1980, transcorreu a descrição da síndrome da imunodeficiência adquirida (aids) pelos Centros de Controle e Prevenção de Doenças dos Estados Unidos da América (CDC, do inglês *Centers for Disease Control and Prevention*), após relatos de que um grupo de homossexuais masculinos de Los Angeles apresentavam sintomas de pneumonia causada por *Pneumocystis jirovecii* (anteriormente denominado *Pneumocystis carinii*) (CDC 1981; Gottlieb et al. 1981) e um câncer raro de pele conhecido como sarcoma de Kaposi (Hymes et al. 1981), usualmente observados em pacientes imunocomprometidos. Posteriormente, relatos de casos similares foram observados em outros grupos sociais, como usuários de drogas intravenosas (Masur et al. 1982; Quagliarello 1982), hemofílicos (CDC 1982b), beneficiários de transfusão sanguínea (CDC 1982c), crianças (CDC 1982a), parceiras sexuais femininas de homens infectados (Masur et al. 1982; CDC 1983b), prisioneiros (CDC 1983a), haitianos (CDC 1982d) e indivíduos da África Central (Clumeck et al. 1983). Essas novas evidências reforçavam que a nova síndrome ocorria através de um agente infeccioso com transmissibilidade sanguínea, sexual e vertical.

O agente etiológico causador da aids foi isolado pouco tempo depois desses primeiros relatos, por dois grupos de pesquisa, liderados por Luc Montagnier (França) e Robert Gallo (EUA), que descobriram, separadamente, que os pacientes com aids poderiam ter sido infectados por um novo retrovírus (Barré-Sinoussi et al. 1983; Gallo et al. 1984; Popovic et al. 1984). Esse retrovírus foi inicialmente denominado como vírus associado à linfadenopatia (LAV) pelo grupo de pesquisa francês (Barré-Sinoussi et al. 1983) e como vírus T-linfotrópico humano do tipo III (HTLV-III) pelo grupo de pesquisa americano (Popovic et al. 1984). Posteriormente, esses dois vírus foram considerados isolados do mesmo retrovírus (Ratner et al. 1985) e em 1986 recebeu sua denominação atual como Vírus da Imunodeficiência Humana (HIV) (Coffin et al. 1986) pelo Comitê Internacional de Taxonomia de Vírus (ICTV, do inglês *International Committee on Taxonomy of Viruses*). No mesmo ano, uma modificação nessa nomenclatura foi necessária após Montagnier e colaboradores isolarem uma

nova variante do HIV em indivíduos de Guiné-Bissau e Cabo Verde (Clavel et al. 1986). Sendo assim, os primeiros isolados virais descobertos foram denominados HIV do tipo 1 (HIV-1) enquanto os novos isolados provenientes da região Ocidental da África como HIV do tipo 2 (HIV-2) (Barin et al. 1985; Clavel et al. 1986).

1.2 Epidemiologia do HIV/aids

O Programa Conjunto das Nações Unidas sobre HIV/AIDS (UNAIDS, do inglês *Joint United Nations Programme on HIV/AIDS*) e a Organização Mundial da Saúde (OMS), estimaram que, até 2017, 78 milhões de pessoas haviam sido infectadas pelo HIV mundialmente e 35 milhões haviam morrido de doenças relacionadas à aids (UNAIDS 2018). No final de 2017, estimou-se que cerca de 37 milhões de pessoas estavam infectadas pelo HIV, incluindo 1,8 milhão de crianças menores de 15 anos. A maioria das infecções globais (53%) ocorrem na África Oriental e Austral, com aproximadamente 20 milhões de casos (Figura 1). Na América Latina, a estimativa foi de 1,8 milhão de pessoas infectadas pelo HIV em 2017 (UNAIDS 2018).

Em 2017, estima-se que ocorreram mundialmente 1,8 milhão de novas infecções e 940 mil mortes por causas relacionadas à aids, o que representa um declínio de 47% de novas infecções comparado a 1996 (3,4 milhões) e de 51% nas mortes por causas relacionadas à aids em relação a 2004 (1,9 milhão) (UNAIDS 2018). As reduções nesses índices foram diretamente impulsionadas por um conjunto de medidas de expansão do acesso a terapia antirretroviral (ART). A maioria das regiões no mundo apresentaram declínio nesses índices entre 2010 e 2017 como África Oriental e Austral (novas infecções: 30%, mortes: 42%), África Ocidental e Central (novas infecções: 8%, mortes: 24%), Ásia e Pacífico (novas infecções: 14%, mortes: 39%), América do Norte e Europa Ocidental e Central (novas infecções: 8%, mortes: 36%), América Latina (novas infecções: 1%, mortes: 12%) e Caribe (novas infecções: 18%, mortes: 23%). Houve uma estabilidade no número de mortes por causas relacionadas à aids na Europa Oriental e Ásia Central desde 2010 enquanto que no Oriente médio e Norte da África esse índice apresentou aumento de 11% (UNAIDS 2018). Além disso, o número anual de novas infecções pelo HIV nessas duas regiões dobrou em menos de 20 anos.

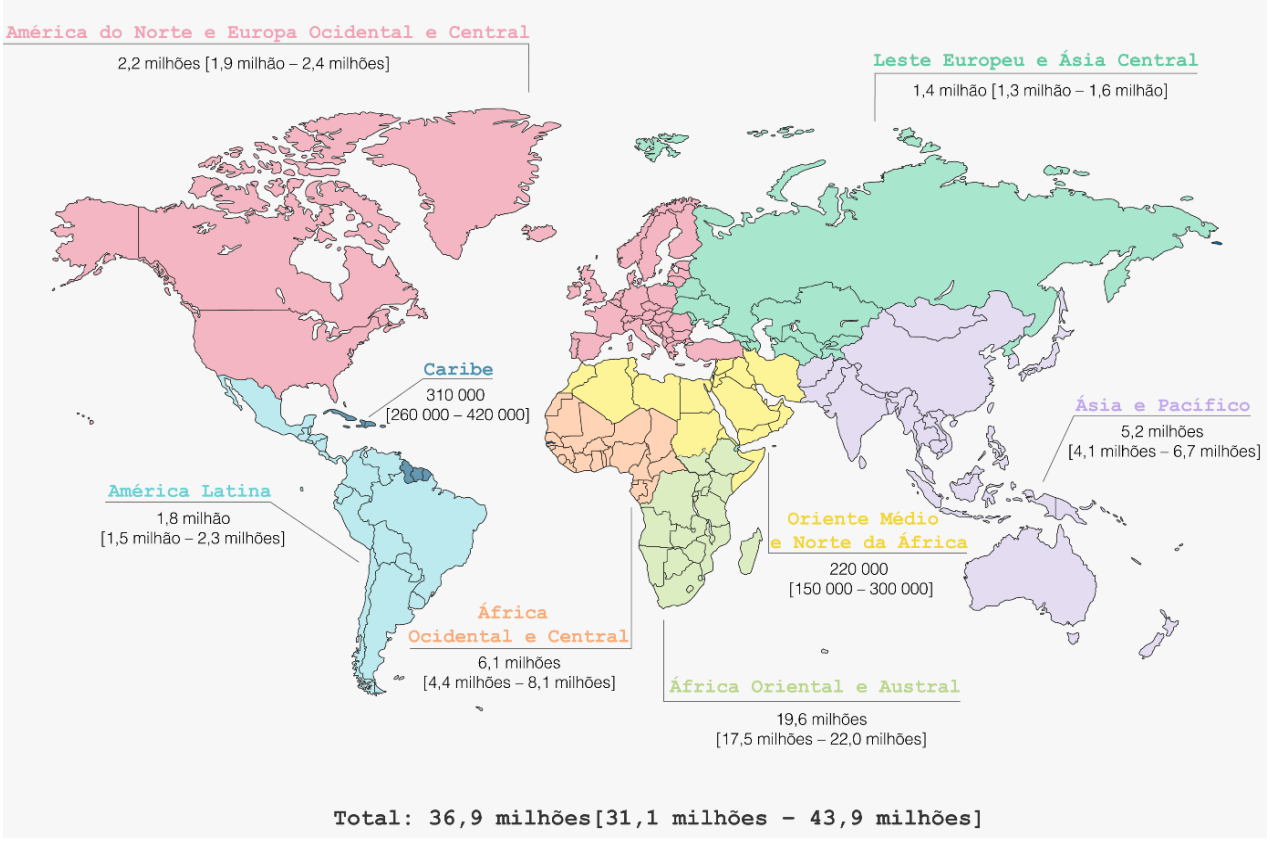

Figura 1: Estimativas globais de pessoas infectadas pelo HIV em 2017. Mapa representativo construído de acordo com as regiões analisadas pela OMS. A cor de cada região e o número estimado de pessoas vivendo com HIV (intervalo de confiança entre colchetes) estão indicadas na figura. Mapa adaptado a partir de dados da UNAIDS, 2018. Mapa disponível em https://www.freepik.com.

Apesar dessa redução global nas novas infecções na população geral, estima-se que em 2017, 47% das novas infecções no mundo foram concentradas nas populações definidas como populações-chave e seus parceiros sexuais (UNAIDS 2018). São consideradas populações-chave os grupos de homens gays e homens que fazem sexo com homens (HSH), usuários de drogas injetáveis (UDI), profissionais do sexo, travestis e transexuais e clientes de profissionais do sexo e outros parceiros sexuais de populações-chave (UNAIDS 2018). As populações-chave e seus parceiros sexuais representaram mais de 95% das novas infecções pelo HIV na Europa Oriental e Central e no Oriente Médio e Norte da África, 90% na Europa Ocidental e Central e na América do Norte, 84% na Ásia e no Pacífico e no Caribe, 77% na América Latina, 40% na África Ocidental e Central, 20% na África Oriental e Austral (UNAIDS 2018). Um dos grandes desafios no controle de novas infecções nessas populações está diretamente relacionada a uma resistência de se identificarem, especialmente em ambientes onde suas ações ou identidades são consideradas socialmente ou religiosamente inaceitáveis, ou são puníveis pela lei local (UNAIDS 2018).

A ART, cada vez mais disponível desde o final da década de 1990, mudou a natureza da infecção pelo HIV, reduzindo significativamente os casos de mortes relacionadas à aids e aumentando a expectativa de vida dos indivíduos infectados pelo HIV por manter suprimida a replicação viral na maioria dos pacientes tratados. Estimativas atuais evidenciam grandes avanços na amplitude do acesso a ART em algumas regiões como América do Norte e Europa Ocidental e Central, África Oriental e Austral, América Latina, Caribe e Ásia e Pacífico, com mais de 50% dos indivíduos infectados pelo HIV com acesso a ART em 2017. Entretanto, algumas regiões como Oriente Médio e Norte da África, Europa Oriental e Ásia Central e África Ocidental e Central ainda apresentam menos de 40% dos indivíduos infectados pelo HIV com acesso a ART (UNAIDS 2018).

Grandes avanços relacionados as estratégias terapêuticas vêm sendo observadas ao longo dos anos. Um desses avanços foi o uso da terapia como prevenção, construída com base nos resultados promissores de estudos observacionais em casais sorodiscordantes (ou seja, um HIV soronegativo e um parceiro soropositivo para o HIV) que demonstrou uma redução substancial (96%) no risco de transmissão sexual do HIV quando o parceiro soropositivo tinha RNA do HIV indetectável no plasma (Cohen et al. 2011, 2016). Outro benefício observado foi com o uso precoce da ART em que estudos demonstraram benefícios clínicos significativos nos indivíduos infectados pelo HIV, reforçando que os efeitos deletérios do vírus começam dias após a infecção, que aumentam o risco de mortalidade e eventos clínicos adversos (INSIGHT START Study Group et al. 2015; O'Connor et al. 2017).

Esses achados provocaram uma mudança nas diretrizes internacionais, que agora recomendam o uso da ART para todos os indivíduos infectados, numa estratégia conhecida como "testar e tratar" (Günthard et al. 2016; WHO 2016) e proporcionaram maior ímpeto para expandir o acesso à ART globalmente. Convergindo com essa estratégia, a UNAIDS propôs em 2014 o estabelecimento de novas metas de controle da infecção pelo HIV que se tornaram o pilar central de um esforço global pelo fim da epidemia de aids até 2030 (UNAIDS 2014). As metas definidas como 90-90-90 estabelecem que até 2020, 90% das pessoas infectadas pelo HIV no mundo sejam diagnosticadas, 90% dos indivíduos diagnosticados estejam em ART e 90% dos indivíduos tratados com ART tenham viremia suprimida. Até 2030, o objetivo é reduzir as novas infecções pelo HIV nos países de baixa e média renda para 200 mil novas infecções, uma redução de 89% em comparação aos índices

atuais, gerando benefícios tanto para a saúde quanto para a economia (UNAIDS 2014).

O Brasil compreende 35% da população total da América Latina e em 2017 conteve quase 50% das novas infecções pelo HIV e 37% das mortes por causas relacionadas à aids na região (UNAIDS 2018). Estima-se que até o final de 2017, cerca de 900 mil pessoas estavam infectadas pelo HIV no Brasil. A região Sudeste compreende 40% das novas infecções, seguida pelas regiões Nordeste (23%), Sul (19%), Norte (10%) e Centro-Oeste (8%) (MS 2018a). Nos últimos anos, tem se observado uma redução de 15% no coeficiente de mortes por causas relacionadas à aids e de 13% na taxa de detecção de novos casos de aids (Figura 2).

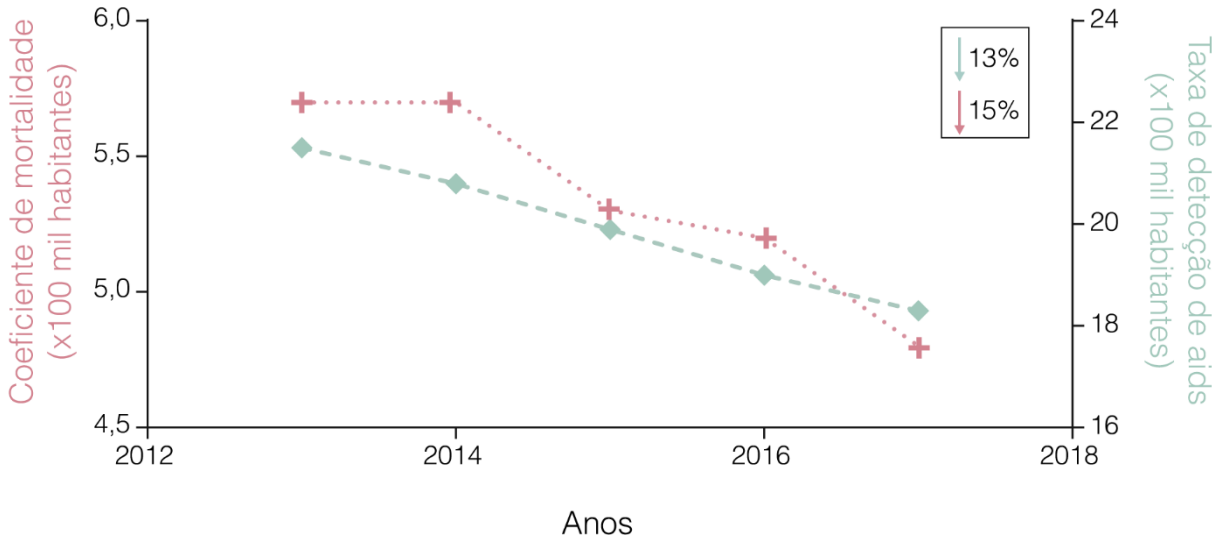

Figura 2: Indicadores epidemiológicos associados a aids no Brasil. Estão demonstrados o coeficiente de mortalidade (rosa) e a taxa de detecção de aids (verde) por 100.000 habitantes no Brasil entre 2013-2017. A caixa no canto superior direito indica as mudanças observadas no intervalo. Dados do Boletim epidemiológico HIV/Aids 2018 (MS 2018b).

A redução desses indicadores pode estar associada ao uso mais intensivo da ART e da ampliação do diagnóstico precoce da infecção no Brasil (MS 2018b). O Brasil foi um dos primeiros países da América Latina a adotar formalmente as metas 90–90–90 da UNAIDS e no início de 2014, recomendou o uso da ART a todos os indivíduos infectados pelo HIV. Atualmente, no entanto, somente 63% dos indivíduos infectados pelo HIV estão em uso da ART (MS 2017). Além do acesso gratuito a ART, o Brasil tem se destacado em iniciativas de prevenção do HIV sendo o único país da América Latina a oferecer gratuitamente a profilaxia pré-exposição (do inglês, *Pre-Exposure Prophylaxis* – PrEP) no setor público de saúde (UNAIDS 2018).

1.3 Origem e Epidemiologia Molecular do HIV-1

Estima-se que pelo menos 13 eventos de transmissão zoonótica do Vírus da Imunodeficiência Símia (SIV) de primatas não humanos para humanos ocorreram na África Centro-Ocidental dando origem as diferentes linhagens que compõe o HIV-1 e o HIV-2 (Keele et al. 2006; Sharp and Hahn 2011; D'arc et al. 2015). O HIV-1 é composto por quatro grupos, M (*major*), N (*non-M*, *non-O*), O (*outlier*) e P (Robertson 2000; Plantier et al. 2009), cada um resultante de um evento independente de transmissão zoonótica de SIV, que infecta chimpanzés da espécie *Pan troglodytes troglodytes* (SIV*cpz-Ptt*, grupos M e N) (Paraskevis et al. 2003; Keele et al. 2006) e gorilas da espécie *Gorilla gorilla* (SIVgor, grupos O e P) (Van Heuverswyn et al. 2006; Takehisa et al. 2009; D'arc et al. 2015). Os outros nove eventos independentes de transmissão zoonótica restantes foram de SIV de macacos *mangabey* (SIVsmm) dando origem aos grupos de A-I do HIV-2, entretanto, somente os grupos A e B causam um número significativo de infecções em humanos (Sharp and Hahn 2011). Enquanto o HIV-2 circula principalmente em países da África Ocidental, incluindo Senegal, Guiné, Gambia e Cabo Verde (Campbell-Yesufu and Gandhi 2011; de Pina-Araujo et al. 2014), e os grupos N, O e P do HIV-1 permanecem principalmente restritos à África Central (Peeters et al. 1997; Plantier et al. 2009; Vallari et al. 2010, 2011), o grupo M representa a forma pandêmica do HIV, infectando milhões de pessoas no mundo (Hemelaar et al. 2019).

O grupo M do HIV-1 se diversificou em diferentes linhagens, denominadas subtipos, designados pelas letras A, B, C, D, F, G, H, J e K, dos quais A e F ainda se subdividem em subsubtipos (A1-A6, F1-F2). Além desses subtipos, formas recombinantes entre os subtipos, designados como formas recombinantes circulantes (CRFs) e formas recombinantes únicas (URFs), também são descritos (Hemelaar et al. 2019). Os CRF são assim denominados quando o genoma completo de três ou mais vírus de indivíduos, não diretamente relacionados epidemiologicamente, são sequenciados e apresentam os mesmos pontos de recombinação. Foram identificados até o momento 98 CRFs (https://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html). As URFs referem-se a sequências recombinantes únicas sem evidência de transmissão posterior (Hemelaar et al. 2019). A variação genética média a nível de aminoácidos entre variantes de um mesmo subtipo (intrasubtipo) está na ordem de 8,2% (5,3 – 10,0%), enquanto que a variação média entre variantes de diferentes subtipos (intersubtipo) é

14.7% (12,2 – 15,8%), podendo variar de acordo com os subtipos e regiões genômicas que são examinadas (Li et al. 2015).

Existe uma grande variação na distribuição global dos subtipos e CRFs do grupo M do HIV-1 (Figura 3). Estimativas recentes mostram que entre 2010 – 2015, o subtipo C foi o responsável pela maioria (46,6%) das infecções pelo HIV-1 no mundo, seguido pelo subtipo B (12,1%), subtipo A (10,3%), CRF02_AG (7,7%), CRF01_AE (5,3%), subtipo G (4,6%) e subtipo D (2,7%). Os subtipos F, H, J e K combinados representaram 0,9% das infecções, enquanto outros CRFs e URFs foram responsáveis, respectivamente, por 3,7% e 6,1% das infecções (Hemelaar et al. 2019). Desta forma, aproximadamente 23% das infecções globais do HIV-1 são atribuídas a vírus recombinantes. Nesse mesmo período, a maioria (76%) das infecções pelo HIV-1 na América Latina foi causada pelo subtipo B, seguido pelos URFs (9,6%), subtipo C (7,3%), subtipo F1 (4,4%) e CRFs (2,5%) (Hemelaar et al. 2019).

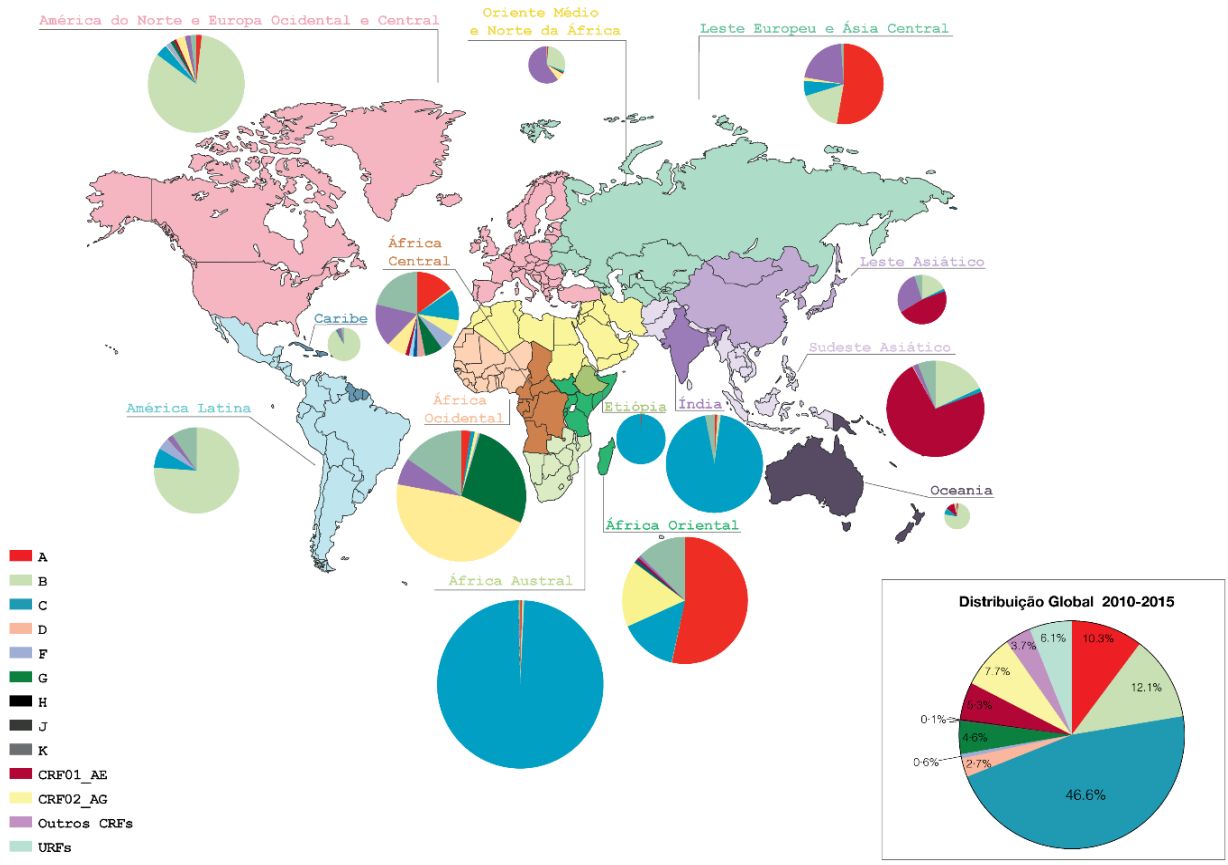

Figura 3: Distribuição global dos subtipos e formas recombinantes do HIV-1. As cores no mapa indicam as regiões geográficas de acordo com a UNAIDS, enquanto os gráficos indicam as prevalências dos subtipos e formas recombinantes em cada região. As cores dos gráficos referem-se aos diferentes clados do HIV-1 de acordo com a legenda à esquerda, e seu tamanho é proporcional ao número de pessoas vivendo com HIV-1 em cada região. O gráfico no canto inferior direito representa a prevalência global dos principais clados do HIV-1. Adaptado de Hemelaar et al. 2019.

No Brasil, a epidemia é composta majoritariamente pelo subtipo B (70 – 90%), seguido pelo subsubtipo F1 (5 – 20%) e subtipo C (1 – 10%), além das formas recombinantes entre eles (Bello et al. 2011; Ferreira 2011; Sanabani et al. 2011; Velasco-de-Castro et al. 2014; da Costa et al. 2016; Gräf et al. 2016; Delatorre et al. 2017). Entretanto, essas prevalências variam nas diferentes regiões do país (Figura 4). O subtipo B predomina na maioria das regiões geográficas brasileiras, exceto no Sul, onde o subtipo C e recombinantes BC alcançam altas prevalências (25-66%) (Passaes et al. 2009; Gräf and Pinto 2013; Gräf et al. 2016). Estudos mostram que houve um aumento na prevalência de subtipos não-B, particularmente de formas recombinantes, na última década no Brasil (Prellwitz et al. 2013; Pessoa et al. 2014; Pessôa et al. 2014; Delatorre et al. 2017; Reis et al. 2017). Também vêm sendo detectados, em diversas regiões no Brasil, o aparecimento de subtipos e formas recombinantes do HIV-1 característicos da epidemia africana, como os subtipos A e D e os recombinantes CRF02_AG e CRF45_cpx (Couto-Fernandez et al. 2006; Eyer-Silva and Morgado 2007; Pimentel et al. 2013; Delatorre et al. 2015, 2016).

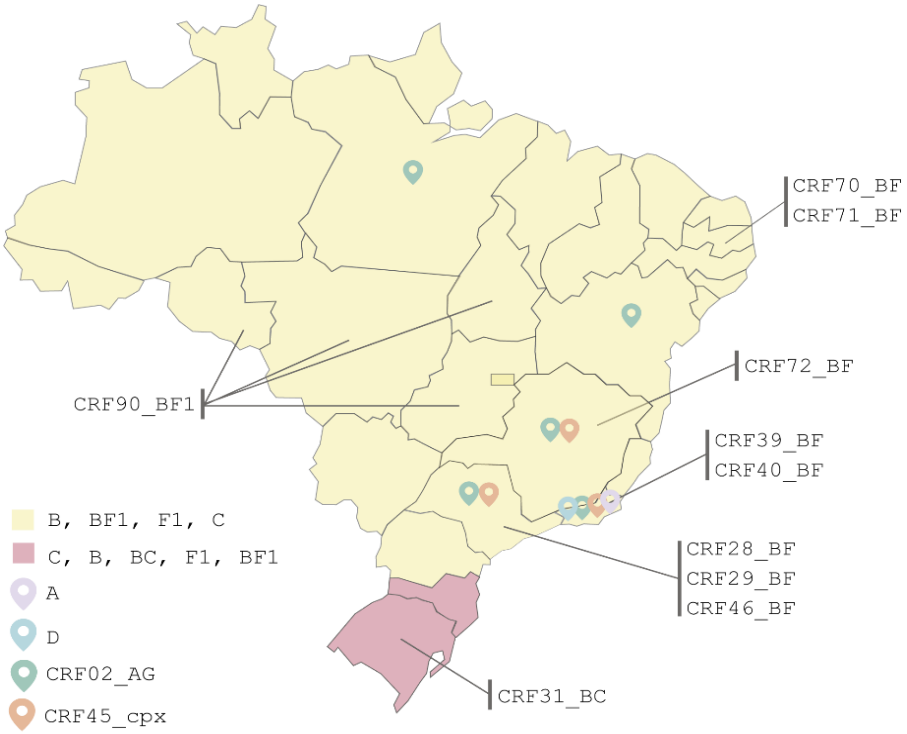

Figura 4: Mapa da distribuição dos clados do HIV no Brasil. Cada cor representa as diferentes prevalências dos subtipos e formas recombinantes presentes em cada estado. A ordem dos subtipos na legenda está organizada da maior para a menor prevalência. As CRFs brasileiras estão assinaladas de acordo com o estado onde ocorreu sua primeira descrição. Os estados com descrição de subtipos divergentes aos mais prevalentes estão indicados de acordo com a legenda. Mapa adaptado a partir dos dados de Bello et al. 2011; Gräf e Pinto 2013 e Los Alamos HIV DB)

1.4 Genoma do HIV e estrutura da partícula viral

O HIV é classificado segundo o Comitê Internacional sobre Taxonomia de Vírus (ICTV) na ordem *Ortervirales*, família *Retroviridae*, subfamília *Orthoretrovirinae* e gênero *Lentivirus* (Krupovic et al. 2018). As principais características dos retrovírus são a presença de um genoma composto por ácido ribonucleico (RNA) de sentido positivo e uma enzima ácido desoxirribonucleico (DNA) polimerase dependente de RNA denominada Transcriptase reversa (TR), que converte o RNA viral em uma forma complementar de DNA (cDNA) de fita dupla (Chiu et al. 1985; Herschhorn and Hizi 2010). Os lentivírus, são assim denominados por apresentarem um longo período entre a infecção inicial e o aparecimento de sintomas graves (período de incubação).

O genoma do HIV tem aproximadamente 10 kilobases (kb) de comprimento e é composto por 9 genes divididos em estruturais (*gag*, *pol* e *env*), acessórios (*nef*, *vif*, *vpr* e *vpu* – HIV-1 ou *vpx* – HIV-2) e regulatórios (*tat* e *rev*), flanqueados por duas regiões não codificadoras denominadas repetições terminais longas (LTR, do inglês *long terminal repeats),* importantes para os eventos de transcrição e integração do genoma viral ao cromossomo da célula hospedeira (Figura 5A). Além desses, os subtipos do grupo M do HIV-1 apresentam também um décimo gene (*asp*) sobreposto inversamente ao gene *env* (envelope) e que codifica a proteína antisenso (ASP). Estudos recentes apontaram a importância do gene *asp* e sua respectiva proteína na disseminação de alguns subtipos do grupo M do HIV-1 no mundo (Cassan et al. 2016), no estabelecimento e a manutenção da latência do HIV-1 e a indução da autofagia *in vitro* (Zapata et al. 2017; Liu et al. 2018).

Todos os retrovírus compartilham os genes *gag*, *pol e env* que codificam poliproteínas precursoras que após a clivagem, pela protease viral (PR) ou por proteases celulares, formam as proteínas estruturais (Herschhorn and Hizi 2010). O gene *gag* (antígeno específico do grupo), através da poliproteína P55, codifica as proteínas da matriz (P17), do capsídeo (P24), do nucleocapsídeo (P7) e P6. O gene *pol* (polimerase), através da poliproteína Gag-Pol, codifica as enzimas TR, PR e integrase (IN). O gene *env*, através da poliproteína GP160 clivada pela protease celular, codifica as glicoproteínas de superfície (GP120) e transmembrana (GP41) do envelope viral (Herschhorn and Hizi 2010).

O RNA genômico dos retrovírus complexos, como o HIV, apresentam múltiplos doadores de splicing alternativos que originam diversos RNAm que codificam as proteínas virais regulatórias (Tat e Rev) e acessórias (Vif, Vpr, Vpu, Vpx e Nef)

(Fanales-Belasio et al. 2010). A proteína Tat ativa a transcrição do genoma viral (Laspia et al. 1989; Karn and Stoltzfus 2012), enquanto a proteína viral Rev medeia o transporte dos RNAm virais para o citoplasma (Pollard and H 1998). As proteínas acessórias Vpr, Vpu, Vif (HIV-1) e Vpx (HIV-2) atuam como adaptadores de substrato para ubiquitina ligases celulares e regulam a degradação e o tráfico de proteínas celulares, sobretudo fatores de restrição (FR) da replicação viral (Malim and Emerman 2008). A proteína Nef é um importante fator de patogenicidade que, entre outras funções, promove a endocitose e degradação do receptor celular CD4 e das moléculas MHC de classe I, e inibe a síntese das moléculas MHC de classe II e de proteínas celulares que participam das cascatas de sinalização intracelular (Malim and Emerman 2008; Pawlak and Dikeakos 2015).

A partícula viral madura do HIV de aproximadamente 120nm de diâmetro é envolta por um envelope glicoproteico derivado da membrana plasmática da célula infectada (Figura 5B). Ancoradas ao envelope estão as espículas virais constituídas cada qual por um trímero das glicoproteínas GP120 e GP41. Abaixo do envelope viral, encontra-se a matriz do vírus, composta por subunidades da proteína P17, e o capsídeo viral de formato cônico, formado pela proteína P24. Protegidos por esse capsídeo, encontram-se as duas fitas simples de RNA genômico de polaridade positiva associadas a proteína P7, junto com as enzimas virais (PR, TR e IN) e as proteínas Vif, Vpr, Nef, Tat, Rev e P6 (Pornillos and Ganser-Pornillos 2013; Santos,Norma Suely de Oliveira; Romanos,Maria Teresa Villela; Wigg 2015).

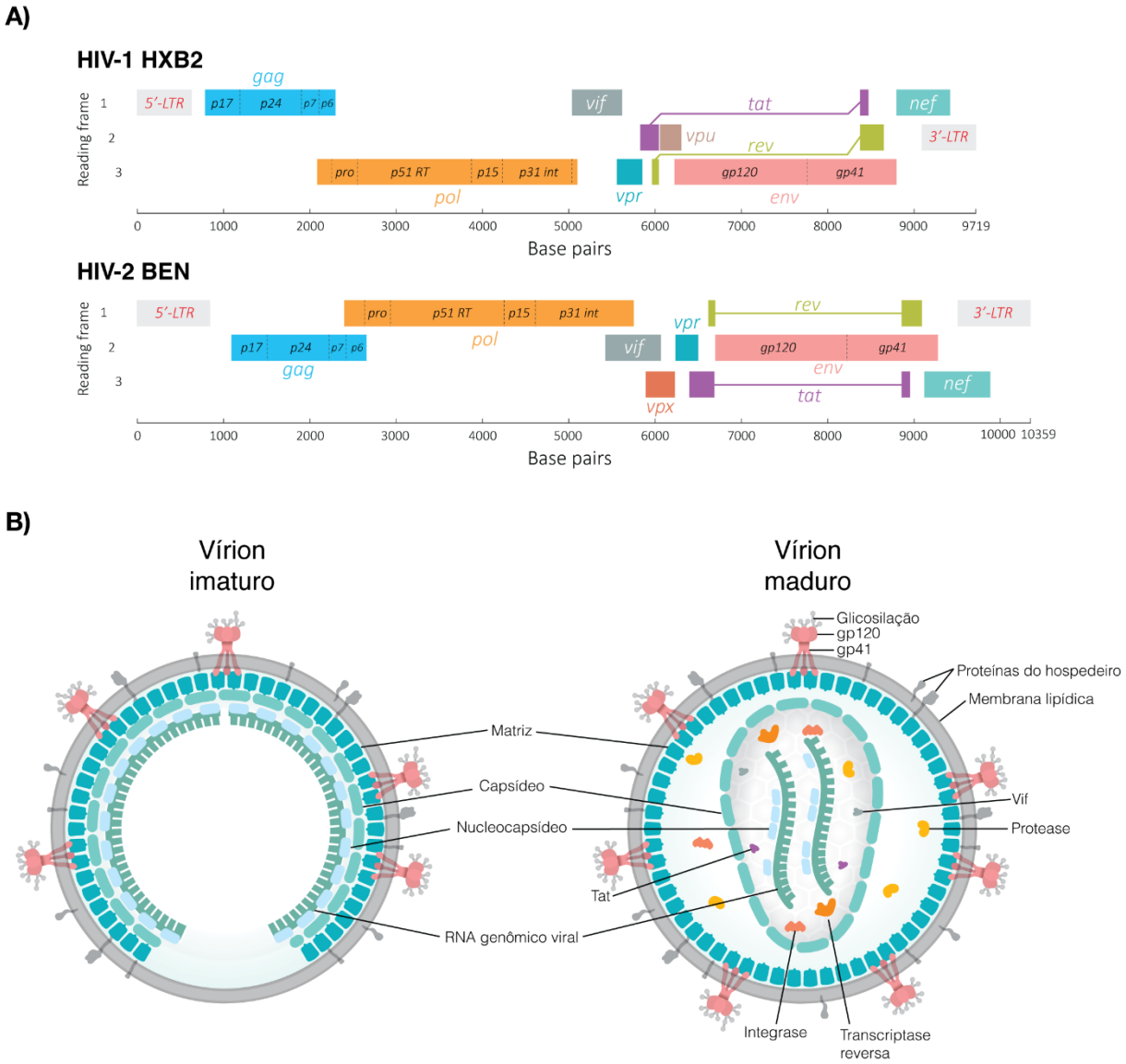

Figura 5: Estrutura do genoma e da partícula viral do HIV. A organização dos genomas do HIV-1 e HIV-2 (A) estão indicadas por retângulos coloridos representando cada gene. A disposição dos genes está de acordo com o frame de leitura onde estão codificados. A partícula viral antes e após a maturação pela ação da protease viral (B) estão indicadas, com seus principais constituintes assinalados. Baseado em https://www.hiv.lanl.gov/content/sequence/HIV/MAP/landmark.html e Pornillos e Ganser-Pornillos et al, 2013.

1.5 Ciclo replicativo do HIV-1

O ciclo replicativo do HIV-1 pode ser dividido em duas fases: inicial e tardia (Freed 2015). A fase inicial começa no processo de adsorção que consiste do reconhecimento inicial do receptor celular CD4 da célula hospedeira, presente principalmente na membrana dos linfócitos T $CD4^+$, macrófagos, células dendríticas (DCs) e a microglia, no sistema nervoso central (Stevenson 2003). Através da glicoproteína GP120 presente no envelope viral, o HIV-1 se liga ao primeiro domínio da molécula CD4, resultando em alterações conformacionais na GP120 que levam a

exposição da porção da alça V3, responsável por interagir com os correceptores celulares, proteínas das famílias CC ou CXC de receptores de quimiocinas, sendo CCR5 e CXCR4 os mais importantes (Wyatt 1998; Fanales-Belasio et al. 2010; Pornillos and Ganser-Pornillos 2013). Essa segunda ligação desencadeia uma mudança conformacional na porção *heptad repeat* 1 (HR1) da glicoproteína GP41, que se insere na membrana da célula alvo. A porção *heptad repeat 2* (HR2) da GP41 também passa por um rearranjo estrutural formando uma estrutura similar a um grampo, que proporciona a aproximação do envelope viral a membrana celular, promovendo a fusão das membranas e permitindo a passagem do nucleocapsídeo viral para o citoplasma (Chan and Kim 1998; Wyatt 1998; Fanales-Belasio et al. 2010).

À medida que o nucleocapsídeo (NC) viral é introduzido no citoplasma da célula, inicia-se o processo de desnudamento, em que a matriz (MA) e o capsídeo viral se dissociam gradativamente, enquanto a TR sintetiza uma fita complementar de DNA (cDNA) a partir do RNA genômico no citosol. Esse complexo viral é referido como complexo da transcrição reversa (RTC) (Campbell and Hope 2015). Durante a síntese do cDNA, a TR degrada o RNA genômico viral por sua atividade RNAse H, e por sua atividade DNA polimerase dependente de DNA, sintetiza a fita senso a partir do cDNA antisenso, formando o cDNA dupla fita viral que será translocado pela membrana nuclear juntamente com as proteínas virais (Vpr, MA, NC e IN) e celulares (principalmente a proteína associada a cromatina LEDGF/p75, do inglês *lens epithelium-derived growth fator*), formando o complexo pré-integração (CPI). A enzima viral IN que compõe o CPI, é a responsável pela integração do cDNA dupla fita ao DNA cromossômico da célula infectada juntamente com LEDGF/p75, estimulando essa transferência do cDNA viral para o DNA cromossômico (Craigie and Bushman 2012; Hu and Hughes 2012; Lusic and Siliciano 2017). A partir dessa etapa, o cDNA viral integrado passa a ser chamado então de provírus, concluindo desta forma a fase inicial do ciclo replicativo do HIV-1 (Li et al. 2011).

A fase tardia compreende a expressão gênica viral, síntese das proteínas virais, automontagem, brotamento e maturação das partículas virais (Freed 2015). Após a integração do cDNA ao DNA cromossômico, inicia-se a transcrição do provírus e a síntese dos RNAm virais pela RNA polimerase II celular, que se liga ao promotor localizado na região 5-LTR. Os LTRs são produzidos nas extremidades do DNA proviral durante o processo de transcrição reversa e apresentam várias sequências promotoras que recrutam fatores celulares, tais como NF-κB (do inglês, *nuclear factor-κB*), Sp-1 (do inglês, *specific factor 1*), C/EBP (do inglês, CCAAT/*enhancer-binding*

protein), NFAT (do inglês, *nuclear factor of activated T cells*) e AP-1 (do inglês, *activator protein 1*), necessários para o início da transcrição. No início, a transcrição é pouco eficiente e origina poucos RNAm, que sofrem múltiplos eventos de *splicing* e dão origem às proteínas Tat, Rev e Nef. A proteína Tat, se liga a uma estrutura secundária no RNA nascente denominada TAR (do inglês, *tat responsive element*) e recruta para o LTR o complexo fator positivo b de elongação da transcrição (P-TEFb, do inglês, *positive transcription elongation factor b*), formando um complexo tripartido P-TEFb-TAR-Tat que aumenta a taxa de processamento da RNA polimerase II. A proteína Rev liga-se a uma região secundária presente nos RNAm virais, denominada RRE (do inglês, *rev responsive element*), impedindo novos eventos de *splicing* e garantindo o transporte para fora do núcleo do RNA genômico viral e dos RNAm parcialmente processados, que codificam as proteínas precursoras virais (Santos,Norma Suely de Oliveira; Romanos,Maria Teresa Villela; Wigg 2015; Chen et al. 2018).

Enquanto o precursor GP160 é traduzido no retículo endoplasmático, as poliproteínas Gag e Gag-Pol são traduzidas pelos ribossomos livres no citoplasma, sendo a poliproteína Gag a responsável pelo recrutamento do RNA genômico e pelo acúmulo dos componentes virais próximo aos locais de brotamento para serem empacotadas como um vírion nascente (Lu et al. 2011; Craigie and Bushman 2012; Sundquist and Kräusslich 2012). Após essa automontagem, ocorre o recrutamento do mecanismo ESCRT celular (complexos de triagem endossômica necessários para o transporte) para entrarem no espaço extracelular, auxiliando na separação da partícula recém montada da membrana celular. Durante ou logo após o brotamento, a protease viral é ativada iniciando o processo de clivagem das proteínas precursoras Gag e Gag-Pol nos componentes estruturais P17, P24 e P7, bem como nas enzimas IN, TR e PR (Sundquist and Kräusslich 2012). Esse processo conhecido como maturação, promove uma reorganização espacial das proteínas virais, em que o capsídeo assume o formato cônico e a partícula se torna infecciosa, capaz de infectar uma nova célula (Sundquist and Kräusslich 2012).

1.5.1 Fatores de restrição (FR) da replicação do HIV

Durante as diferentes fases do ciclo viral na célula, diversas proteínas intracelulares comumente conhecidas como fatores de restrição (FR) atuam como uma primeira linha de defesa, na tentativa de interferir em etapas críticas da replicação viral e evitar a sua propagação (Figura 6) (Colomer-Lluch et al. 2018). Alguns desses

FR também atuam como sensores inatos desencadeando respostas inatas contra as infecções. Diversos FR já foram descritos como inibidores a replicação do HIV, sendo constitutivamente expressos em alguns tipos celulares, alguns FR podem ainda ser estimulados principalmente por interferon do tipo 1 (IFN-I), como APOBEC3G, SAMHD1, Teterina (BST-2), motivo tripartido 5 (TRIM5α), Mx2, proteínas transmembranas induzidas por interferon (IFITMs) e Schlafen 11 (SFLN11) (Kluge et al. 2015).

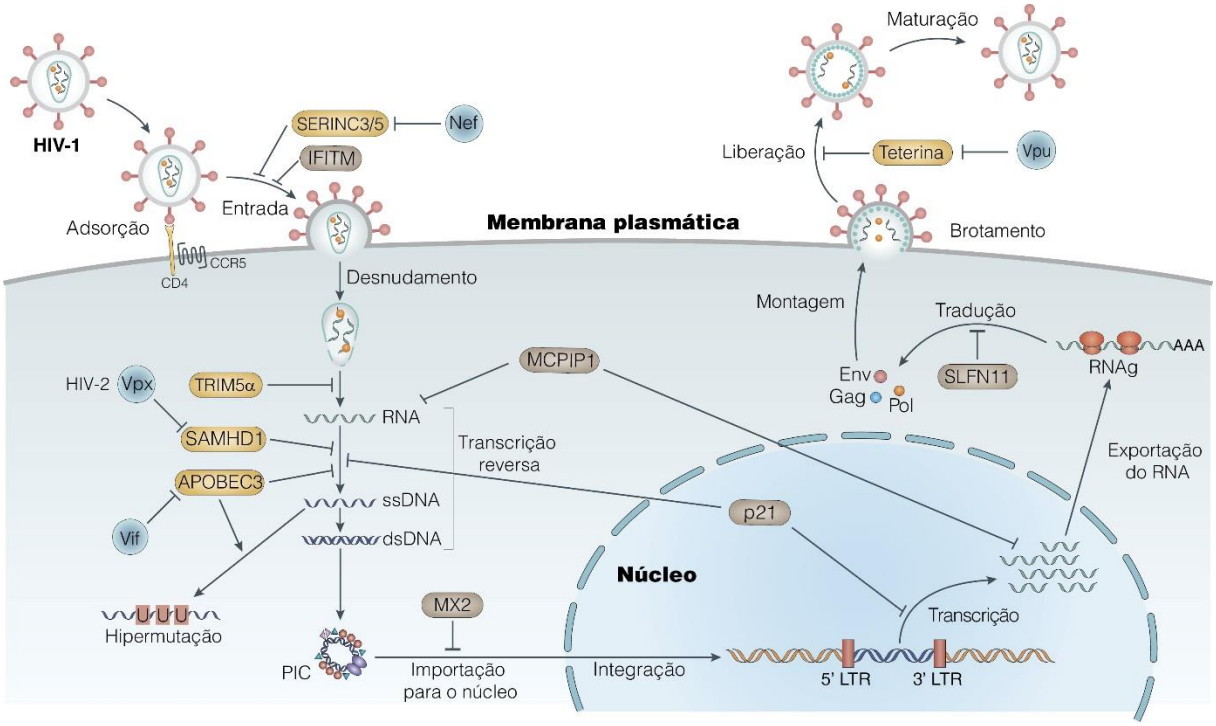

Figura 6: Disrupção do ciclo replicativo do HIV por diferentes fatores de restrição. Cada etapa do ciclo replicativo com atuação de algum fator de restrição estão indicados. Aqueles FR que possuem proteínas contrarreguladoras virais estão indicados em amarelo, enquanto a proteína viral está em azul. SERINC3/5 e IFITM interferem na etapa de entrada. TRIM5α atua no desnudamento, enquanto SAMHD1, APOBEC3 e p21 interferem na transcrição reversa. Mx2 desempenha um papel na prevenção da importação do complexo pré-integração (PIC) ao núcleo. p21, por seu papel no controle do ciclo celular, pode atuar na inibição da transcrição do genoma viral pela RNA polimerase II, enquanto MCPIP1 reduz RNAm virais e SLFN11 previne a tradução de proteínas virais. Teterina atua na última etapa do ciclo, interferindo com a liberação de novas partículas virais. Adaptado de Barré-Sinoussi et al. 2013 e Doyle et al. 2015.

1.5.1.1 Famílias IFITM e SERINC bloqueiam a entrada do HIV na célula

Duas famílias de proteínas celulares são responsáveis por restringir a entrada do HIV na célula hospedeira. As proteínas transmembranas induzidas por interferon (do inglês, *interferon induced transmembrane* – IFITMs) são encontradas na membrana celular de diferentes tipos celulares e apresentam ampla atividade contra vírus envelopados como vírus influenza A, vírus da hepatite C, vírus ebola, vírus da

dengue, HIV-1, entre outros (Shi et al. 2017). Três proteínas desta família (IFITM1, IFITM2 e IFITM3) apresentam atividade antiviral em humanos (Bailey et al. 2014). Acredita-se que essa atividade ocorra através de mudanças conformacionais tanto na membrana da célula do hospedeiro como na membrana dos vírions, como o aumento da curvatura, diminuição da fluidez e alterações na composição, impedindo assim a fusão entre as membranas celulares e virais (Foster et al. 2018). Esse bloqueio promovido pelas proteínas IFITMs parece depender também do tropismo viral para diferentes co-receptores celulares, sendo os vírus trópicos de CCR5 mais sensíveis à ação de IFITM1, enquanto os vírus trópicos de CXCR4 são mais sensíveis à ação de IFITM2 e IFITM3 (Foster et al. 2016, 2018).

Outra família de proteínas celulares, as incorporadoras de serina (SERINC) foram recentemente descobertas como fatores de restrição viral, atuando contra diversos vírus do gênero *Lentivirus* (HIV, SIV e vírus da anemia infecciosa equina) e *Gammaretrovirus* (vírus da leucemia murina) (Gonzalez-Enriquez et al. 2017). As proteínas SERINC fazem parte de uma família de proteínas transmembrana presentes em todas as células eucarióticas, compostas de cinco proteínas (SERINC1-5), entretanto apenas as SERINC3 e principalmente SERINC5 parecem atuar como FR da replicação viral (Gonzalez-Enriquez et al. 2017). Podendo também ser incorporados na membrana dos vírions, essas proteínas impedem a correta interação entre o envelope viral e a membrana celular. Estudos em células primárias demonstraram que essa inibição ou a redução da fusão das membranas pode ser mediado por três distintos mecanismos: alterando o tamanho do poro de fusão e impedindo a conformação correta da proteína viral GP41, mantendo a GP41 exposta por um longo período e tornando-a susceptível a ação de anticorpos neutralizantes, e/ou promovendo mudanças estruturais nas glicoproteínas do envelope viral que impedem a entrada do vírus (Gonzalez-Enriquez et al. 2017). Para evadir esse FR, a proteína Nef do HIV regula o transporte de SERINC3/5 e outras proteínas da membrana plasmática e endossomos para compartimentos como outros endossomos ou lisossomas para sua posterior degradação, impedindo dessa maneira a incorporação dessas proteínas nos vírions (Usami et al. 2015).

1.5.1.2 Trim5α, família APOBEC, SAMHD1 e p21 bloqueiam a transcrição reversa do HIV

Após a entrada da partícula viral na célula, uma proteína celular pertencente à família de proteínas tripartidas, a proteína contendo motivo tripartido 5 (TRIM5α) atua

como um FR, bloqueando a replicação do HIV pós-entrada (Colomer-Lluch et al. 2018). As infecções virais são detectadas na célula por componentes do sistema imune inato, chamados receptores de reconhecimento de padrões (PRRs), que reconhecem características químicas e estruturais conservadas de patógenos, chamados de padrões moleculares associados ao patógeno (PAMPs) (Takeuchi and Akira 2010). TRIM5α atua como um PRR específico para o capsídeo viral e inicia a sinalização da resposta imune inata mediada pela cascata de IFN-α, aumentando a expressão de genes pró-inflamatórios e citocinas e potencializando a restrição viral (Pertel et al. 2011; Doyle et al. 2015). Essa proteína também atua restringindo a infecção diretamente de diferentes retrovírus (principalmente SIV e HIV) em várias linhagens de células de mamíferos. Entre os três domínios da proteína, o domínio RING possui atividade ubiquitina-ligase E3 e essa atividade é amplificada após interações entre TRIM5α – capsídeo viral, direcionando o capsídeo viral para a degradação, promovendo um desnudamento precoce desse capsídeo e uma transcrição reversa ineficiente (Santos,Norma Suely de Oliveira; Romanos,Maria Teresa Villela; Wigg 2015). O TRIM5α humano é incapaz de restringir eficientemente o HIV-1. O mecanismo pelo qual o HIV-1 neutraliza a ação da TRIM5α em humanos não está inteiramente esclarecido, mas aminoácidos específicos na proteína do capsídeo estariam envolvidos no bloqueio da ação de TRIM5α durante o desnudamento/transcrição reversa (Javanbakht et al. 2006; Speelmon et al. 2006).

Durante a transcrição reversa viral, proteínas da família de enzimas editoras de Apolipoproteína B mediante RNAm semelhante ao polipeptídeo catalítico (APOBEC) também atuam na contenção da replicação do HIV. Essa família compreende as citidinas desaminases APOBEC1, APOBEC2, APOBEC3A-H e APOBEC4. A subfamília de APOBEC3 (A3A-H) exibem diferenciados níveis de atividade antiviral, sendo as proteínas A3G e A3F as que exercem uma restrição ao HIV mais potente (Simon et al. 2015; Grant and Larijani 2017). O aumento na expressão de A3G/F ocorre por vários mediadores da inflamação, como o IFNα (Sarkis et al. 2006), indicando que essas proteínas são codificadas por genes estimulados por IFN (do inglês, *interferon-stimulated genes* – ISGs) (Colomer-Lluch et al. 2018). O mecanismo de ação dessas proteínas é exercido após A3G/F sejam empacotadas no vírion, manifestando-se apenas no próximo ciclo de infecção (Simon et al. 2015). A ação de A3G/F promove a desaminação de citosina na fita negativa do DNA viral convertendo-a em uracila, que ao ser lida pela TR para síntese da fita positiva do DNA viral resulta na hipermutação de guanina para adenina. Dependendo do grau de desaminação, as

moléculas de DNA viral podem ser reconhecidas como aberrantes e degradadas pela célula, ou podem ser integrados ao DNA genômico, mas não serem capazes de produzir progênie infecciosa uma vez que as hipermutações podem comprometer a codificação de proteínas virais funcionais. Para neutralizar a atividade antiviral de A3G, a proteína viral Vif induz a sua degradação antes do seu empacotamento no vírion através do recrutamento de um complexo E3 ubiquitina ligase contendo a proteína CUL5 e os adaptadores de substrato ELOB e ELOC. A formação desse complexo de ubiquitina – A3G promove a rápida degradação dessas proteínas pelo proteassoma (Simon et al. 2015).

Nessa mesma etapa do ciclo viral, os desoxinucleosídeos trifosfatos (dNTPs) são requeridos como substratos para a síntese do cDNA viral pela TR. A disponibilidade desses dNTPs na célula é mediada pela proteína 1 contendo o domínio HD e SAM (SAMHD1) (Chen et al. 2019). SAMHD1 é uma enzima citosólica com atividade de fosfohidrolase de dNTPs (Goldstone et al. 2011; Laguette et al. 2011), sendo expressa em leucócitos $CD4^+$ periféricos, incluindo células mieloides (macrófagos e células dendríticas) e linfócitos T $CD4^+$ em repouso (Laguette et al. 2011). Essa proteína também apresenta atividade exonuclease, podendo degradar os ácidos nucléicos virais (Choi et al. 2015). SAMHD1 desempenha papel crucial na manutenção de um pool baixo de dNTPs celulares em células que estão em estágio de repouso (Amie et al. 2013), enquanto que em células em divisão, que necessitam de um pool maior de dNTPs, a atividade enzimática de SAMHD1 é inibida pela fosforilação mediada pelas cinases dependente de ciclinas 1 e 2 (CDK1, CDK2) (Soper et al. 2018). Alguns fatores de autorregulação desse processo podem potencialmente bloquear a replicação do HIV-1, como é o caso do inibidor de cinase dependente de ciclina (CDKN1A/p21) que ao interromper o ciclo celular bloqueia a biossíntese de dNTP através da inibição direta das CDK, contribuindo indiretamente para a atividade antiviral de SAMHD1 (Pauls et al. 2014). Para antagonizar SAMHD1, um mecanismo mediado pela proteína Vpx presente apenas no HIV-2 e alguns SIVs induz a ubitiquinação e degradação de SAMHD1 através do recrutamento de um complexo E3 ubiquitina ligase, direcionando a proteína antiviral a degradação via proteossoma (Laguette et al. 2011).

1.5.1.3 Mx2 bloqueia a importação do PIC para o núcleo

Antes da integração do genoma viral ao DNA cromossômico da célula, a proteína de resistência à Myxovírus do tipo 2 ou B (Mx2/B) localizada na membrana

nuclear, no complexo poro-nuclear (Doyle et al. 2015), atua inibindo a importação nuclear do PIC e a subsequente integração do provírus (Soper et al. 2018). Embora induzida por IFNα, a expressão do gene Mx, diferentemente de outros ISGs, não é induzida por outras citocinas ou diretamente por estímulos virais (Haller 2013). Apesar do mecanismo antiviral mediado por Mx2 não esteja completamente elucidado, sabe-se que interações diretas com a proteína do capsídeo viral (Doyle et al. 2015) estão envolvidos no processo e que essa atividade é dependente de ciclofilina a (CypA), um fator celular que potencializa a infecção pelo HIV-1 por guiar o capsídeo viral para as nucleoporinas, facilitando a importação do genoma viral para o núcleo (Staeheli and Haller 2018).

1.5.1.4 MCPIP1, Schlafen11 e p21 reduzem os níveis de RNAm/proteínas virais

O FR conhecido como proteína induzida por proteína quimiotática de monócito 1 (MCPIP1), atua como um regulador crítico da resposta inflamatória e homeostase imune, possuindo uma expressão enriquecida em leucócitos (Fu and Blackshear 2017). Além do domínio dedo de zinco, esta proteína também possui um domínio RNAse, um domínio associado a ubiquitina na porção N-terminal e um domínio rico em serinas na porção C-terminal (Yokogawa et al. 2016). MCPIP1 atua como uma RNase, promovendo a degradação do RNAm de algumas citocinas inflamatórias (Matsushita et al. 2009; Iwasaki et al. 2011) e clivagem de precursores de miRNAs (Suzuki et al. 2011). No caso da infecção pelo HIV-1, MCPIP1 pode atuar indiretamente, limitando a infecção nos linfócitos T $CD4^+$, ao suprimir o estado geral de ativação celular através da inibição da ativação do NF-κB e da via de sinalização do receptor de linfócitos T, quanto diretamente ao reduzir os níveis de múltiplas espécies de RNAm viral (Liu et al. 2013). A proteína p21 também pode restringir a transcrição dos RNAm virais inibindo a enzima CDK9, requerida para otimizar o enlongamento desses transcritos ou pelo bloqueio da biossíntese de dNTPs, principalmente através da supressão da subunidade RNR2 da enzima ribonucleotídeo redutase (Chowdhury et al. 2003). Este segundo mecanismo restringe não somente a transcrição de RNAm virais, como também a própria etapa de transcrição reversa do HIV.

A quantidade de proteínas virais que são produzidas na célula infectada pode ser limitada pela ação da proteína celular induzida por IFN-I, Schlafen 11 (SLFN11) (Soper et al. 2018). Devido ao viés do uso de códons do HIV-1, que possui grande quantidade de códons ricos em A/U, o vírus estimula que a célula produza os RNAt

correspondentes para favorecer a tradução de proteínas virais. Este mecanismo é parcialmente limitado por SLFN11, que se liga aos RNAt de uma maneira códon específica (Seissler et al. 2017). Este mecanismo foi proposto originalmente em linhagens de células humanas e em linfócitos T $CD4^+$ primários ativados (Li et al. 2012).

1.5.1.5 Teterina impede o brotamento de novas partículas do HIV

A teterina (também conhecida como BST-2 - antígeno estromal da medula óssea 2) é uma proteína transmembrana induzível por IFN-I, capaz de ancorar potentemente partículas virais envelopadas em brotamento nas células infectadas (Colomer-Lluch et al. 2018). Esta proteína possui uma topologia única, composta por uma porção citoplasmática curta seguida por um domínio transmembrana na porção N-terminal e uma âncora de glicosil-fosfatidilinositol na porção C-terminal, que permite que uma extremidade fique ligada a membrana plasmática e a outra ao envelope viral (Yang et al. 2010). Os vírions retidos por esta proteína são então internalizados e degradados através da via endossomal/lisossomal (Perez-Caballero et al. 2009). A teterina também pode atuar como um sensor viral da imunidade inata, por conta da indução de respostas pro-inflamatórias devido ao acúmulo de vírions retidos na membrana plasmática (Galão et al. 2012), assim como um modulador da imunidade celular, através da melhora na ativação das células dendríticas e na apresentação de antígenos por MHC-II (Li et al. 2016). Uma vez que a teterina não possui como alvo proteínas virais específicas, e sim interage com o envelope viral que deriva em grande parte da célula hospedeira, esta proteína exerce atividade antiviral contra um amplo espectro de vírus envelopados (Tokarev et al. 2009). Entretanto, alguns lentivírus de primatas desenvolveram diferentes mecanismos para escapar da restrição de teterina. A proteína Vpu do HIV-1 sobrepuja a restrição da teterina humana através de três mecanismos: (i) promovendo a poli-ubiquitinação do seu domínio transmembranar e induzindo sua degradação protéica, (ii) regulando negativamente a concentração de teterina na superfície celular e (iii) sequestrando teterina em compartimentos endossomais que levam a sua degradação lisossômica (Colomer-Lluch et al. 2018).

1.5.2 Terapia antirretroviral (ART) e o controle exógeno da replicação do HIV-1

Para a maioria dos indivíduos infectados pelo HIV-1, a única possibilidade de supressão da replicação viral e da progressão da infecção pelo HIV-1 é através da terapia antirretroviral. Além disso as estratégias terapêuticas atualmente adotadas não

visam somente a redução ao risco de progressão da doença como também é utilizada como medida de prevenção da transmissão do vírus (Pau and George 2014; Cohen et al. 2016).

Ao longo dos anos, desde os primeiros casos de infecção pelo HIV-1, diversos antirretrovirais (ART) têm sido aprovados pela agência americana de regulação FDA (do inglês, *Food and Drug Administration*) e estão disponíveis para o tratamento dos pacientes infectados pelo HIV (FDA 2018). Esses fármacos, foram desenvolvidos para atuar bloqueando diferentes etapas do ciclo do HIV, de forma que a terapia antirretroviral encontra-se dividida em seis principais classes: inibidores da transcriptase reversa análogos de nucleosídeos e nucleotídeos (do inglês, *nucleoside/nucleotide reverse transcriptase inhibitors* – NRTI), inibidores não nucleosídeos da transcriptase reversa (do inglês, *non-nucleoside reverse transcriptase inhibitors* – ITRNN), inibidores de protease (do inglês, *protease inhibitors* PI), inibidores de integrase (do inglês, *integrase inhibitors* – IIN), inibidores de fusão (do inglês, *fusion inhibitors* – FI) e antagonistas do correceptor CCR5 (Arts and Hazuda 2012).

No Brasil, o esquema terapêutico preferencialmente sugerido como inicial deve sempre incluir combinações de três fármacos, sendo dois pertencentes as classes NRTI/ NNRTI associados a uma outra classe de antirretrovirais (NNRTI, PI ou IIN). Atualmente têm sido indicados como primeira linha terapêutica os fármacos lamivudina (3TC) e tenofovir (TDF), ambos da classe dos NRTI, associados com dolutegravir (DTG), inibidor da integrase (MS 2018b). Além do tratamento clássico, o Brasil foi um dos pioneiros no uso de medidas de prevenção, oferecendo gratuitamente a profilaxia pré (PrEP) e pós- exposição (PEP) (MS 2018d). A PreP, composta pelos medicamentos TDF e entricitabrina (FTC) é uma maneira adicional de prevenção para pessoas não infectadas pelo HIV, mas que se apresentam em potencial risco de contrair a infecção. Enquanto que a PEP, composta pelos medicamentos TDF + 3TC (Truvada) + DTG, é indicada as pessoas que foram potencialmente expostas ao HIV nas últimas 72 horas (MS 2018c).

1.6 Imunopatogênese da infecção pelo HIV-1

1.6.1 Transmissão

O HIV-1 pode ser transmitido pelo contato sexual através das superfícies mucosas dos tratos genital ou retal, pela exposição materno-infantil (transmissão vertical) e pela inoculação percutânea, sendo a transmissão sexual responsável por cerca de 70% das infecções mundiais (Shaw and Hunter 2012). Na ausência da ART, a probabilidade de transmissão por contato sexual vaginal não chega a 0,1% (Patel et al. 2014). Nas relações sexuais, entretanto, o risco de transmissão do HIV-1 é aumentado substancialmente na presença de outras infecções sexualmente transmissíveis, pela viremia plasmática do HIV-1 aumentada, pela taxa de exposição a múltiplos parceiros e por fim, em casos de relações anais receptivas (Galvin and Cohen 2004). Por conta da ausência de uma barreira física, a transmissão intravenosa apresenta um risco estimado de >1% (Royce et al. 1997), enquanto que a transmissão da mãe para o filho durante a gravidez, o parto e durante a amamentação são estimados em 25%-40% (Prendergast et al. 2007).

Sendo a transmissão sexual a maior responsável pelas infecções, a transmissão do HIV através das mucosas é geralmente estabelecida por um único vírus fundador, que possui propriedades únicas, como o uso preferencial do correceptor celular CCR5 (variantes R5-trópicas) em vez de CXCR4 (variantes X4-trópicas) (Keele et al. 2008). Essa transmissão preferencial por vírus R5-trópicos pode ser atribuída, em parte, à expressão aumentada de CCR5 em células T $CD4^+$ que estão presentes na lâmina própria das mucosas (Lackner et al. 2012) como também pela capacidade diferencial das DCs de capturar e transferir passivamente os vírus R5-trópicos mais eficientemente do que os vírus X4-trópicos (Parrish et al. 2013; Maartens et al. 2014). Além disso, estudos mostram que variantes R5, podem apresentar resistência ao IFN-α, favorecendo o estabelecimento da infecção nos estágios iniciais (Parrish et al. 2013; Maartens et al. 2014). Entretanto a transmissão de múltiplas variantes do HIV-1 já foi observada (Sagar et al. 2004, 2006; Abrahams et al. 2009; Kearney et al. 2009; Novitsky et al. 2011) assim como a transmissão de vírus X4-trópicos (de Mendoza et al. 2007; Poveda et al. 2007; Sierra-Enguita et al. 2014).

1.6.2 Características clínicas da infeção pelo HIV-1 na ausência da ART

A história natural da infecção pelo HIV-1 é conceitualmente dividida em três fases: aguda, crônica (assintomática) e aids (Maartens et al. 2014) (Figura 7). A fase aguda inicia-se imediatamente após a transmissão, quando a maioria dos pacientes desenvolve sintomas clínicos inespecíficos da infecção pelo HIV-1, que duram geralmente por cerca de duas semanas e coincidem com um pico de viremia plasmática e um declínio acentuado dos linfócitos T $CD4^+$ periféricos (Coffin and Swanstrom 2013). A medida que os sintomas desaparecem, a viremia decai abruptamente, atingindo um patamar estável, conhecido como set point viral (Little et al. 1999; Coffin and Swanstrom 2013). Esse período marca o início da fase crônica, de duração variável entre os indivíduos infectados, que normalmente se estende por 8-10 anos, na ausência de sintomas. No entanto, durante a fase crônica o vírus continua replicando e se observa uma perda gradual dos linfócitos T $CD4^+$ periféricos. Tipicamente, após um longo período de infecção e na ausência de ART, as contagens de linfócitos T $CD4^+$ declinam para níveis críticos (abaixo de 200 células/mm^3), tornando os indivíduos infectados mais vulneráveis a infecções oportunistas, marcando a entrada na fase de aids e posterior progressão para a morte (Coffin and Swanstrom 2013).

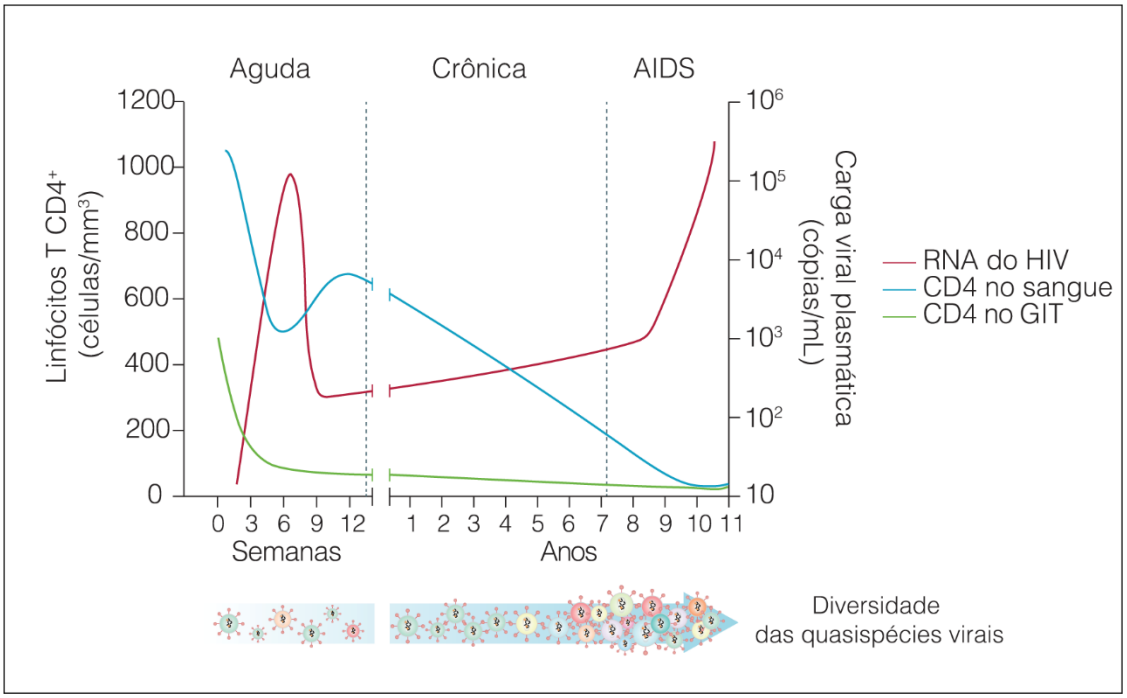

Figura 7: História natural da infecção pelo HIV. Mudanças dinâmicas na viremia plasmática (linha vermelha), nas contagens de células T CD4⁺ no sangue (linha azul) e no trato gastro-intestinal (GIT, linha verde) durante a progressão da doença estão indicados em função do tempo. Cada linha do gráfico representa uma das características indicadas de acordo com as cores. Após a fase aguda da infecção, a viremia plasmática é reduzida e a contagem de células CD4⁺ é recuperada parcialmente. À medida que a fase crônica avança, o controle imune é gradualmente perdido até que o indivíduo entre na fase final da infecção pelo HIV, conhecida como aids. Diferenças nas taxas de diversidade das quasispécies virais ao longo do tempo estão representadas esquematicamente na parte inferior do gráfico. Adaptado de Maartens et al. 2014.

1.6.2.1 Fase aguda

Na transmissão sexual, o HIV-1 infecta inicialmente as DCs intraepiteliais e submucosas, além de células T CD4⁺ presentes nas mucosas (genital ou retal) que expressam o receptor CD4 e o correceptor CCR5 (Hladik et al. 2007). As DCs infectadas migram para os linfonodos, onde encontram células T CD4⁺ ativadas que são altamente permissivas à infecção (Pan et al. 2013). Como a infecção é estabelecida no tecido linfoide secundário, a replicação acontece livremente se espalhando do local inicial da infecção para outros tecidos e órgãos. Nos primeiros 10 dias de infecção, denominada fase de eclipse, a viremia é indetectável e sem sintomas clínicos aparentes (Coffin and Swanstrom 2013). Após esse breve período, a replicação viral aumenta exponencialmente, com a viremia plasmática atingindo um pico de ~10^7 cópias de RNA viral por mililitro de sangue e grandes proporções de células T CD4⁺ infectadas no sangue e nos linfonodos após três a quatro semanas. É possível observar nesse período sintomas semelhantes aos da gripe (febre, aumento

dos gânglios linfáticos). No momento em que a viremia atinge altos níveis, a resposta imune surge tanto na forma de células T $CD8^+$, específicas contra os antígenos do HIV-1 expressos na célula infectada, quanto de anticorpos contra as proteínas virais (Koup et al. 1994; Pantaleo et al. 1994; Coffin and Swanstrom 2013). No final da fase aguda, há um declínio acentuado da viremia plasmática decorrente do controle parcial da replicação viral pelo sistema imunológico, como também do esgotamento das células-alvo ativadas. Esse controle parcial é um forte preditor da progressão subsequente da infecção (Coffin and Swanstrom 2013).

1.6.2.1.1 Infecção precoce no GALT

Os tecidos linfoides associado ao intestino (GALT) representam o maior componente do sistema linfoide, abrigando grande parte dos linfócitos e macrófagos totais do corpo (Karris and Smith 2011; Lackner et al. 2012; Thompson et al. 2017). As populações de linfócitos podem ser divididas entre as constituintes do epitélio e da lâmina própria, sendo esse último um sítio anatômico que abriga um vasto reservatório de células T $CD4^+$ (Lackner et al. 2012). Em condições normais, o GALT mantém um estado de inflamação "fisiológica" que ocorre devido à contínua estimulação antigênica luminal, capaz de manter a produção de citocinas e quimiocinas pró-inflamatórias (Monteleone et al. 2002; McGowan et al. 2004; Karris and Smith 2011). Além disso, em contraste com o sistema imunológico sistêmico, grande parte das células T $CD4^+$ presentes no GALT são células de memória efetoras que expressam CCR5 (Lackner et al. 2012) e são, portanto, altamente vulneráveis à infecção pelo HIV. Estima-se que 60% das células T $CD4^+$ de memória presente no GALT sejam infectadas em poucos dias após a infecção, no momento de pico da viremia (Mattapallil et al. 2005; Lackner et al. 2009, 2012). Isto resulta numa acentuada depleção dos linfócitos T $CD4^+$ no GALT que persiste durante todo o curso da infecção (Mehandru et al. 2004; Brenchley and Douek 2008; Thompson et al. 2017). Essa depleção de células T $CD4^+$ no GALT é decorrente tanto dos efeitos citopáticos diretos da infecção viral, quanto pela morte de linfócitos T $CD4^+$ não infectados decorrente da ativação, proliferação e expansão exacerbada de linfócitos T $CD4^+$ e do aumento da prevalência de células T $CD8^+$ no GALT, na tentativa de conter a infecção (Dandekar 2007; Février et al. 2011) A depleção de células T $CD4^+$ no GALT afeta uma subpopulação relevante de células T $CD4^+$, denominada células T helper (Th17). As células T helper medeiam a imunidade contra patógenos nas superfícies das mucosas, secretando citocinas efetoras (IL-17 e IL-22), e são fundamentais para

manter a integridade do epitélio intestinal (Karris and Smith 2011; Sandler and Douek 2012). A depleção desta subpopulação celular na fase aguda da infecção pelo HIV, resulta num aumento da permeabilidade do epitélio intestinal e no extravasamento de produtos microbianos do lúmen intestinal para a circulação (translocação microbiana), que por sua vez contribui com a manutenção de um estado de ativação crônico do sistema imune ao longo de todo o curso clínico (Sandler and Douek 2012) (Figura 8).

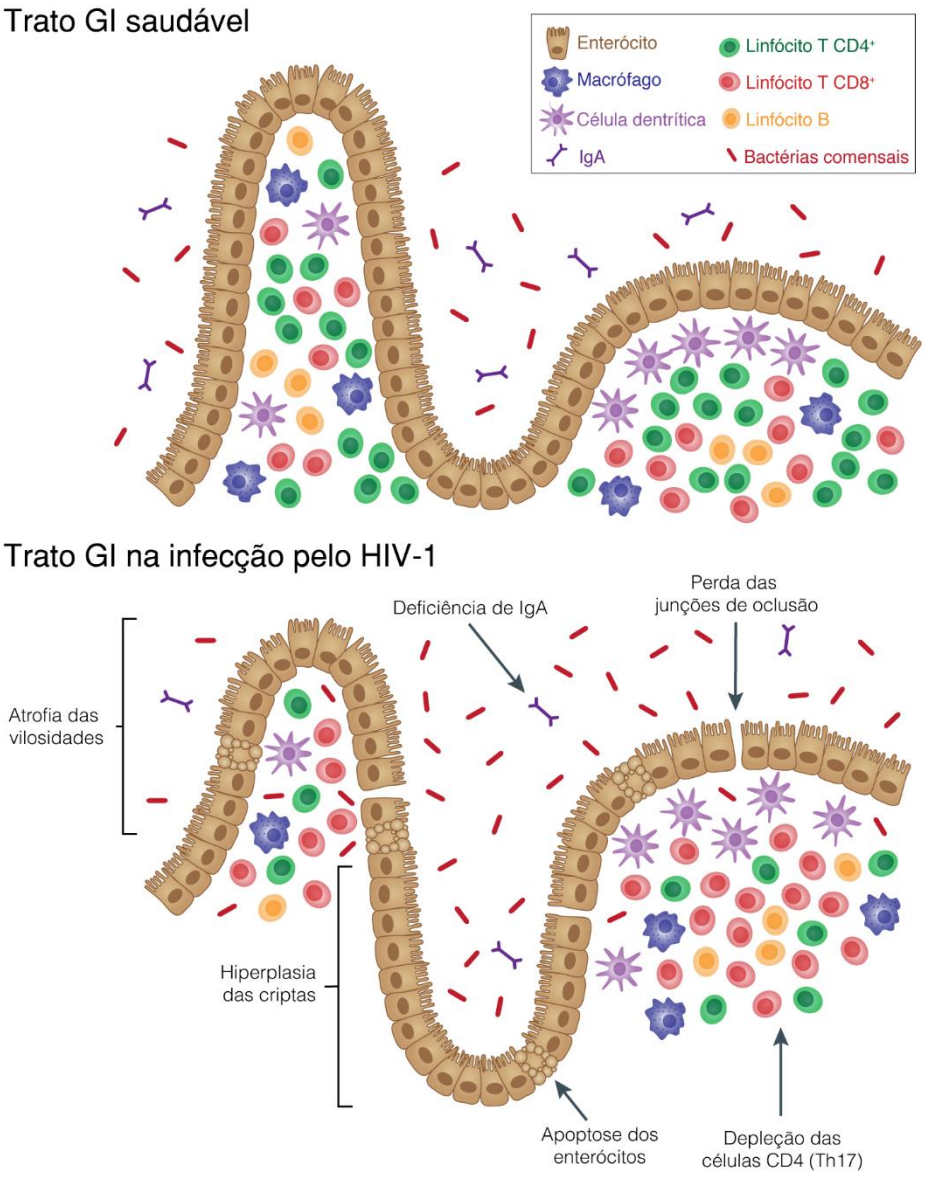

Figura 8: Patologia da infecção pelo HIV na mucosa gastrointestinal. A mucosa saudável (parte superior) se caracteriza por altos números de linfócitos T CD4⁺, particularmente das subpopulações Th17/22, que contribuem para a manutenção da integridade epitelial. Junções de oclusão entre os enterócitos mantém as bactérias confinadas ao lúmen intestinal. Durante a infecção pelo HIV (parte inferior) a população de linfócitos T CD4⁺ é perdida de forma massiva, levando a enteropatia sustentada. Constituintes microbianos entram na submucosa e na circulação, disparando a ativação imune sistêmica. Modificado de Sandler e Douek 2012 e Prebensen 2017.

1.6.2.2 Fase crônica

A fase crônica, ou também conhecida como fase de latência clínica, é definida por uma viremia estável (*set point* viral) por longos períodos (Coffin and Swanstrom 2013). O tempo de duração dessa fase na ausência de ART é variável entre os indivíduos infectados pelo HIV (Pantaleo and Fauci 1996). Na maioria dos indivíduos infectados pelo HIV-1 (70-80%), denominados “Progressores Típicos” (PT), a fase crônica se estende por um período de oito anos. Alguns indivíduos, denominados Progressores Rápidos (RP) (10-15%), tem uma fase crônica mais curta e evoluem para aids em até três anos após infecção. Alguns poucos indivíduos (<5%) denominados “Progressores Lentos” ou “LTNP” (do inglês *long-term nonprogressors*), no entanto, mantêm altas contagens de linfócitos T CD4$^+$ e permanecem clinicamente assintomáticos por mais de 10 anos de infecção (Figura 9) (Pantaleo et al. 1995; Pantaleo and Fauci 1996).

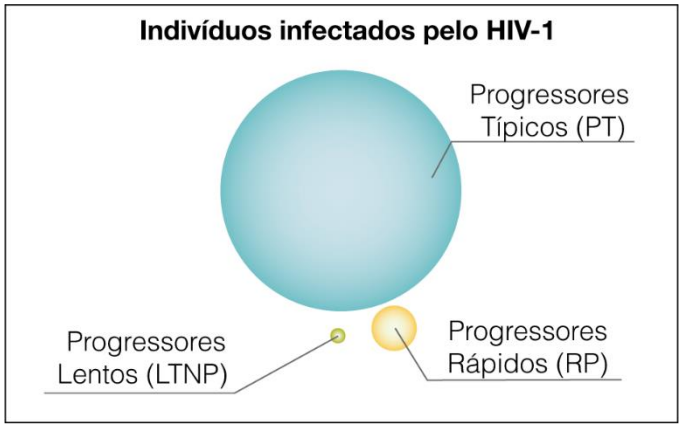

Figura 9: Proporção de indivíduos apresentando os diferentes perfis de progressão. Cada círculo é representam subconjuntos de cada perfil de controle e seu diâmetro é proporcional a representatividade de cada perfil.

Durante a fase crônica, a replicação viral contínua e a translocação de antígenos bacterianos através da mucosa intestinal, resultam em um estado de ativação imunológica crônica, evidenciada pelo aumento da expressão de marcadores de ativação (CD38$^+$ e HLA-DR$^+$) e “turnover” (Brenchley et al. 2006; Février et al. 2011; Maartens et al. 2014) nas células T, assim como pelos níveis elevados de citocinas pró-inflamatórias (IL-1β, IL-6, fator de necrose tumoral [TNF]-α, e IFN-α) e de marcadores de inflamação periféricos (CD14 solúvel, proteína induzida por interferon gama 10 [IP-10]) (Fauci 1996; Stylianou et al. 2000; Neuhaus et al. 2011) (Figura 10). A ativação imunológica crônica seria a responsável pela depleção de células T CD4$^+$ e progressão para aids (Deeks et al. 2004). Desta forma, tanto o *set point* viral quanto

os níveis de ativação imunológica durante a fase crônica da infecção são marcadores preditivos da taxa de declínio de células T CD4^{+} e da duração da fase crônica em cada indivíduo infectado (Deeks et al. 2004).

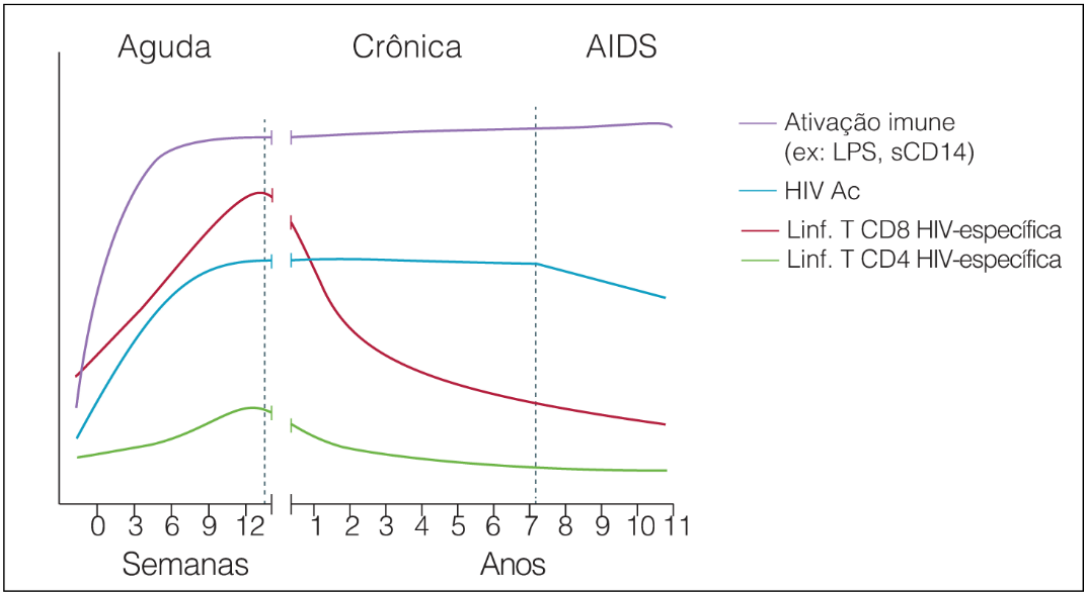

Figura 10: Ativação imunológica nas diferentes fases da história natural da infecção pelo HIV-1. Cada linha representa diferentes marcadores associados a ativação immune desencadeada pela infecção, como também dos níveis de anticorpos anti-HIV, sendo coloridas de acordo com a legenda a direita. Adaptado de Maartens et al, 2014.

1.6.3 Diversidade das quasispécies do HIV

A transmissão do HIV-1 através dos tecidos da mucosa é geralmente estabelecida por um (ou uns poucos) vírus fundador(es), que infecta (m) uma pequena população de células da mucosa (Swanstrom and Coffin 2012; Maartens et al. 2014). Durante a fase crônica da infecção, os vírus transmitidos evoluem e acumulam mutações como resultado de características inerentes ao seu ciclo replicativo, assim como pela contínua pressão seletiva do sistema imune do hospedeiro, originando assim um conjunto de variantes geneticamente distintas dentro da população viral de um único indivíduo, denominada quasispécie viral (Swanstrom and Coffin 2012; Deeks et al. 2015). Durante a fase crônica nos PT, a região mais variável do genoma que corresponde ao gene *env*, acumula mutações em relação ao vírus fundador a uma taxa aproximada de 1% ao ano (Shankarappa et al. 1999).

A elevada variabilidade genética das quasispécies do HIV ao longo da fase crônica é resultado de três fatores principais: 1) a baixa fidelidade da enzima TR durante a transcrição do RNA para DNA que resulta numa taxa de mutação na ordem de 3×10^{-5} mutações por nucleotídeo por ciclo replicativo (Swanstrom and Coffin

2012); 2) a elevada frequência de recombinação (8-10 vezes por ciclo replicativo) entre os dois genomas virais empacotados na mesma partícula durante a transcrição reversa (Rhodes et al. 2005); e 3) a hipermutação do genoma viral promovido pelo fator do hospedeiro APOBEC3(G/F) (Kim et al. 2014). Esses fatores combinados ao grande tamanho da população viral replicante (são gerados aproximadamente 10^{10}-10^{12} diferentes vírions por dia) (Perelson et al. 1996) e a uma replicação viral contínua garantem a geração de um grande número de variantes virais, geneticamente distintas dentro de um mesmo hospedeiro. Essa diversidade genética intra-hospedeiro também é mantida e favorecida por conta da ressemeadura das populações provirais presentes nos reservatórios majoritariamente composto por células T $CD4^+$ de memória.

A diversidade genética intra-hospedeiro do HIV pode também ser aumentada como resultado da coinfecção (CO, quando duas ou mais variantes virais infectam um indivíduo simultaneamente ou antes da soroconversão) ou da superinfecção (SI, quando a segunda variante infecta o indivíduo após a soroconversão) (Figura 11) (Smith et al. 2005). A CO e SI pode também gerar vírus heterozigotos contendo duas cópias distintas de RNA (cada uma proveniente de um vírus parental distinto), empacotadas na mesma partícula, que ao infectar uma célula geram vírus recombinantes intersubtipo (quando as moléculas de RNA empacotadas são de diferentes subtipos) ou intrasubtipo (quando as moléculas de RNA empacotadas são do mesmo subtipo) (Hu et al. 1990; Chin et al. 2005).

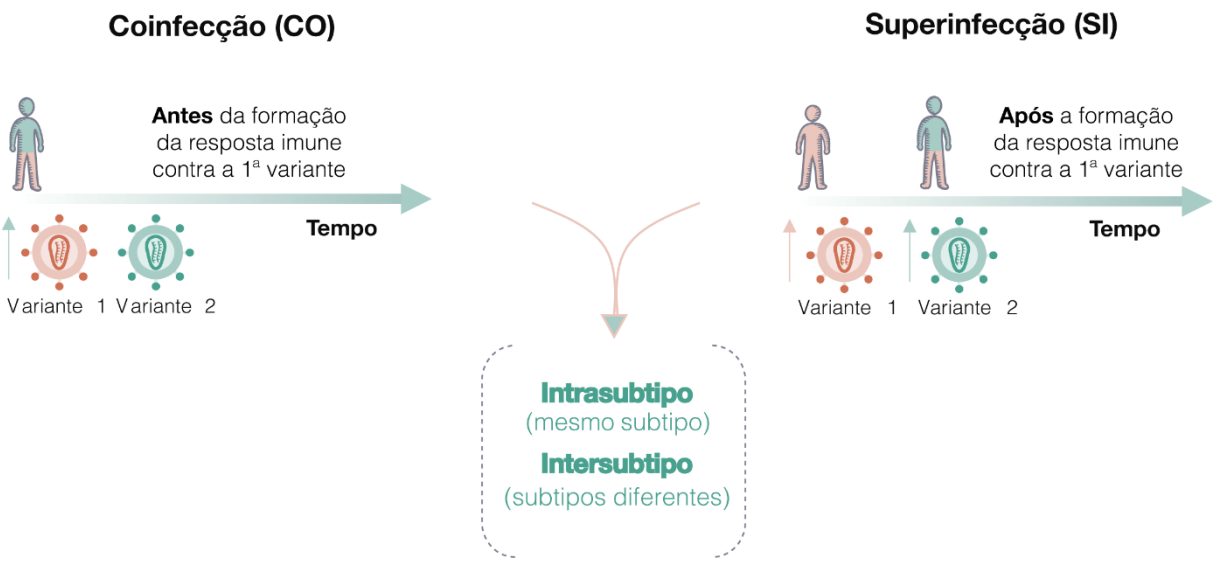

Figura 11: Esquema para classificação da coinfecção ou superinfecção pelo HIV. Para cada variante viral foi assinalada uma cor. A coloração de cada indivíduo representa a(s) variante(s) infectantes naquele momento. Adaptado de Smith et al, 2005.

1.7 Controle da replicação do HIV: cura esterilizante vs cura funcional

Com o amplo acesso a ART, é possível manter níveis indetectáveis de carga viral na maioria dos indivíduos infectados pelo HIV, evitando desta forma a evolução para a fase de aids. Entretanto, não existe ainda uma estratégia universal para erradicação do vírus (cura esterilizante). O primeiro e até o momento único paciente a alcançar a cura esterilizante é o conhecido como paciente de Berlim (Hütter et al. 2009). Esse paciente foi submetido ao transplante de células-tronco hematopoiéticas para tratar uma leucemia mieloide aguda tendo como doador um indivíduo que possuía uma deleção de 32pb (CCR5Δ32) no gene do correceptor CCR5, que confere resistência à infecção pelo HIV (Rong Liu et al. 1996). Após o transplante o vírus não foi mais detectado, mesmo na ausência de ART e segue assim a dez anos após o procedimento (Hütter et al. 2009). Mais recentemente, após dez anos do primeiro transplante que culminou com a cura da infecção pelo HIV no paciente de Berlim e após outras duas tentativas sem o mesmo desfecho (Henrich et al. 2014), um segundo caso de possível cura foi reportado com o paciente de Londres (Gupta et al. 2019). Esse paciente foi submetido a um transplante de células-tronco hematopoiéticas para tratamento do Linfoma de Hodgkin tendo também como doador um indivíduo portador da deleção no gene CCR5 e até o momento apresenta remissão da infecção pelo HIV

após 18 meses do procedimento (Gupta et al. 2019). Esses relatos bem-sucedidos apontam que estratégias baseadas na prevenção da expressão do CCR5 podem ser um caminho promissor para se atingir a cura esterilizante universal.

Um dos principais obstáculos da cura esterilizante é a formação dos reservatórios virais latentes de longa vida no início da infecção, sobretudo em células T $CD4^+$ de memória (Chun et al. 1998; Finzi et al. 1999). Recentes abordagens terapêuticas vêm sendo testadas com o objetivo de atingir a cura esterilizante, como a estratégia “shock and kill” (Deeks 2012). Esta estratégia tem como objetivo tornar os provírus integrados em células de longa vida (reservatório latente) transcricionalmente ativos, e então eliminá-los através dos efeitos combinados da terapia atual e das células T $CD8^+$ citotóxicas específicas para o HIV-1(Deeks 2012; Passaes and Sáez-Cirión 2014; Pham and Mesplède 2018).

Uma alternativa a cura esterilizante, é a denominada cura funcional que tem como objetivo limitar a formação dos reservatórios latentes e minimizar seu tamanho, conseguindo assim uma remissão duradoura da replicação viral na ausência de ART, mesmo que o nível de provírus do HIV permaneça detectável (Pham and Mesplède 2018). Uma das estratégias de cura funcional mais bem-sucedidas é a iniciação precoce da ART, ainda na fase aguda da infecção. O estudo VISCONTI com uma coorte de indivíduos adultos diagnosticados na fase aguda, observou que a ART iniciada em até 10 semanas após a infecção e mantida por uma média de três anos resultou no controle sustentado da viremia, por uma média de sete anos após a suspensão da ART em 14 indivíduos, que foram denominados Controladores do HIV pós-tratamento (Sáez-Cirión et al. 2013). Outro exemplo de controle pós-tratamento foi obtido no caso conhecido como “Bebê do Mississippi”. Esse bebê contraiu o HIV por transmissão vertical e iniciou a ART 30 horas após o nascimento. Após 18 meses, a ART foi suspensa e carga viral se manteve indetectável por quase dois anos (Persaud et al. 2013). No entanto, após esse período, a criança apresentou um rebote da carga viral (Siliciano and Siliciano 2014). Esses exemplos demonstram claramente que a ART precoce pode restringir o tamanho dos reservatórios virais e minimizar as suas chances de reativação, mas não os erradicar.

1.7.1 Controladores do HIV: um modelo de cura funcional

Após o advento dos testes de quantificação da viremia plasmática em meados de 1990, foi observado que um subgrupo de indivíduos infectados pelo HIV, na sua maioria LTNP, era capaz de manter a carga viral a níveis baixos ou indetectáveis por

mais de 2-10 anos na ausência da ART, sendo denominados de Controladores do HIV (HIVc) (Deeks and Walker 2007a; O'Connell et al. 2009a). Aqueles indivíduos capazes de manter as cargas virais inferiores a 2.000 cópias/mL foram denominados Controladores Virêmicos (VC), enquanto que uma parcela mais rara (<1%) dos indivíduos infectados pelo HIV capazes de manter a carga viral indetectável (<50-80 cópias/mL) foi denominada Controladores de Elite (EC) (Figura 12) (Okulicz et al. 2009).

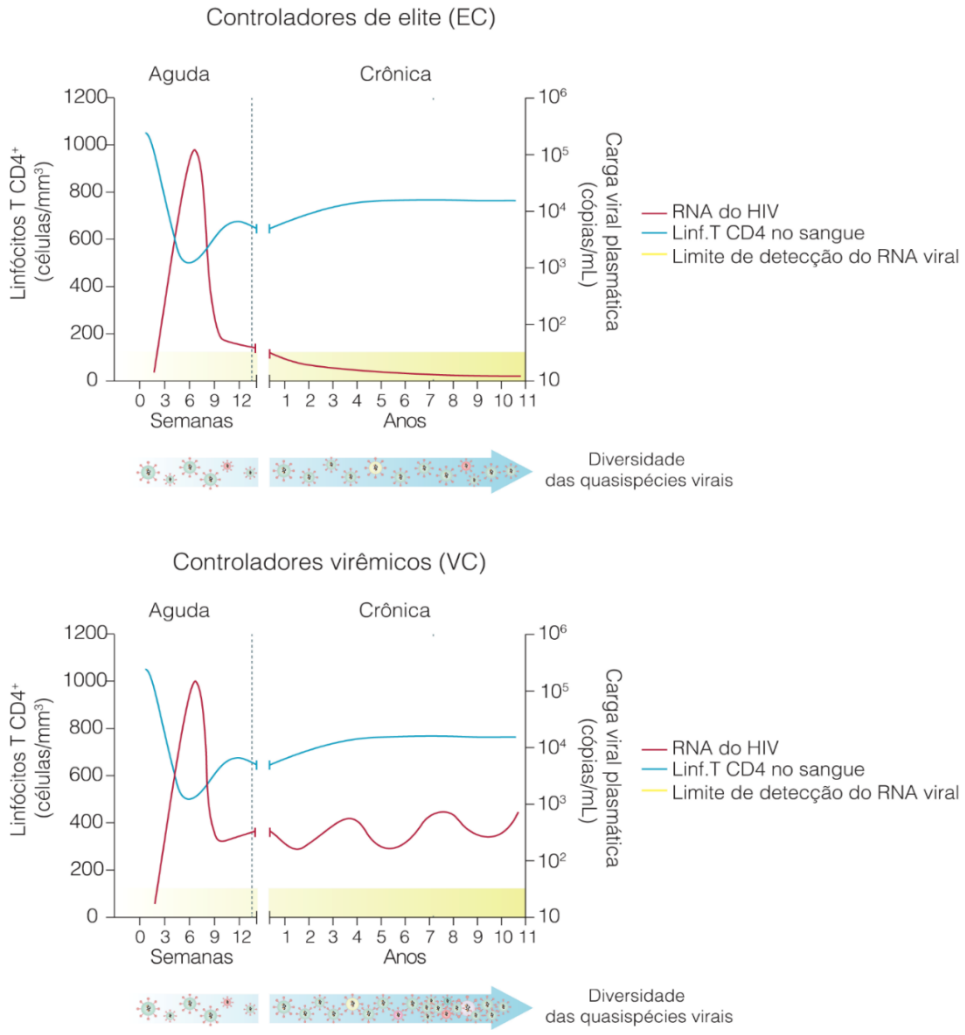

Figura 12: História natural da infecção pelo HIV em controladores do HIV. Tanto em EC quanto VC, a contagem de linfócitos T CD4⁺ no sangue (linha azul) são mantidas durante a fase crônica da infecção. Entretanto, enquanto EC mantém os níveis de RNA do HIV-1 (linha vermelha) indetectáveis, viremia em VC é detectável, porém mantida em níveis baixos. Dados de Okulicz et al. 2009. Figura adaptada de Maartens et al. 2014.

Os EC foram apontados como um modelo natural de cura funcional (Autran et al. 2011). Ao longo dos anos, a caracterização de fatores do hospedeiro (genético e

imunológico) e/ou do vírus foram importantes para traçar os fatores que estariam relacionados ao controle da infecção pelo HIV nesses indivíduos. Sabe-se que fatores epidemiológicos, como gênero, origem étnica e modo de infecção, não parecem impactar no desenvolvimento de um perfil de EC ou VC nos indivíduos infectados (Deeks and Walker 2007a; Migueles and Connors 2010), entretanto o que esses estudos demonstraram é que o cenário que pode explicar essa capacidade extraordinária de manter a supressão do HIV por longos períodos é complexa e provavelmente multifatorial (Walker and Yu 2013).

Estudos recentes mostraram que os EC podem apresentar fenótipos distintos e essas características que os diferenciam são importantes para delinear quais desses subgrupos são os melhores modelos de estudo da cura funcional. Dentre essas características distintas, um estudo observou dois subgrupos de EC, um deles apresentando uma frequência de células T $CD8^+$ ativadas e de biomarcadores inflamatórios acima dos valores normais, e um outro subgrupo que apresentou níveis de marcadores inflamatórios e de ativação indistinguíveis dos indivíduos não infectados pelo HIV-1, representando estes últimos o melhor modelo de estudo da cura funcional da infecção pelo HIV (Côrtes et al. 2018). Além disso, níveis elevados e a capacidade polifuncional da resposta de células T HIV-específica e um baixo reservatório proviral também são características que foram distintas em alguns EC (Pernas et al. 2017).

Dessa forma, identificar as características presentes em EC que são capazes de controlar persistentemente o HIV pode ser importante para definir os verdadeiros indivíduos modelo de cura funcional, auxiliando no entendimento dos mecanismos responsáveis por essa remissão a longo prazo, pode fornecendo informações únicas para estratégias de cura do HIV.

1.7.1.1 Características virológicas

A hipótese inicial para explicar os baixos níveis de viremia observados nos EC foi a infecção com vírus defectivos/atenuados, que seriam incapazes de replicar corretamente. De fato, a presença de vírus defectivos ou atenuados por mutações e/ou deleções em genes estruturais e acessórios já foi observado em alguns indivíduos LTNP ou EC (Michael et al. 1995; Huang et al. 1998; Kirchhoff et al. 1999; Alexander et al. 2000; Lum et al. 2003; Mologni et al. 2006; Rajan et al. 2006), mas essa não é uma característica observada em todos os indivíduos com fenótipo de

controle da replicação viral. Alguns estudos demonstram que a maioria dos indivíduos EC são infectados por vírus competentes e patogênicos (Huang et al. 1995; Blankson et al. 2007; Lamine et al. 2007; Miura et al. 2008). Outra característica virológica singular dos EC é a baixa diversidade genética do reservatório viral periférico, muitas vezes mantido apenas por expansão clonal das células T $CD4^+$ de memória infectadas (Boritz et al. 2016). A principal diferença dos HIVc e os PT, é que nesse último há uma elevada taxa de evolução viral durante o período assintomático, resultando na emergência de variantes virais mais citopáticas (Tersmette et al. 1989; Maas et al. 2000) e/ou de escape frente à resposta imune do hospedeiro (Koenig et al. 1995). Estudos demonstraram que os EC mantêm um reservatório viral periférico extremamente homogêneo (≤ 2% de diversidade em *env*) e sem aumento mensurável da divergência ao longo do tempo (**Figura 12**). No entanto, a presença de variantes atenuadas e a baixa diversidade do reservatório latente, podem ser o resultado do fenótipo EC, e não a causa do mesmo (Wang et al. 2003a; Bello et al. 2005, 2007a, Bailey et al. 2006b, 2006a; O'Connell et al. 2010; Salgado et al. 2014; Boritz et al. 2016).

1.7.1.2 Características genéticas do hospedeiro

A busca por fatores genéticos associados ao controle da replicação viral evidenciou que a heterozigose da deleção de 32 pares de bases no gene do receptor de quimiocina CCR5 estaria associada ao perfil LTNP (Cohen et al. 1997; Quillent et al. 1998; Pastori et al. 2010), sendo também observada em coortes de EC (Cohen et al. 1997; Pastori et al. 2010). Outro fator genético associado ao perfil LTNPs/EC é a presença dos alelos do sistema antígeno leucocitário humano (HLA, do inglês *Human leukocyte antigen*) de classe I B*27 (McNeil et al. 1996), B*57 (Migueles et al. 2000; Lécuroux et al. 2014) e B*52 (Teixeira et al. 2014).

1.7.1.3 Características da resposta imune inata

A primeira linha de defesa contra a infecção, a imunidade inata fornece uma resposta imediata contra as infecções, e por esse motivo e por ser uma resposta de curta duração é considerada uma resposta ampla contra qualquer patógeno, porém inespecífica (Mogensen et al. 2010). A importância da imunidade inata na infecção pelo HIV, ou qualquer infecção, está relacionada ao recrutamento de células imunes através da secreção de citocinas e quimiocinas, ativando o sistema imunológico de longa duração e específico através da apresentação de antígeno (McMichael et al.

2010). Embora a infecção pelo HIV seja uma doença crônica e, portanto, parece mais relevante estudar a imunidade adaptativa, existem propriedades únicas da resposta imune inata nos EC que foram descritas no contexto do controle natural (Deeks and Walker 2007b; Walker and Yu 2013).

1.7.1.3.1 Resposta mediada por células dendríticas (DCs)

As células apresentadoras de antígenos, denominadas células dendríticas (DCs) processam os antígenos virais para posteriormente apresentá-los aos linfócitos T, que então serão ativados. Um subconjunto dessas células, as DCs plasmocitoides (pDCs), respondem rapidamente contra infecções virais secretando quantidades significativas de IFN do tipo 1, sendo uma célula altamente produtora de IFN-α em resposta ao estímulo viral (Fitzgerald-Bocarsly and Jacobs 2010), sendo importante elo de comunicação entre as respostas imune inata e adaptativa (McKenna et al. 2005). As pDCs também são capazes de produzir várias outras citocinas e quimiocinas pró-inflamatórias (TNF-α, RANTES, IL-10, IP-10, IL-6 e a proteína inflamatória macrofágica 1 [MIP-1α/b]), após ativação de NF-κB através do estímulo viral, promovendo uma redução da replicação viral (Megjugorac 2003; Fuchsberger et al. 2005). Embora ocorra uma depleção extensiva de células imunes durante a infecção aguda pelo HIV-1, as pDCs apresentam funcionalidade preservada e nível de produção diferenciada de IFN-α em ECs, podendo reduzir a replicação viral (Barblu et al. 2012; Machmach et al. 2012).

1.7.1.3.2 Resposta mediada por células Natural Killers (NK)

As células natural killer (NK) apresentam atividade citotóxica contra células infectadas por vírus, produzindo citocinas pró-inflamatórias e moléculas citotóxicas. Estudos têm mostrado associação entre alelos dos receptores expressos nas superfícies de NK, as moléculas KIR (do inglês, *killer immunoglobulin-like receptor*) e o controle da replicação. Em particular, os alelos KIR3DL1 e KIR3DS1 foram associados com uma progressão mais lenta para aids ao interagir com os alelos HLA-B (López-Vázquez et al. 2005; Alter et al. 2007; Kamya et al. 2011). O controle da replicação mediado por NK também tem sido observado em indivíduos EC/VC, expressando o alelo HLA-Bw4*80I nas células alvo e o receptor KIR3DL1 nas células NK, cuja citotoxidade foi mais forte do que aquela medida para células T $CD8^+$ (Alter et al. 2007; Tomescu et al. 2012). Entretanto, um estudo demonstrou que a capacidade antiviral das células NK não é particularmente forte e nem está presente

em todos os ECs, indicando que a inibição da replicação viral mediada por células NK não é necessária para a manutenção do controle viral (O'Connell et al. 2009b).

1.7.1.3.3 Fatores de Restrição (FR) da replicação viral

A expressão elevada de determinados FR pode ser um importante mecanismo intrínseco capaz de restringir a replicação do HIV-1 em PBMCs e linfócitos T $CD4^+$ de indivíduos EC (Buzon et al. 2011; Chen et al. 2011; Saez-Cirion et al. 2011). Ao avaliar a infecção *in vitro* de linfócitos T $CD4^+$, foram observados baixos níveis de replicação viral nas células dos EC quando comparados as células de PT ou de indivíduos não infectados pelo HIV-1 (Chen et al. 2011; Saez-Cirion et al. 2011). Entretanto, a restrição a replicação pelo HIV não parece ser observada em todos os indivíduos EC, uma vez que outros estudos demonstraram que linfócitos T $CD4^+$ desses indivíduos podem ser susceptíveis a infecção experimental *in vitro* pelo HIV-1 (Julg et al. 2010; O'Connell et al. 2011; Rabi et al. 2011). Estudos avaliando a expressão de FR clássicos em EC também apresentaram resultados controversos. Um estudo descreve altos níveis de expressão de APOBEC3G, Teterina e TRIM5α em linfócitos T $CD4^+$ de EC quando comparados a indivíduos com viremia suprimida pela ART e indivíduos não infectados pelo HIV-1, e níveis similares/reduzidos quando comparados com PT (Abdel-Mohsen et al. 2013). Alguns estudos já observaram altos níveis de expressão de outros FR em linfócitos T $CD4^+$ de EC, como Schlafen11 (Abdel-Mohsen et al. 2013), Blimp-1 (De Masson et al. 2014), SAMHD1 e p21 (Chen et al. 2011; Riveira-Muñoz et al. 2014) em comparação a indivíduos PT ou não infectados pelo HIV-1. Outros estudos, no entanto, não foram capazes de confirmar tais associações (Buzon et al. 2011). Em outro estudo, os níveis de expressão dos FR em linfócitos T $CD4^+$ definiu dois subgrupos de EC: um subgrupo com perfil de expressão similar a indivíduos não infectados pelo HIV-1 e outro com expressão similar ao observado em indivíduos com viremia suprimida pela ART (Vigneault et al. 2011).

1.7.1.4 Características da resposta imune adaptativa

Componentes da imunidade adaptativa como anticorpos neutralizantes e células T $CD4^+$ e $CD8^+$ são os fatores dominantes no controle da replicação do HIV durante a fase aguda. A importância destas respostas no fenótipo de EC, no entanto, é controversa (Walker and Yu 2013; Zaunders and van Bockel 2013).

1.7.1.4.1 Resposta mediada por anticorpos

No caso do HIV, por conta da sua grande diversidade genética, se torna necessário a geração de anticorpos capazes de neutralizar diversas cepas do HIV, denominados anticorpos amplamente neutralizantes (bNAbs), para que a replicação viral seja efetivamente controlada. Entretanto, alguns trabalhos demonstraram que somente uma pequena fração dos HIVc são capazes de produzir bNAbs contra o HIV apesar da baixa replicação viral (O'Connell et al. 2009a; González et al. 2018). Apesar disso, também foi observado que o potencial de citotoxicidade mediada por células dependente de anticorpos (ADCC), tanto em relação a qualidade dos anticorpos não neutralizantes quanto a expressão dos receptores Fcγ na superfície celular parece ser maior nos HIVc do que em indivíduos PT (Lambotte et al. 2009) , indicando que os mecanismos de defesa imunológica baseados no ADCC podem contribuir para o controle natural da replicação do HIV-1.

1.7.1.4.2 Resposta mediada por Linfócitos T CD4$^+$

A principal consequência da infecção pelo HIV é a perda progressiva de linfócitos T CD4$^+$, principal célula alvo do vírus. Os linfócitos T CD4$^+$ são populações celulares que auxiliam outros componentes do sistema imunológico como os linfócitos B na produção de anticorpos como também na ativação e funcionamento das células T CD8$^+$ citotóxicas e macrófagos (Castellino and Germain 2006). Durante a infecção pelo HIV, o tamanho e a composição dos linfócitos T CD4$^+$ é drasticamente reduzido tanto na periferia como no GALT (Guadalupe et al. 2003; Brenchley et al. 2004), afetando a homeostase e a função dessas células (McCune 2001). As subpopulações de linfócitos T CD4$^+$ que reconhecem os peptídeos do HIV, denominados linfócitos T CD4$^+$-específicos para o HIV, são os mais afetados (Douek et al. 2002). Esses desempenham respostas polarizadas com perfil Th1, com produção de INF-γ, TNF-α e IL-2 (Pitcher et al. 1999). Nos indivíduos PT a capacidade proliferativa e de produção dessas citocinas é comprometida (Wilson et al. 2000; Harari et al. 2004). Entretanto, estudos mostram que EC mantêm respostas robustas e polifuncionais de linfócitos T CD4$^+$-específicos para o HIV quando comparados com PT, secretando altos níveis de IL-2 melhorando a atividade citotóxica das células T CD8$^+$ (Porichis and Kaufmann 2011). A incapacidade de restauração dessa resposta T CD4$^+$-específica após a supressão da viremia com a ART (Wilson et al. 2000; Harari et al. 2004), sugere que a resposta exercida em EC pode ter um papel fundamental no controle viral.

1.7.1.4.3 Resposta mediada por Linfócitos T CD8$^+$

Diversos estudos com coortes de LTNP/EC tem confirmado que as células T CD8$^+$ desses indivíduos apresentaram maior concentração de perforina e granzima B, quando estimuladas com peptídeos de Gag e, consequentemente, uma maior capacidade de lise de células alvo infectadas, quando comparadas com células T CD8$^+$ de indivíduos PT (Migueles et al. 2008). Além disso, frente ao estímulo com células T CD4$^+$ infectadas ou peptídeos do HIV-1, as células T CD8$^+$ de indivíduos EC possuem maior capacidade de proliferação, como também um perfil polifuncional, com maior frequência de células produzindo diferentes citocinas e quimiocinas – IL-2, IFN-γ, MIP 1β (CCL4), TNF-α – junto com uma maior capacidade de degranulação, quando comparadas com as de indivíduos PT (Migueles et al. 2002; Betts et al. 2006; Sáez-Cirión et al. 2007; Owen et al. 2010; Card et al. 2012). Outro aspecto da resposta de células T CD8$^+$ que tem sido fortemente associada ao controle da replicação em EC é a capacidade de supressão da replicação viral *in vitro,* sem a necessidade de estímulos exógenos (Sáez-Cirión et al. 2007, 2009). No entanto, essas características não estão presentes em todos os EC, o que evidencia mais uma vez a heterogeneidade existente entre esses indivíduos (Sáez-Cirión et al. 2009).

1.7.1.5 Fatores associados à perda do controle em HIVc

Alguns estudos mostraram que após certo tempo de controle da replicação viral (2-10 anos), alguns HIVc apresentam uma progressão virológica (definida como um aumento sustentado da viremia plasmática acima de 50 cópias/mL para os EC e acima de 2.000 cópias/mL para os VC) e/ou uma progressão imunológica (definida pelo declínio significativo de linfócitos T CD4$^+$) (Andrade et al. 2008; Okulicz et al. 2009; Sajadi et al. 2009; Boufassa et al. 2011; Yang et al. 2012; Olson et al. 2014; Leon et al. 2016; Chereau et al. 2017).

Um dos fatores associados tanto com a progressão virológica quanto imunológica em alguns HIVc é a SI. Independentemente da capacidade do indivíduo infectado de controlar a primoinfecção, a aquisição de uma nova variante viral tem sido associada ao aumento da viremia plasmática e ao rápido declínio dos linfócitos T CD4$^+$ (Gottlieb et al. 2004; Jurriaans et al. 2008; Cornelissen et al. 2012; Ronen et al. 2014; Luan et al. 2017), levando a perda do controle em HIVc, independentemente de envolver variantes do mesmo subtipo ou de subtipos diferentes (Rachinger et al. 2008; Braibant et al. 2010; Clerc et al. 2010). No entanto, outros HIVc parecem manter o controle virológico após a SI por uma variante viral do mesmo subtipo (Casado et al.

2007; Lamine et al. 2007; Pernas et al. 2013). Estes estudos indicam que a resposta imune gerada pela primeira infecção não é capaz de evitar a SI dos HIVc, e não seriam portanto um modelo adequado para o desenvolvimento de vacinas profiláticas (Gao et al. 2017). Alguns HIVc capazes de conter a replicação de uma segunda variante, no entanto, podem representar um excelente modelo para o desenvolvimento de vacinas terapêuticas.

A presença de níveis elevados de marcadores associados a inflamação e a ativação de células T tem sido apontado como marcadores preditivos de progressão imunológica e virológica em EC e VC (Andrade et al. 2008; Hunt et al. 2008; Noel et al. 2014, 2015; Pernas et al. 2017; Rodríguez-Gallego et al. 2018). Um estudo apontou que tanto IP-10 quanto a ativação sistêmica de células T $CD8^+$ são biomarcadores discriminatórios para detecção de HIVc em risco de progressão imunológica (Noel et al. 2014). Episódios intermitentes de viremia (blips) também foram associados a progressão imunológica e a uma maior ativação sistêmica (Noel et al. 2015) enquanto que níveis elevados de RNA viral plasmático e de DNA viral total associado a célula foram detectados entre um e dois anos antes da confirmação da progressão virológica em HIVc (Noel et al. 2015). Estudos mais recentes apontaram outros fatores como biomarcadores discriminatórios da progressão virológica em HIVc, incluindo uma diversidade genética elevada do DNA viral, além de níveis elevados da citocina pró-inflamatória CCL5 (RANTES) e da proteína galectina-3 no plasma, um ano antes da perda do controle (Pernas et al. 2017; Rodríguez-Gallego et al. 2018).

O modelo atualmente aceito indica que a replicação viral não parece ser completamente suprimida nos EC e a existência de uma replicação viral residual estaria associada tanto a um aumento da diversidade genética do reservatório viral quanto ao aumento da ativação e inflamação, que por sua vez resultaria na progressão virológica e/ou imunológica observada em alguns indivíduos EC (Boritz et al. 2016; Pernas et al. 2017). Identificar aqueles HIVc com um risco aumentado de perder o controle virológico e imunológico é fundamental para o manejo clínico destes indivíduos, assim como para a definição do melhor modelo natural de cura funcional.

2 OBJETIVOS

2.1 Objetivo Geral

Investigar os possíveis mecanismos envolvidos no controle da replicação do HIV-1 e da evolução para aids, assim como as características do reservatório viral em uma coorte de indivíduos soropositivos para o HIV-1 com o perfil de HIVc.

2.2 Objetivos Específicos

Artigo 1:

- Caracterizar geneticamente o HIV presente no reservatório proviral em PBMCs de HIVc, no sentido de entender os mecanismos de persistência do vírus no contexto de diferentes níveis de supressão viral sistêmica.

Artigo 2:

- Avaliar o impacto da superinfecção intersubtipo do HIV-1 na viremia, ressemeadura do reservatório, evolução viral e progressão da doença em HIVc com diferentes perfis de controle.

Artigo 3:

- Avaliar a importância da diversidade proviral do gene *env* do HIV-1 como possível marcador prognóstico da progressão virológica e/ou imunológica, em indivíduos EC com diferentes níveis de diversidade das quasispécies provirais.

Artigo 4:

- Analisar o perfil de expressão de fatores de restrição celulares previamente associados com a inibição da replicação de retrovírus nos PBMCs, a fim de verificar o papel destes fatores no controle da replicação do HIV-1 nos HIVc.

3 ARTIGOS

3.1 ARTIGO 1

Highly divergent patterns of genetic diversity and evolution in proviral quasispecies from HIV controllers.

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Periódico: Retrovirology, 2017.

Resumo:

A evolução intra-hospedeiro do HIV-1 tem sido observada em indivíduos que naturalmente suprimem a viremia a baixos níveis (HIVc), através da análise do RNA viral no compartimento plasmático. A detecção da evolução no compartimento proviral em controladores do HIV, contudo, tem sido mais desafiadora e a precisa correlação entre o nível de supressão viral sistêmica e a taxa de renovação do reservatório nesses indivíduos não são totalmente compreendidos. Dessa maneira, nós examinamos as quasispécies provirais através do método de amplificação do genoma único do gene *env,* em uma coorte de 23 indivíduos controladores do HIV no Brasil divididos em três grupos, de acordo com o nível de supressão viral sistêmica: 1) Controladores de elite (EC) que apresentam níveis de carga viral indetectáveis persistentes (PEC, n=6); 2) Controladores de elite com episódios ocasionais de viremia transitória (51-400 cópias/ml) (EEC, n=7); e Controladores virêmicos que apresentam uma viremia detectável persistente de baixo nível (80-2.000 cópias/ml) (VC, n=10). A diversidade das quasispécies provirais do HIV-1 nas células mononucleares do sangue periférico (PBMC) em EC foi significativamente ($P<0.01$) menor do que em VC, mas não significativamente diferente entre os grupos PEC e EEC. Nós detectamos uma variação considerável na média da distância nucleotídica pareada e na proporção de sequências únicas nas quasispécies provirais de PEC e EEC. Alguns indivíduos PEC e EEC apresentaram populações provirais altamente homogêneas com grande número de sequências idênticas, enquanto outros exibiram populações provirais relativamente diversas com uma alta proporção de sequências únicas, comparáveis aos indivíduos do grupo VC. O acompanhamento a longo prazo (10-15 anos) das populações provirais do HIV-1 revelaram uma ausência de evolução das populações virais em um indivíduo PEC e taxas de divergência mensuráveis em um indivíduo EEC [3,1 (1,2-5,6) × 10^{-3} subst./sítio/ano] e em um indivíduo VC [2,9 (0,7-

5,1) × 10^{-3} subst./sítio/ano]. Não existe uma relação simples entre a supressão viral sistêmica e a diversidade proviral intra-hospedeira ou a taxa de nova propagação do reservatório, em controladores do HIV cronicamente infectados. Nossos resultados demonstram que padrões muito divergentes de diversidade viral e divergência intra-hospedeiro podem ser detectados no cenário de supressão natural da replicação do HIV-1, e que a evolução e a ressemeadura do reservatório proviral no PBMC ocorre em alguns controladores de elite.

de Azevedo et al. Retrovirology (2017) 14:29
DOI 10.1186/s12977-017-0354-5

Retrovirology

RESEARCH Open Access

Highly divergent patterns of genetic diversity and evolution in proviral quasispecies from HIV controllers

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Abstract

Background: Ongoing intra-host HIV-1 evolution has been shown in individuals that naturally suppress the viremia to low levels (HIV controllers) by the analysis of the RNA in plasma compartment. Detection of evolution at the DNA proviral compartment in HIV controllers, however, has been more challenging and the precise correlation between the systemic viral suppression level and rate of reservoir's reseeding in those individuals is not fully understood. In this sense, we examined the proviral DNA quasispecies by single genome amplification of the *env* gene in a cohort of 23 HIV controllers from Brazil, divided in three groups, according to the level of systemic viral suppression: (1) elite controllers with persistent undetectable viral load (PEC, $n = 6$); (2) elite controllers with occasional episodes of transient (51–400 copies/mL) viremia (EEC, $n = 7$); and (3) viremic controllers with persistent low-level (80–2000 copies/mL) viremia (VC, $n = 10$).

Results: The HIV-1 diversity of the PBMC-associated proviral quasispecies in EC was significantly ($P < 0.01$) lower than in VC, but not significantly different between PEC and EEC groups. We detected a considerable variation in the average pairwise nucleotide distance and proportion of unique sequences in the HIV-1 proviral quasispecies of PEC and EEC. Some PEC and EEC displayed highly homogenous proviral populations with large clusters of identical sequences, while others exhibited relatively diverse proviral populations with a high proportion of unique sequences comparable to VC subjects. The long-term (10–15 years) follow-up of the HIV-1 proviral populations revealed a complete evolutionary stasis in one PEC and measurable divergence rates in one EEC [3.1 (1.2–5.6) $\times 10^{-3}$ substitutions/site/year and one VC [2.9 (0.7–5.1) $\times 10^{-3}$ substitutions/site/year].

Conclusions: There is no simple relationship between systemic viral suppression and intra-host proviral diversity or rate of reservoir's reseeding in chronically infected HIV controllers. Our results demonstrate that very divergent patterns of intra-host viral diversity and divergence could be detected in the setting of natural suppression of HIV-1 replication and that ongoing evolution and reseeding of the PBMC proviral reservoir occurs in some elite controllers.

Keywords: HIV-1, Elite controllers, Viremic controllers, Reservoir, Diversity, Evolution, Reseeding

Background

The natural history of human immunodeficiency virus type-1 (HIV-1) infections may display very divergent patterns among individuals. Most HIV-1 infected individuals, termed typical progressors (TP), display high plasma viral loads and progress to AIDS without treatment after 5–10 years of infection [1]. Some individuals, termed long-term non-progressors (LTNPs), display longer asymptomatic periods (>10 years) and keep normal CD4$^+$ T cell counts in the absence of treatment [1]; while others, termed HIV controllers, exhibit a durable

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control of viral replication maintaining at very low levels during chronic infection [2]. Among HIV controllers, the viremic controllers (VC) suppress the viremia to levels <2000 HIV-1 RNA copies/mL and the elite controllers (EC) to levels <50-80 HIV-1 RNA copies/mL.

Intra-host HIV-1 evolution in TP follows a consistent pattern of temporal changes in viral diversity and divergence during the course of infection, that affect both proviral DNA populations in peripheral blood mononuclear cells (PBMC) and viral RNA populations in plasma [3]. According to that pattern, infection is usually initiated by a relatively homogeneous viral population (with less than 1% envelope [*env*] diversity) that diversifies during the asymptomatic phase, reaching a peak of population diversity (up to 10% at the *env* gene) and divergence before leveling off or decrease towards the AIDS phase. A roughly similar pattern of intra-host HIV-1 evolution was described for LTNPs and HIV controllers in the plasma compartment [3]. LTNPs display HIV-1 RNA populations that continuously evolve during chronic infection and reach an overall diversity comparable to that observed in TP [4]. Several studies also demonstrate ongoing evolution and divergence of HIV-1 RNA sequences from most EC [5–12], although the mean diversity of plasma populations in EC is significantly lower than that observed for TP at chronic infection [8].

The HIV-1 diversity and divergence pattern of PBMC-associated proviral sequences from LTNPs and HIV controllers, however, differed strikingly from that observed in the plasma virus. In some LTNPs, DNA proviral populations are composed of a complex mixture of archival (dating close to the patient's seroconversion time) and recent (dating close to the sampling time) variants [13] and displayed no temporal structure in the changes of diversity and divergence during chronic infection [14]. In all chronically infected EC and some VC, DNA proviral populations are extremely homogenous (with less than 2% *env* diversity), mostly composed by ancestral sequences and with no measurable divergence over time [5, 9, 10, 12, 15–19]. A recent study demonstrates that most proviral sequences detected in PBMC from HIV controllers are largely representative of archival variants probably integrated during primary infection and propagated by clonal expansion of the memory $CD4^+$ T cell latent reservoir, although rare proviral clones of recent origin could be detected in some patients [12].

These observations suggest that the virus is evolving in HIV controllers, but most evolving plasma viruses do not replenish the PBMC reservoir and the majority of PBMC-associated proviral sequences detected in chronically infected HIV controllers represent ancestral variants. The precise correlation between the systemic viral suppression level and the rate of reservoir's reseeding in HIV controllers, however, is not fully understood. Furthermore, previous studies may have failed to detect proviral sequence replenishment and ongoing evolution in HIV controllers because of the narrow follow-up time (usually 2–6 years). To answer these questions, we performed a cross-sectional analysis of the DNA proviral quasispecies diversity at the *env* gene in 23 HIV controllers with different levels of systemic viral suppression and we also recover the long-term (10–15 years) pattern of changes of HIV-1 proviral populations in the setting of low/undetectable viremia.

Methods

Study subjects

A cohort of 23 HIV controllers, defined as subjects infected with HIV-1 for at least 5 years and maintaining RNA viral loads of <2000 copies/mL without antiretroviral therapy, has been followed-up at the Instituto Nacional de Infectologia Evandro Chagas (INI), Rio de Janeiro, Brazil. These subjects were classified in two categories according to the plasmatic viral load (VL) during follow-up [20]: (1) elite controllers (EC) if most (≥70%) plasma VL determinations were below the limit of detection for the respective available assay (<50–80 copies/mL) (n = 13) and (2) viremic controllers (VC) if most (≥70%) VL determinations were between 80 and 2000 copies/mL (n = 10). The EC were further subdivided in two subgroups [21]: persistent elite controllers (PEC) if 100% of VL measures were below the limit of detection (n = 6) and (2) ebbing elite controllers (EEC) if subjects had occasional (<30% of frequency) episodes of transient low-level (51–400 copies/mL) viremia (n = 7). Patients were followed at least once every 6–12 months to perform infection-monitoring tests such as RNA viral load quantification and $CD4^+$ T lymphocyte count. In each visit, PBMC were obtained by Histopaque-1077 (Sigma, USA) density gradient and stored in liquid nitrogen until use. The present work was approved by the Brazilian National Human Research Ethics Committee (CONEP 14430/2011) and all subjects gave written informed consent.

$CD4^+$ T cell counts and plasma HIV-1 RNA quantification

Absolute $CD4^+$ T cell counts were obtained using the MultiTest TruCount-kit and the MultiSet software on a FACSCalibur flow cytometer (BD Biosciences San Jose, CA). Plasma VL were measured according to the Brazilian Ministry of Health guidelines, with methodologies being updated overtime to improve sensitivity: Nuclisens HIV-1 RNA QT assay (Organon Teknika, Durham, NC, limit of detection: 80 copies/mL) from 1999 to 2007; the Versant HIV-1 3.0 RNA assay (bDNA 3.0, Siemens, Tarrytown, NY, limit of detection: 50 copies/mL) from 2007

to 2013; and the Abbott RealTime HIV-1 assay (Abbott Laboratories, Wiesbaden, Germany, limit of detection: 40 copies/mL) from 2013 to 2016.

HIV-1 DNA extraction and single genome sequencing

Cryopreserved PBMC were thawed, washed and immediately after, the total genomic DNA was isolated with addition of the DNAzol® Reagent (Invitrogen, USA) as described [22]. To limit template resampling, single genome amplification (SGA) was performed by limiting dilution nested PCR at a concentration of DNA that would produce less than 40% of positive PCR reactions, providing a >70% probability that a positive PCR originates from a single molecule [23]. A fragment of nearly 600 bp of the HIV-1 *env* gene (including the C2–C4 regions of gp120) was amplified by PCR using AmpliTaq Gold® 360 DNA Polymerase (Applied Biosystems, USA) as described [17]. The final PCR products were purified using the Illustra GFX PCR DNA purification kit (GE Healthcare, USA) and directly sequenced using the ABI BigDye Terminator v.3.1 reaction Kit (Applied Biosystems, Foster City, CA) in an ABI PRISM 3100 automate sequencer (Applied Biosystem). Chromatograms were assembled into contigs using the SeqMan 7.0 software (DNASTAR Inc., Madison, WI). Sequences resulting from low-quality chromatograms, from chromatograms with double peaks (indicative of more than one template per sequencing reaction), or showing APOBEC3G/F-mediated hypermutation as determined using Hypermut software [24] were discarded.

HIV-1 subtyping

Env sequences from HIV controllers were aligned with HIV-1 subtype reference sequences using ClustalW and then manually edited, yielding a final alignment covering positions 7008–7650 relative to the HXB2 reference genome. Maximum-likelihood (ML) phylogenetic trees were reconstructed with the PhyML 3.0 program [25] using the most appropriate nucleotide substitution model selected using program jModeltest v. 3.7 [26], the SPR branch swapping heuristic tree search algorithm, and the approximate likelihood-ratio test (aLRT) [27] for branch support.

Prediction of coreceptor usage and CCR5 genotyping

The V3 region of *env* sequences was translated using MEGA7 [28] and viral tropism was predicted using Geno2pheno (http://coreceptor.bioinf.mpisb.mpg.de/cgi-bin/coreceptor.pl) with a false positive rate (FPR) cutoff of 5% [29]. The presence of the Δ32 variant in CCR5 was assessed by PCR amplification/agarose gel electrophoresis as previously described [21].

Analyses of viral diversity and divergence

The complexity of proviral quasispecies was characterized using two indices: the mean nucleotide diversity (π) and the normalized Shannon entropy (H_{SN}). The π measures the average number of nucleotide differences between any two sequences of the quasispecies obtained at the same time point and was calculated using MEGA7 [28] as described previously [17]. The H_{SN} provides a measure of haplotype (mutant) frequencies and was calculated by using the R package, Vegan [30], after rarefaction of samples to the small sample size ($n = 10$) for bias correction of sample size differences [31]. The divergence rate of proviral *env* sequences was estimated for three patients (one PEC, one EEC and one VC) with available sequences sampled between 5 and 15 years ago [17]. ML phylogenetic trees were reconstructed for each patient as described above and linear regression analysis of the root-to-tip distances against sampling time were performed using program Tempest [32] to verify the temporal structure of the datasets. The intra-host viral evolutionary (divergence) rate was then directly estimated from the sampling date of the sequences for those datasets with a good temporal structure using program BEAST v1.8 [33]. Analyses were performed using the most appropriate nucleotide substitution model for each patient, a relaxed uncorrelated lognormal molecular clock model [34] with a CTMC rate reference prior [35] and a Bayesian coalescent tree prior [36]. Three MCMC chains were run for 1×10^7 generations and then combined. Effective Sample Size (ESS) and 95% Highest Probability Density (HPD) values were inspected using Tracer v1.6 (http://tree.bio.ed.ac.uk/software/tracer/) to assess the convergence and uncertainty of parameter estimates.

Statistical analysis

Statistical analyses were performed using GraphPad v6 (Prism Software, USA). The Mann–Whitney test was use to compare the quasispecies diversity, the time since HIV-1 diagnosis and the $CD4^+$ T cell counts between subjects groups. Tests were considered significant if the P value was ≤ 0.05.

Results

Epidemiological, clinical and virological characteristics of HIV controllers

The main clinical and epidemiological characteristics of our HIV controllers' cohort are shown in Table 1. Female gender (61%) was more frequent than male (39%), 70% of the patients identified themselves as heterosexual and 22% as men who have sex with men (MSM), while information regarding exposure behavior was not available for 9%. A higher proportion of females (77 vs 40%) and

Table 1 Clinical and epidemiological characteristics of HIV controllers

Patient	Birth date (year)	Gender	Exposure category	Last HIV negative test (year)	First HIV positive test (year)	Median HIV RNA VL (range)	Median CD4$^+$ T cells (IQR)
PEC02	1963	Female	HET	ND	1997	<LD	1272 (1128–1425)
PEC52	1971	Female	HET	ND	1997	<LD	1391 (1343–1461)
PEC30	1983	Male	HET	ND	2009	<LD	842 (669–968)
PEC35	1980	Female	HET	2004	2011	<LD	859 (767–943)
PEC38	1976	Female	Unknown	ND	2011	<LD	1080 (1020–1230)
PEC39	1944	Female	Unknown	ND	2011	<LD	1411 (1072–1640)
EEC09	1969	Male	MSM	ND	2001	<LD (<LD–388)	932 (807–1069)
EEC11	1967	Female	HET	1989	1995	<LD (<LD–580)	1127 (1007–1301)
EEC42	1954	Female	HET	1992	1993	<LD (<LD–341)	991 (924–1118)
EEC17	1950	Female	HET	ND	2000	<LD (<LD–96)	1874 (1674–2132)
EEC18	1933	Female	HET	ND	2001	<LD (<LD–300)	694 (667–809)
EEC19	1968	Male	HET	ND	2006	<LD (<LD–73)	889 (820–973)
EEC36	1976	Female	HET	2005	2010	<LD (<LD–61)	945 (937–1157)
VC04	1965	Female	HET	ND	2008	557 (108–4407)	779 (689–811)
VC05	1964	Male	HET	ND	1991	241 (55–800)	1254 (1101–1410)
VC06	1978	Male	MSM	1999	2000	169 (<LD–405)	1093 (960–1215)
VC14	1970	Female	HET	1996	1999	106 (55–782)	702 (688–757)
VC15	1974	Female	HET	ND	2001	855 (510–2052)	699 (681–825)
VC16	1967	Male	MSM	ND	1998	240 (<LD–1683)	556 (532–608)
VC23	1971	Male	HET	2004	2008	628 (139–1842)	635 (569–671)
VC31	1963	Male	MSM	2006	2006	1558 (587–10,026)	733 (654–814)
VC32	1978	Male	MSM	2004	2005	153 (<LD–722)	641 (564–709)
VC43	1973	Female	HET	ND	2008	232 (66–864)	850 (775–911)

MSM men who have sex with men, *ND* not determined, *VL* viral load (copies/mL), *LD* limit of detection, *IQR* interquartile range, median CD4$^+$ T cell (cells/μL)

heterosexuals (69 vs 40%) was observed in the EC group than in the VC group. Participants had a median age of 49 years (IQR: 41–53 years old) and had documented HIV infection for a median of 11 years (IQR: 6–15 years). The EC and VC groups have a similar median age (52 vs 46 years, respectively) and median documented time of HIV infection (9 vs 10 years, respectively) at sampling time (Additional file 1: Figure S1A, B). None of the HIV controllers exhibited AIDS-related conditions and the CD4$^+$ T cell counts were ≥500 cells/μL during follow-up (Fig. 1). Most of them (83%) also had documented HIV-infection for over 8 years, thus being classified as LTNPs. EC, however, displayed a higher median CD4$^+$ T-cell count than VC at sampling time (1202 vs 735 cells/μL., respectively) (Additional file 1: Figure S1C). No significant differences in clinical and epidemiological characteristics were observed between PEC and EEC subgroups (data not shown).

A cross-sectional analysis of the HIV-1 proviral quasispecies in the 23 HIV controllers was performed by SGA of the *env* gene at between 5 and 20 years after HIV-diagnosis (Fig. 1). Similar median numbers of *env* clones per sample were obtained in EC (16, IQR: 14–18) and VC (18, IQR: 14–21) groups, as well as in PEC (15; IQR: 14–16) and EEC (17; IQR: 15–21) subgroups (Table 2). ML phylogenetic analysis revealed that *env* sequences from most individuals ($n = 20$, 87%) clustered by subject in highly supported (bootstrap >95%) monophyletic lineages (Fig. 2), thus supporting infection by a single variant. For three individuals (EEC09, VC06 and VC32), however, the *env* sequences branched in two independent monophyletic clades (Fig. 2), indicating

(See figure on next page.)

Fig. 1 Clinical follow-up of the 23 HIV-1 controllers. Plasma RNA viral load (copies/mL, *circles*) and CD4$^+$ T cell counts (cells/μL, *squares*) values over time (years) are shown on the *left* and *right Y* axis respectively. RNA viral loads below or above the detection limit are colored *black* and *red*, respectively. The limit of detection of RNA viral load varied over time according to the methodology used. *Shaded areas* indicate the time points selected in this study (*gray*) and previously (*green*) [17] for the DNA quasispecies analysis. Patient identification is shown in the *upper left corner* of each graph

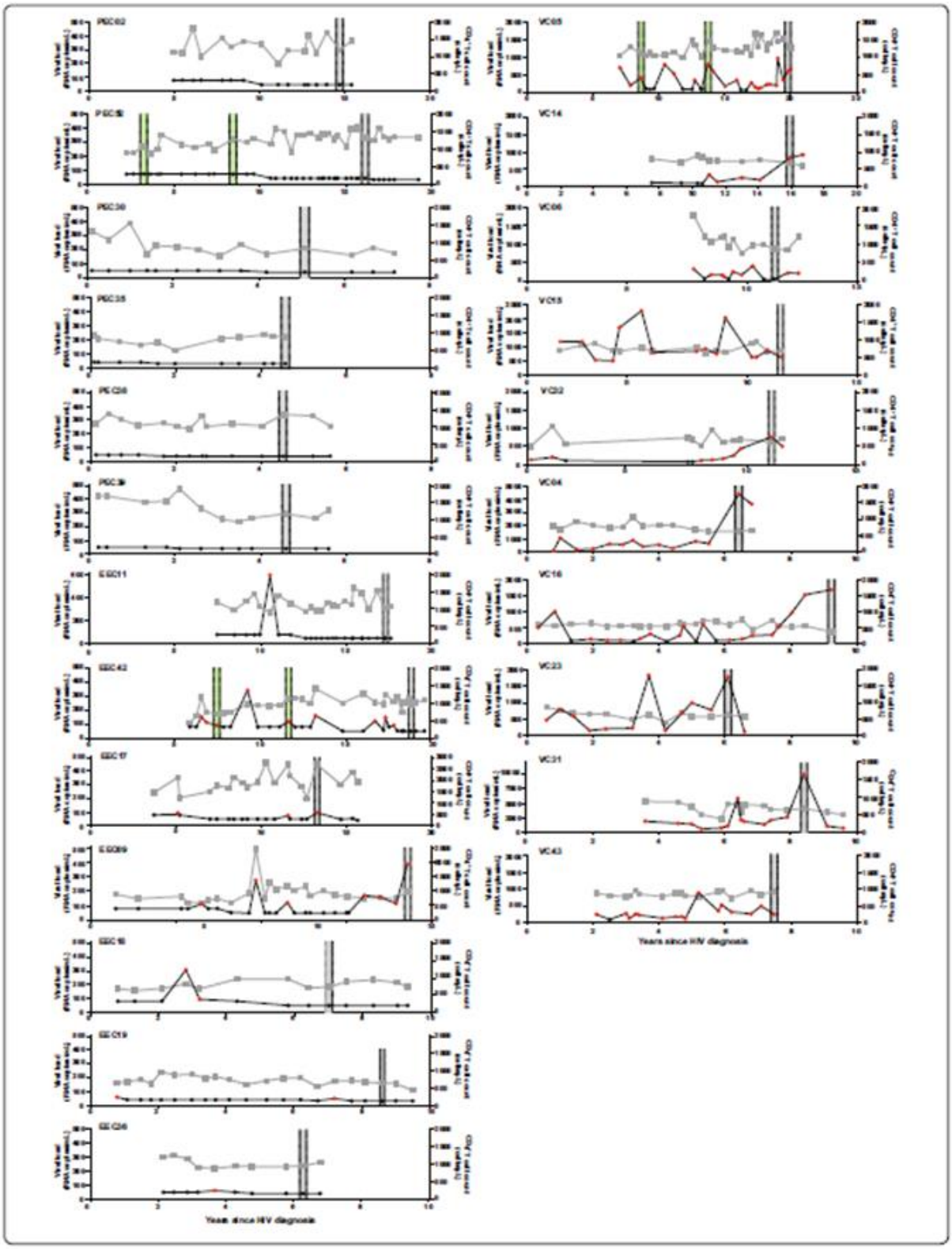

Years since HIV diagnosis
Years since HIV diagnosis

Table 2 Virological characteristics of HIV controllers

Patient	Number of *env* clones	Subtype	HIV-1 tropism[a]		π (%)	H_{SN}	Proportion of unique sequences (%)
PEC02	14	B	100% R5		4.4	0.72	57
PEC52	16	B	100 X4		0.1	0.36	18
PEC30	11	B	55% R5	45% X4	2.9	0.88	81
PEC35	19	F1	100% R5		0.1	0.23	21
PEC38	15	A	100% R5		3.9	0.80	60
PEC39[a]	14	B	100% R5		4.2	0.97	93
EEC09	12	B	100% R5		0.5	–	45
	21	F1	100% R5		0.2	0.59	38
EEC11[a]	13	B	100% R5		0.2	0.57	38
EEC42	16	B	100% R5		1.9	1.00	100
EEC17	10	B	100% R5		4.6	0.94	90
EEC18	20	B	95% R5	5% X4	2.7	0.87	75
EEC19	17	B	100% R5		0.6	0.26	23
EEC36	21	B	100% R5		1.9	0.82	62
VC04	24	C	100% R5		4.8	1.00	100
VC05	14	B	100% R5		5.9	0.93	86
VC06[a]	24	B	100% R5		2.1	0.92	70
	5	B	100% R5		0.02	–	60
VC14	12	F1	100% R5		4.4	0.99	96
VC15	14	B	100% R5		4.4	1.00	100
VC16	21	B	95% R5	5% X4	4.4	1.00	100
VC23	20	C	100% R5		2.5	1.00	100
VC31[a]	15	B	100% R5		6.4	0.98	93
VC32	17	B	100% R5		3.5	0.98	94
	2	F1	100% R5		0.2	–	100
VC43	17	B	70% R5	30% X4	4.1	0.96	88

[a] Heterozygous patients for Δ32 CCR5 allele

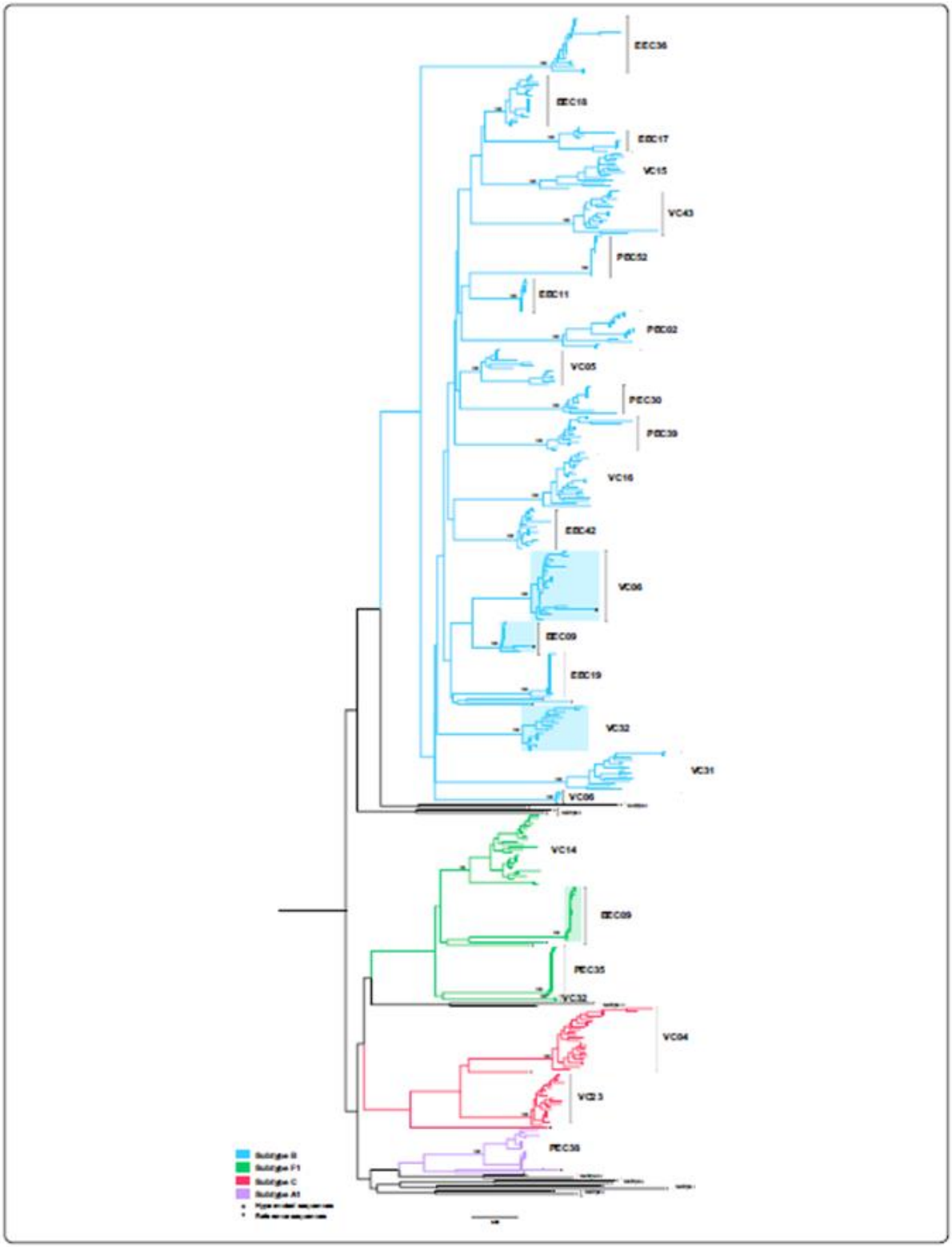
dual infection. The subject VC06 was double infected by two HIV-1 subtype B variants, while individual EEC09 and VC32 were double infected by HIV-1 subtypes B and F1 variants. A second sample from these three individuals was analyzed confirming the previous result (data not shown). Overall, subtype B (74%) was the most frequent HIV-1 variant detected in our cohort, followed by subtypes F1 (13%), C (9%), and A1 (4%) (Fig. 2; Table 2). Prediction of coreceptor usage showed that most individuals (78%) presented only R5-tropic viral clones, two individuals (one EEC and one VC) presented a low frequency (5%) of X4-tropic clones, two individuals (one PEC and one VC) displayed a high frequency (30–45%) of X4-tropic viruses, and one PEC had only X4-tropic viral clones (Table 2). None of the subjects with high frequency of X4-tropic viruses is homozygous/heterozygous for the CCR5/Δ32 genotype (Table 2). Hypermutated proviral sequences were detected at a very low frequency (<5%) in only two individuals (Fig. 2).

Diversity of proviral quasispecies in HIV controllers

To address the potential relationship between systemic viral suppression level and reservoir's reseeding among the 23 HIV controllers of our cohort, we calculated π and H_{SN} indices that measure the average pairwise nucleotide distance and the mutant frequencies (proportion of unique sequences) in the set of aligned sequences of each individual, respectively. For double-infected patients,

(See figure on next page.)

Fig. 2 ML phylogenetic tree of *env* sequences from HIV-1 controllers and HIV-1 subtype reference sequences. Branches were colored according to the subtype assignment as shown in the legend at *bottom left*. The individual's identification is displayed on the right side of the clusters. Sequence clusters from dual infected individuals (EEC09, VC06 and VC32) are indicated by *shaded boxes*. Bootstrap support for each individual cluster is shown. *Black circles* point to the reference sequences and *asterisks* highlight the sequences with APOBEC3G-mediated G to A hypermutations. Horizontal branch lengths are proportional to the *bar at the bottom* indicating nucleotide substitutions per site


EEC36
EEC18
EEC17
VC15
VC43
PEC52
EEC11
PEC02
VC05
PEC30
PEC39
VC16
EEC42
VC06
EEC09
EEC19
VC32
VC31
VC06
VC14
EEC09
PEC35
VC32
VC04
VC23
PEC36

only sequences of the prevalent HIV-1 variant were considered. VC displayed quite diverse (π > 2%) and complex (H_{SN} > 0.90) proviral quasipecies that were mostly (>70%) composed by unique sequences (Table 2; Additional file 2: Figure S2). The overall mean π and H_{SN} estimated for HIV-1 quasispecies in the VC group were significantly higher than those estimated for the EC group ($P < 0.01$), despite the fact that the time since HIV-diagnosis was comparable among groups (Fig. 3). This supports that the PBMC reservoir of VC display higher rate of evolution and reseeding than that of EC.

A closer inspection of the EC group, however, reveals that both diversity and complexity of HIV-1 quasispecies extensively varied among subjects (Table 2; Fig. 3). The combined analysis of π and H_{SN} allow us to detect two divergent patterns of intra-host viral diversity within the EC group (Fig. 4; Additional file 2: Figure S2). The first group (G1) comprises five EC (two PEC and three EEC) that present highly homogenous (π < 1%) proviral quasispecies with large clusters of identical sequences (H_{SN} < 0.6). The second group (G2) comprises eight EC (four PEC and four EEC) showing relatively diverse (π ≥ 2%) proviral populations with high proportion

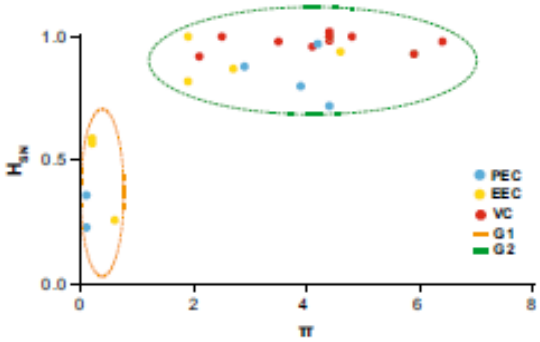


Fig. 4 Identification of divergent patterns of intra-host viral diversity among EC subjects. The normalized Shannon entropy (H_{SN}, *y* axis) of each subject's proviral *env* quasispecies was plotted against the corresponding mean nucleotide diversity (π, *x* axis). The colors of the *circles* represent the different levels of systemic viral suppression in HIV-1 controllers as indicated in the legend. *Dashed circles* identify the two main patterns of intra-host viral diversity: proviral quasispecies of individuals from group 1 (G1, *dashed orange line*) displayed low diversity (π < 1%) and a high proportion of identical sequences (H_{SN} < 0.6); proviral quasispecies of individuals from group 2 (G2, *dashed green line*) exhibited larger diversity (π ≥ 2%) and a higher frequency of unique sequences (H_{SN} > 0.7)

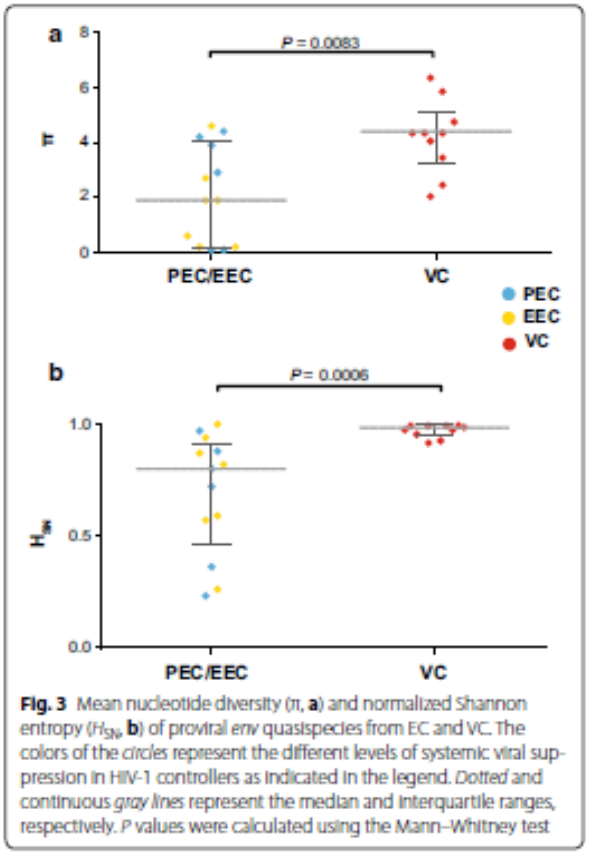


Fig. 3 Mean nucleotide diversity (π, **a**) and normalized Shannon entropy (H_{SN}, **b**) of proviral *env* quasispecies from EC and VC. The colors of the *circles* represent the different levels of systemic viral suppression in HIV-1 controllers as indicated in the legend. *Dotted* and continuous *gray lines* represent the median and interquartile ranges, respectively. *P* values were calculated using the Mann–Whitney test

of unique sequences (H_{SN} > 0.7), comparable to those observed among VC subjects. Similar median values of π and H_{SN} were estimated for EC with or without detection of occasional viremia above the limit of detection (Additional file 3: Figure S3). Quasispecies diversity and complexity were also not correlated with time since HIV diagnosis in EC (Additional file 4: Figure S4).

Rates of evolution of proviral quasispecies in HIV controllers

The pattern of intra-host viral diversity observed in EC-G1 is consistent with amplification of viral reservoir mostly by clonal expansion of infected memory $CD4^+$ T cells; whereas the pattern observed in EC-G2 and VC patients supports a continuous reseeding of the proviral reservoir. To confirm that hypothesis, we investigated the long-term evolution of the PBMC proviral compartment in three individuals from groups EC-G1 (PEC52), EC-G2 (EEC42) and VC (VC05) by combining the *env* proviral sequences obtained in the present study with those obtained from the same patients 10–13 years ago and that were described previously [17]. ML phylogenetic trees were reconstructed for each patient and the root-to-tip distances were plotted against sampling time. Despite the very long follow-up time (13 years), proviral *env* sequences of patient PEC52 were mostly identical and with no evidence of increasing root-to-tip distance over time (Fig. 5), thus confirming absence of

reseeding and evolution of the PBMC reservoir in this patient. All proviral *env* sequences from patient EEC42 and most (70%) *env* sequences from patient VC05 sampled at the most recent time-point, by contrast, were different from those sampled 11–12 years earlier and with clear evidence of evolution (increasing root-to-tip distance over time) (Fig. 5). To estimate the intra-host HIV-1 evolutionary rate in subjects EEC42 and VC05, *env* sequences from different time points were analyzed using the BEAST program. For each cluster of identical sequences, only those *env* sequences sampled at the earliest time point were retained to reduce the impact of latency on intra-host evolutionary rate estimations. According to these analyses, the mean intra-host evolutionary rate of proviral *env* sequences estimated for patient EEC42 was 3.1×10^{-3} subst/site/year (95% HPD: $1.2–5.6 \times 10^{-3}$ subst/site/year) and for patient VC was 2.9×10^{-3} subst/site/year (95% HPD: $0.7–5.1 \times 10^{-3}$ subst/site/year).

Discussion

In this study, we examined the DNA proviral quasispecies diversity at the *env* gene in 23 chronically infected HIV controllers with different levels of systemic viral suppression. Most HIV controllers included in our cohort were females (61%) and this percentage was higher for EC (77%) than for VC (40%). This may be a consequence of the greater frequency of women seeking health services for routine and preventive exams than men, enabling the diagnosis of HIV even in the absence of symptoms [37], and/or may reflect gender-specific differences in the plasma HIV-1 RNA levels [38–40]. Although no HIV controllers exhibited AIDS-related conditions and had $CD4^+$ T cell counts ≥500 cells/μL

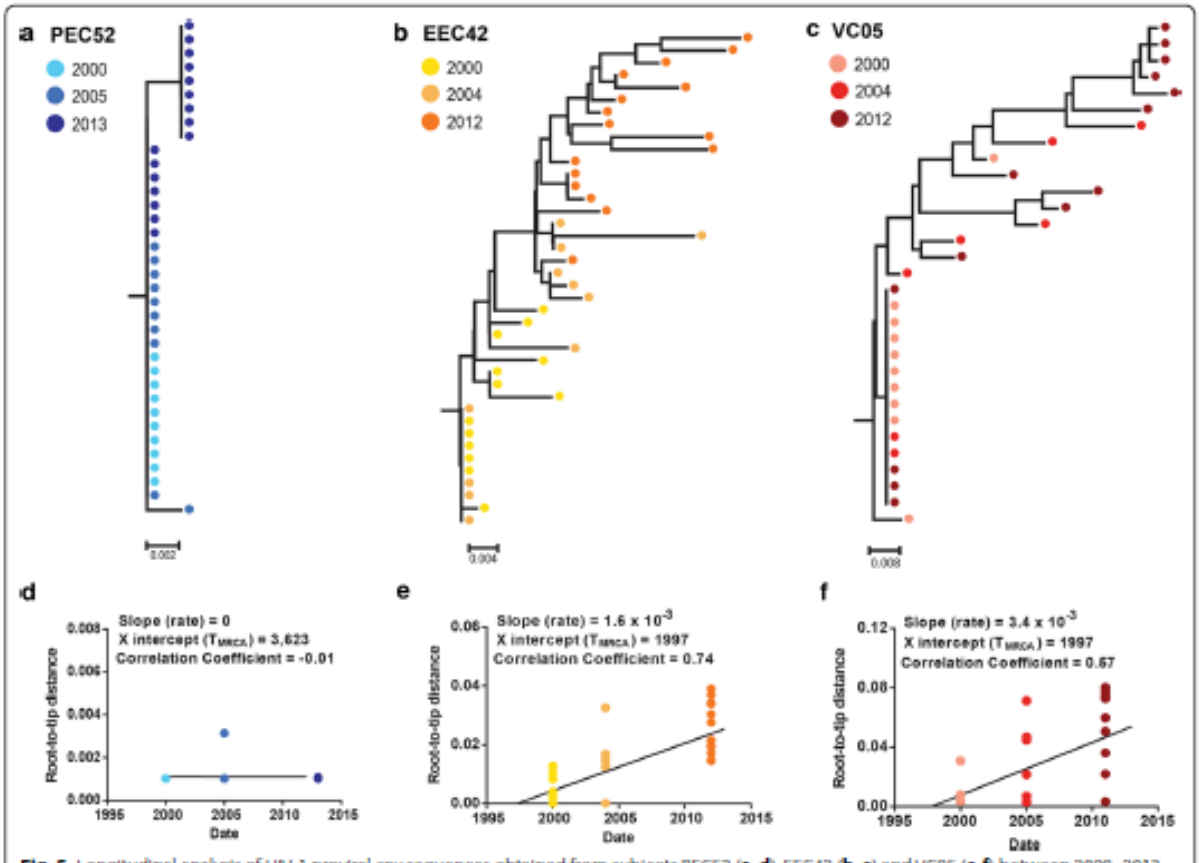


Fig. 5 Longitudinal analysis of HIV-1 proviral *env* sequences obtained from subjects PEC52 (**a**, **d**), EEC42 (**b**, **e**) and VC05 (**c**, **f**) between 2000–2013, 2000–2012 and 2000–2011, respectively. **a**–**c** ML phylogenetic trees for each individual are shown, in which horizontal branch lengths are drawn to scale with the *bar at the bottom* indicating nucleotide substitutions per site. **d**–**f** Plots of the root-to-tip distance against sequence sampling time are shown below each subject tree. The slope, coefficient of regression and X intercept of linear regression analysis is indicated. The colors of the *circles* in phylogenetic trees and plots represent the sampling year at which sequences were obtained

during follow-up, EC (1202 cells/μL) displayed a significantly higher median CD4$^+$ T cell counts than VC (735 cells/μL) at sampling time, supporting the relevance of persistent low-level viremia on the long-term CD4$^+$ T cell decline [20, 41].

Analysis of proviral *env* sequences from HIV controllers revealed a diverse molecular epidemiologic profile with detection of HIV-1 subtypes B (74%), F1 (13%), C (9%) and A1 (4%). While subtypes B, F1 and C are common HIV-1 clades circulating in Brazil [42], subtype A1 has been only described in one case [43]. Three individuals (one EC and two VC) were dually infected with strains of the same (B) or different (B and F1) subtypes, resulting in a prevalence of dual HIV-1 infection (13%) comparable to that previously estimated in a Spanish cohort of LTNP-EC (20%) [44]. Prediction of coreceptor usage further revealed a significant frequency (30–100%) of X4-tropic clones in proviral quasispecies of two PEC and one VC. Reanalysis of proviral *env* sequences from EC and VC already published [12, 16, 18] showed that high frequency (>30%) of X4-tropic clones is a rare phenomenon, being detected in only one out of 25 subjects analyzed (data not shown). These results demonstrate that natural suppression of HIV-1 viremia below 2000 copies/mL can be achieved in the context of either single or dual HIV-1 infections, regardless of the subtype and coreceptor usage of infecting virus.

The HIV-1 proviral population continuously diversifies during untreated asymptomatic infection, although the rate of diversification greatly varies among individuals. In TP with RNA viral loads above 10,000 copies/mL, *env* gene diversity increases at a mean rate of 1%/year and reaches a peak ($\pi = 6$–10%) after 5–10 years post-infection [3]. High levels of *env* proviral diversity ($\pi = 4$-8%) have been also described in samples taken 10–15 years after HIV diagnosis from LTNPs with plasma viremia between 2000 and 10,000 copies/mL [4, 16, 17, 19, 45]. Much lower levels of *env* proviral diversity ($\pi = 0.1$–6%), by contrast, were detected here in samples taken between 5 and 20 years after HIV diagnosis from HIV controllers (RNA viral load lower than 2000 copies/mL). This is consistent with previous studies [15–19] and with the notion that no viral diversification is expected when the host immune response greatly reduces the HIV-1 replication limiting the selection of escape mutants [46].

A closer inspection of the quasispecies diversity in different HIV controller groups here studied, however, revealed a more complex scenario. Particularly, the mean *env* diversity of proviral quasispecies in EC subjects varied over a large range (0.1–4.6%) and two distinct patterns of intra-host viral diversity were observed in that group. While some EC subjects (EC-G1) displayed highly homogeneous proviral populations ($\pi < 1\%$) mainly composed by large clusters of identical sequences ($H_{SN} < 0.6$), other EC subjects (EC-G2) showed more diverse ($\pi \geq 2\%$) proviral populations comprising high proportions of unique sequences ($H_{SN} > 0.7$), comparable to those observed in VC subjects. Thus, contrary to initial expectations, the presence of a highly homogenous PBMC-associated HIV-1 proviral population is not a common characteristic of all EC subjects and no linear correlation could be observed between proviral quasispecies diversity and systemic viral suppression in HIV controllers.

Analysis of the long-term evolution of proviral populations revealed that the distinct patterns of intra-host viral diversity observed in HIV controllers might reflect different driving forces for the maintenance of the viral reservoir. Proviral *env* sequences of individual PEC52 (EC-G1 group) taken over a period of 13 years were mostly identical and displayed no evidence of divergence over time, demonstrating that most PBMC-associated proviral sequences detected in this chronically infected HIV controller represent ancestral variants that persist for >10 years of infection. This pattern supports the notion that the proviral reservoir, in some EC subjects, is mostly maintained by the clonal expansion of CD4$^+$ T lymphocytes. Those cells were probably latently infected at the initial stage of infection, culminating in the absence of evolution and the preservation of a highly homogenous proviral population, similar to those observed in the majority of acutely infected patients [47–54].

In sharp contrast to patient PEC52, proviral populations of subjects EEC42 (EC-G2 group) and VC05 (VC group) displayed an increasing divergence and a partial or complete replacement of sequence variants over time. Although the mean *env* intra-host divergence rate here estimated for HIV controllers ($\sim 3 \times 10^{-3}$ subst/site/year) was much lower than that previously estimated for TP ($\sim 10 \times 10^{-3}$ subst/site/year) [3], the pattern observed is fully consistent with a continuous reseeding of the PBMC proviral reservoir in those HIV controllers. While several studies already demonstrate ongoing evolution and divergence of HIV-1 RNA sequences from the plasma compartment in VC and EC [5–12], this is the first study to quantify the intra-host divergence rate of DNA proviral sequences in the setting of undetectable viremia. These observations demonstrate that the HIV-1 in VC and in some EC is not only evolving, but also that the PBMC reservoir is continuously being resseded at a low, but measurable, rate leading to the partial or complete substitution of ancestral variants over time.

The divergent patterns of genetic diversity and evolution of proviral populations from EC here observed may

be due to: (1) different levels of systemic suppression, (2) diverse mechanisms of natural control of HIV-1 replication, and/or (3) differences in the transmitted virus populations. Although a previous study conducted by our group demonstrated that rare episodes of detectable viremia in EC are associated to higher levels of systemic immune activation and a stronger HIV-1 specific immune response [21], pointing to lower levels of systemic viral supression in EEC than in PEC, we found no significant difference in the quasispecies diversity between both EC subgroups. It is possible that EC-G1 subjects display more efficient control mechanisms, capable of limiting new rounds of infection, particularly in the lymph nodes, than those present in EC-G2 subjects. Finally, it is also possible that the high proviral diversity detected in some EC was not due to intra-host evolution, but was present since the beginning of infection. Indeed, it was demonstrated that a substantial fraction of subjects (20–30%) displayed heterogeneous (2–5% *env* diversity) proviral populations in PBMC before seroconversion, most likely resulting from transmission of multiple HIV-1 variants [47–54]. If differences observed arise from multiple underlying mechanisms, definition of homogenous EC subgroups could become increasing challenging as more subjects are characterized.

Conclusions

These results reveal that very divergent patterns of intra-host viral diversity and divergence could be detected in the setting of natural suppression of HIV-1 replication, suggesting that HIV-1 may evolve differently in every patient. We found no simple relationship between systemic viral suppression and intra-host proviral diversity or rate of reservoir's reseeding in chronically infected HIV controllers, although the influence of some potential confounding factors such as the transmission of multiple HIV-1 variants in some EC cannot be ruled out. Our study also demonstrates that ongoing evolution and reseeding of the PBMC proviral reservoir is possible in some EC. The long-term longitudinal follow-up of more EC patients will be important to elucidate the major driving forces of the different intra-host evolutionary patterns here detected as well as their impact on the long-lasting control of HIV-1 replication and disease progression.

Additional files

Additional file 1: Figure S1. Median of the Age (A), time since HIV diagnosis (B) and CD4+ T cell count (C) of HIV-1 controllers at the sampling point. The colors of the circles represent the different levels of systemic viral suppression in HIV-1 controllers as indicated in the legend. Dotted and continuous gray lines represent the median and interquartile ranges, respectively. *P* values were calculated using the Mann–Whitney test.

Additional file 2: Figure S2. ML phylogenetic trees of HIV-1 proviral *env* sequences obtained by SGA from PBMC of EC-G1, EC-G2 and VC subjects. Each tree represents the sequences from an individual. Presence of black and white circles in subjects EEC09, VC06 and VC32 is indicative of dual infection. Trees were rooted at the midpoint. Horizontal branch lengths are drawn to scale with the bar at the bottom indicating nucleotide substitutions per site. Sequences with G-to-A hypermutations were removed from this analysis.

Additional file 3: Figure S3. Mean nucleotide diversity (π, A) and normalized Shannon entropy (H_{Sn}) (B) of proviral *env* quasispecies from PEC and EEC. The colors of the circles represent the different levels of systemic viral suppression in HIV-1 controllers as indicated in the legend. Dotted and continuous gray lines represent the median and interquartile ranges, respectively. *P* values were calculated using the Mann–Whitney test.

Additional file 4: Figure S4. Mean nucleotide diversity (π, A) and normalized Shannon entropy (H_{Sn}, B) of proviral *env* quasispecies from PEC and EEC plotted against time since HIV diagnosis. The *P* value of linear regression analysis is indicated in each plot. The colors of the circles represent the subject classification according to the pattern of intra-host viral diversity (G1 and G2) described in Figure 4, as indicated in the legend at the right.

Authors' contributions
SSDA conducted most of the experiments and analyzed the data. DGC participated in DNA extraction and single genome sequencing. FHC participated in sample processing and determination of $CD4^+$ T-cell counts. SLMT and KSS conducted the CCR5 genotyping. BH, BG and VGV conducted the patient recruitment and follow-up. MGM participated in study design and supervised the project. GB conceived and designed the study and supervised the experiments. SSDA and GB wrote the first draft. All authors read and approved the final manuscript.

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Acknowledgements
We thank Dr. Edson Delatorre for assisting in the Vegan analysis, Dr. Vera Bongertz for review of the manuscript and the Plataforma Genômica de Sequenciamento de DNA-RPT01A-PDTIS/FIOCRUZ for the nucleotide sequencing. We also thank all patients whose participation and commitment made this work possible.

Competing interests
The authors declare that they have no competing interests.

Availability of data and materials
HIV-1 sequences generated during the current study were deposited in Genbank under the Accession Numbers KY852518—KY852939.

Ethics approval and consent to participate
The present work was approved by the Brazilian National Human Research Ethics Committee (CONEP 14430/2011) and all subjects gave written informed consent.

Funding
SSDA is supported by funding from the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq). This work was supported by Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro—FAPERJ (Grant Number E-26/110.123/2014).

Publisher's Note

Received: 31 March 2017 Accepted: 25 April 2017
Published online: 02 May 2017

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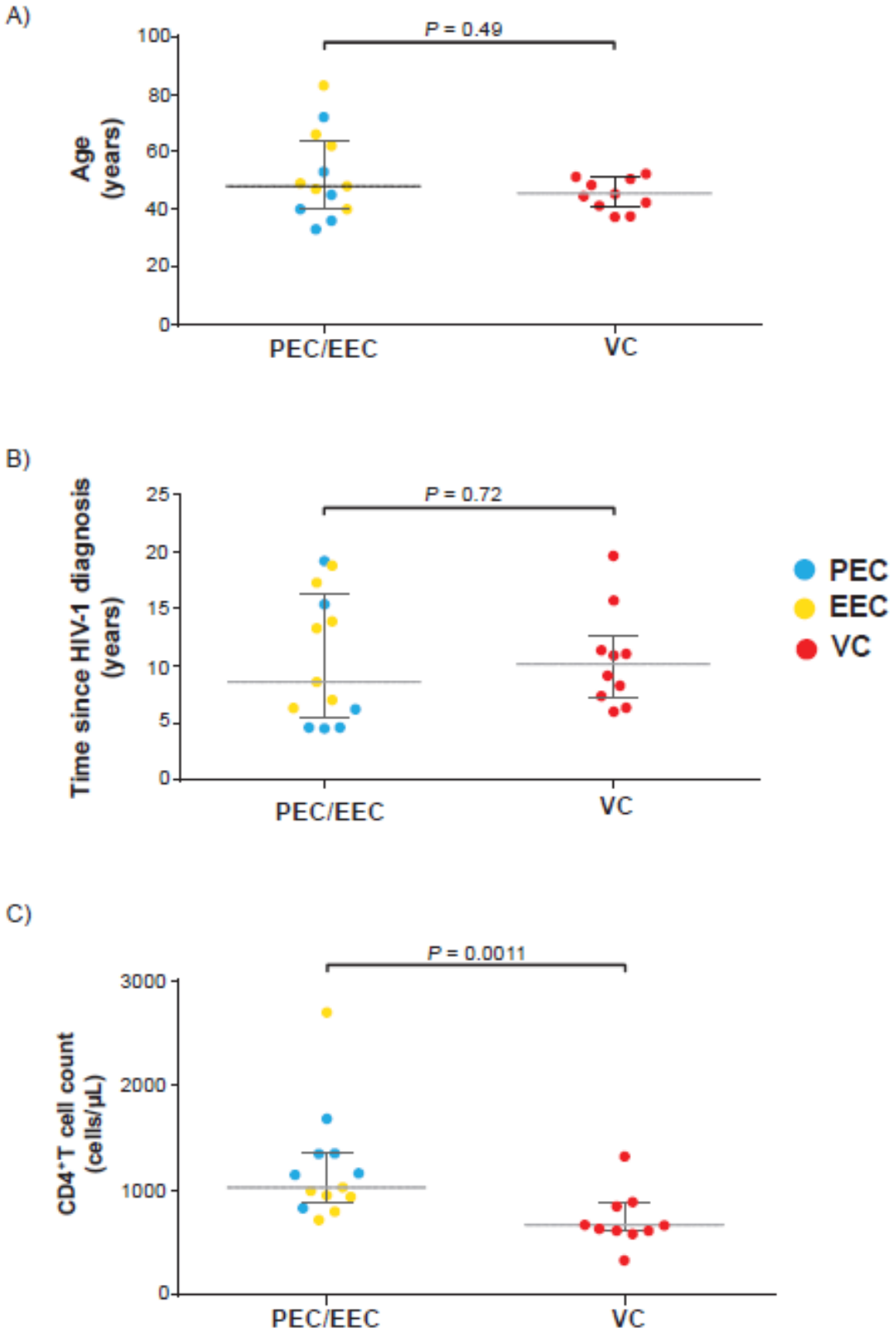

Figure S1. Median of the Age (A), time since HIV diagnosis (B) and CD4+ T cell count (C) of HIV-1 controllers at the sampling point. The colors of the circles represent the different levels of systemic viral suppression in HIV-1 controllers as indicated in the legend. Dotted and continuous gray lines represent the median and interquartile ranges, respectively. P values were calculated using the Mann–Whitney test.

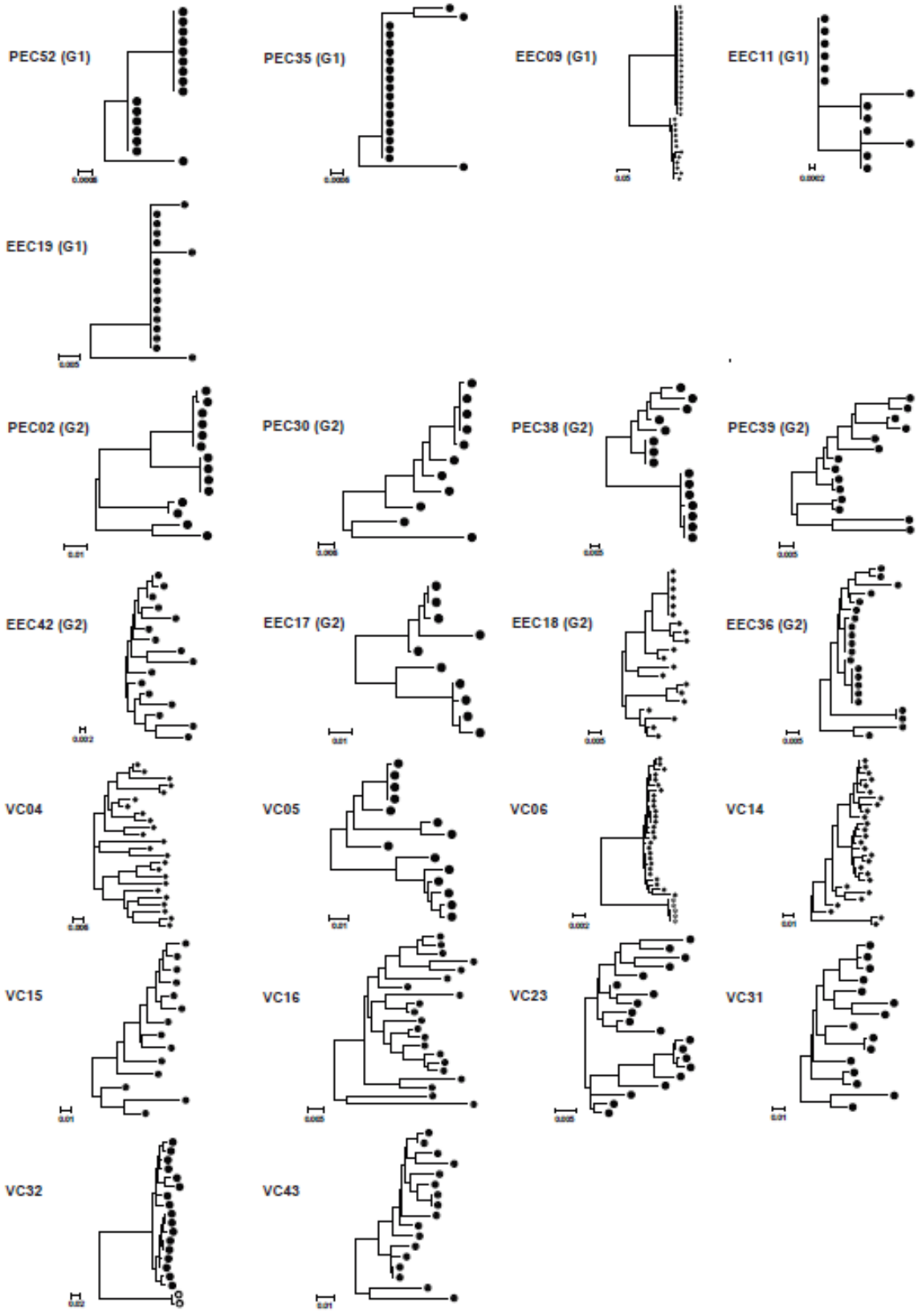

Figure S2. ML phylogenetic trees of HIV-1 proviral env sequences obtained by SGA from PBMC of EC-G1, EC-G2 and VC subjects. Each tree represents the sequences from an individual. Presence of black and white circles in subjects EEC09, VC06 and VC32 is indicative of dual infection. Trees were rooted at the midpoint. Horizontal branch lengths are drawn to scale with the bar at the bottom indicating nucleotide substitutions per site. Sequences with G-to-A hypermutations were removed from this analysis.

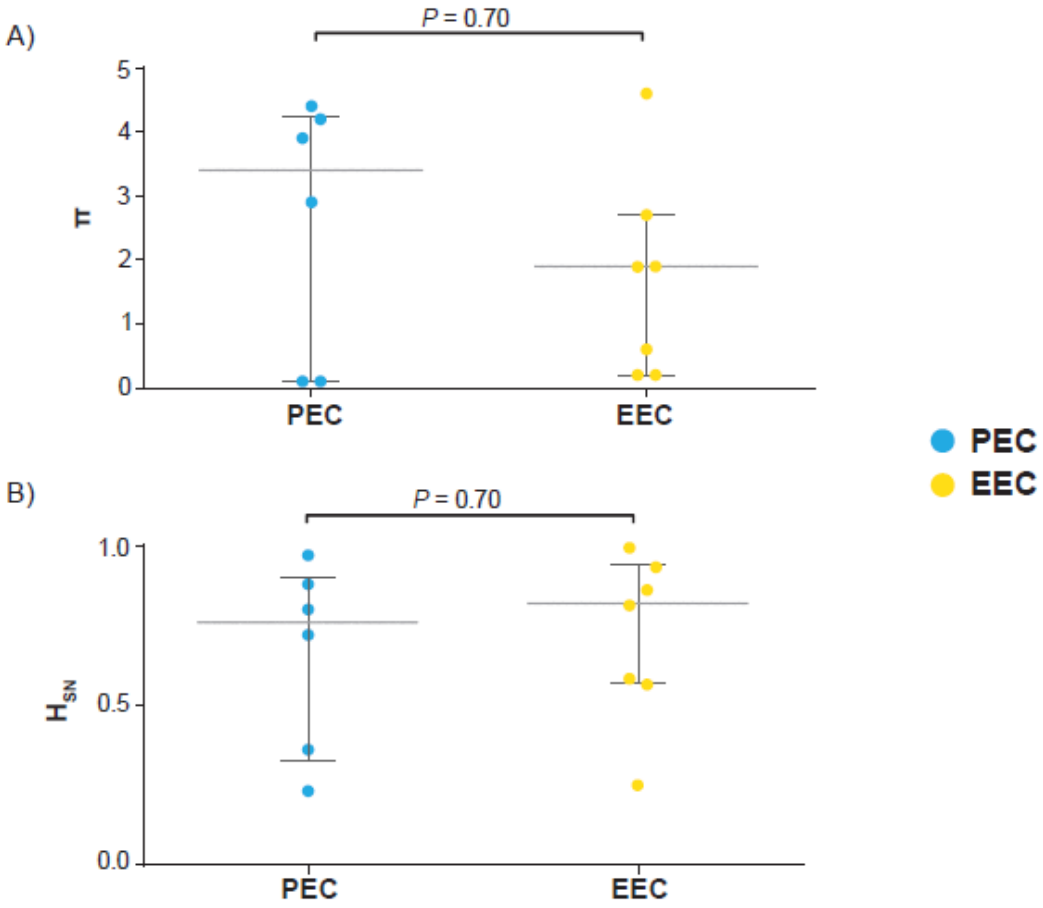

Figure S3. Mean nucleotide diversity (π, A) and normalized Shannon entropy (H SN) (B) of proviral env quasispecies from PEC and EEC. The colors of the circles represent the different levels of systemic viral suppression in HIV-1 controllers as indicated in the legend. Dotted and continuous gray lines represent the median and interquartile ranges, respectively. P values were calculated using the Mann–Whitney test.

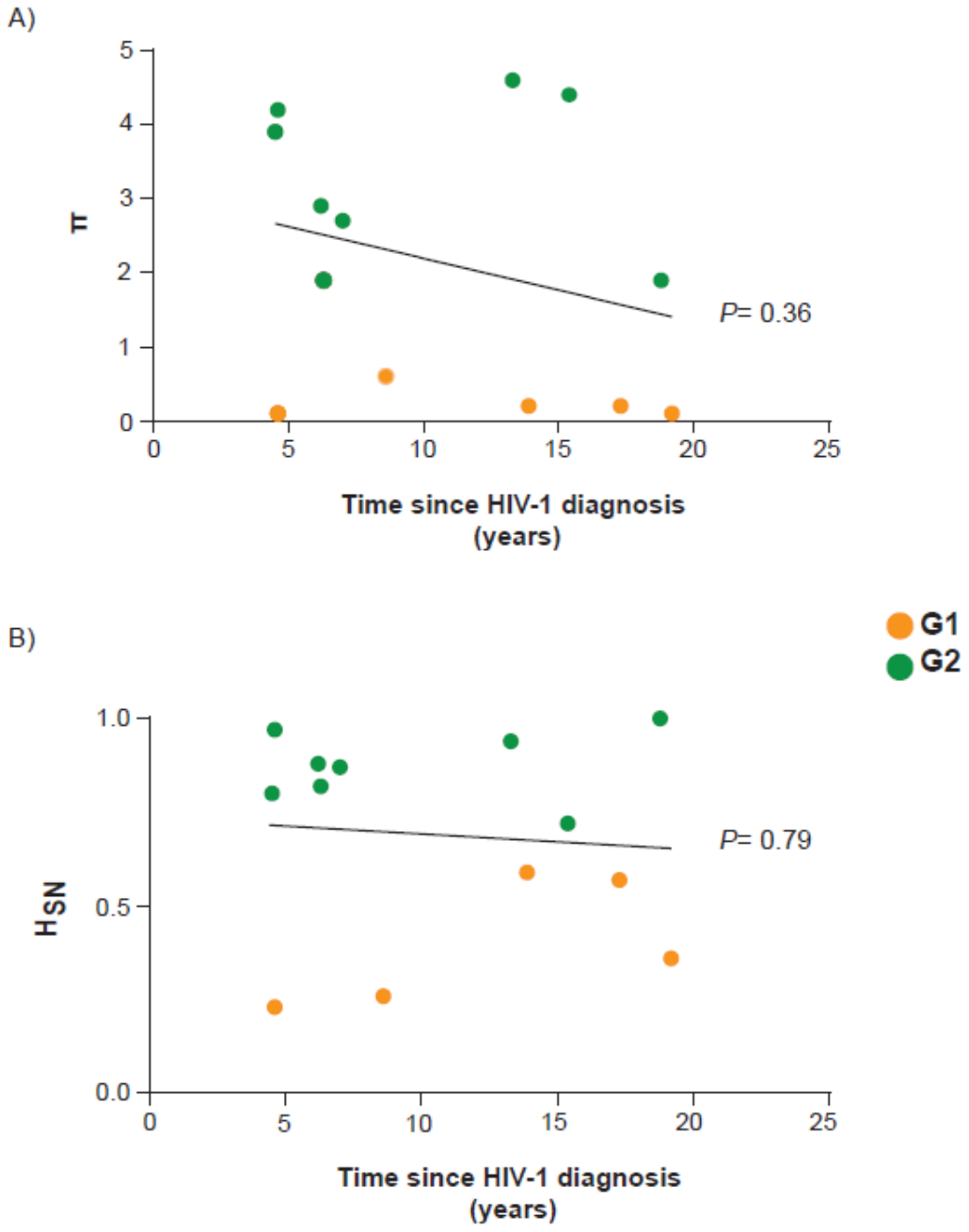

Figure S4. Mean nucleotide diversity (π, A) and normalized Shannon entropy (HSN, B) of proviral *env* quasispecies from PEC and EEC plotted against time since HIV diagnosis. The P value of linear regression analysis is indicated in each plot. The colors of the circles represent the subject classification according to the pattern of intra-host viral diversity (G1 and G2) described in Figure 4, as indicated in the legend at the right.

3.2 ARTIGO 2

HIV controllers suppress viral replication and evolution and prevent disease progression following intersubtype HIV-1 superinfection.

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Periódico: AIDS, 2018.

Resumo:

Controladores do HIV (HIVc), são indivíduos que naturalmente suprimem a replicação do HIV-1 a níveis baixos por muitos anos. Se esses indivíduos são capazes de controlar a viremia e manter a estabilidade dos parâmetros imunológicos após a superinfecção (SI) do HIV-1 não está bem elucidado. O objetivo deste estudo foi investigar o impacto da superinfecção intersubtipo do HIV-1 na viremia, na ressemeadura do reservatório, na evolução viral e na progressão da doença em controladores de HIV (HIVc). Para tanto, conduzimos uma análise longitudinal de dois HIVc brasileiros (EEC09 e VC32), previamente identificados como duplamente infectados com vírus dos subtipos B e F1. Alterações na viremia plasmática, nos níveis totais de DNA do HIV-1, na contagem de células T CD4 e na composição das quasispécies do HIV-1 foram medidas ao longo do tempo. A diversidade do *env* de HIV-1 em amostras de células mononucleares do sangue periférico (PBMC) e plasma foi acessada por amplificação de genoma único e sequenciamento de nova geração, respectivamente. A evolução viral foi avaliada pela estimativa da diversidade e divergência nucleotídica. O indivíduo EEC09 provavelmente se infectou inicialmente com uma cepa do HIV-1 do subtipo B, com tropismo para CCR5 e sequencialmente se superinfectou com uma cepa do subtipo B com tropismo para CXCR4 e novamente com uma cepa do subtipo F1. O indivíduo VC32 se infectou inicialmente com uma cepa do subtipo B e se superinfectou com uma cepa do subtipo F1. Os eventos de superinfecção intersubtipo levaram a um aumento moderado da viremia e a um extenso *turnover* da população viral no plasma, mas causaram um impacto divergente no tamanho e composição da população de DNA do HIV-1 associado às células. Ambos os indivíduos mantiveram o controle virológico (<2.000 cópias/ml) e não apresentaram evidência de evolução viral ou progressão imunológica, por pelo menos

2 anos após o evento de superinfecção intersubtipo. Estes dados revelaram que alguns HIVc são capazes de limitar repetidamente a replicação e a evolução de cepas virais superinfecciosas de um subtipo diferente, sem sinais de progressão da doença.

HIV controllers suppress viral replication and evolution and prevent disease progression following intersubtype HIV-1 superinfection

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Objective: The aim of this study was to investigate the impact of intersubtype HIV-1 superinfection on viremia, reservoir reseeding, viral evolution and disease progression in HIV controllers (HIC).

Design: A longitudinal analysis of two Brazilian HIC individuals (EEC09 and VC32) previously identified as dually infected with subtypes B and F1 viruses.

Methods: Changes in plasma viremia, total HIV-1 DNA levels, $CD4^+$ T-cell counts and HIV-1 quasispecies composition were measured over time. HIV-1 *env* diversity in peripheral blood mononuclear cell (PBMC) and plasma samples was accessed by single genome amplification and next-generation sequencing approaches, respectively. Viral evolution was evaluated by estimating nucleotide diversity and divergence.

Results: Individual EEC09 was probably initially infected with a CCR5-tropic subtype B strain and sequentially superinfected with a CXCR4-tropic subtype B strain and with a subtype F1 variant. Individual VC32 was infected with a subtype B strain and superinfected with a subtype F1 variant. The intersubtype superinfection events lead to a moderate increase in viremia and extensive turnover of viral population in plasma but exhibited divergent impact on the size and composition of cell-associated HIV DNA population. Both individuals maintained virologic control (<2000 copies/ml) and presented no evidence of viral evolution or immunologic progression for at least 2 years after the intersubtype superinfection event.

Conclusion: These data revealed that some HIC are able to repeatedly limit replication and evolution of superinfecting viral strains of a different subtype with no signs of disease progression.

AIDS 2019, **33**:399–410

Keywords: disease progression, HIV controllers, reservoir, superinfection, viral load

Introduction

By definition, HIV-1 infection with more than one strain is classified as a coinfection or as a superinfection when the acquisition of a new variant occurs prior/simultaneously to or after seroconversion, respectively. Multiple HIV-1 infections are typically associated with increased viral load levels and faster decline in $CD4^+$ T cells [1–13]. There is some debate, however, on the impact of superinfection on HIV controllers

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Received: 13 August 2018; accepted: 26 October 2018.

DOI:10.1097/QAD.0000000000002090

ISSN 0269-9370

(HIC), a rare group of individuals who can efficiently suppress systemic HIV-1 replication and maintain RNA viral loads in the undetectable (<50–80 copies/ml; elite controllers) or detectable (50–2000 copies/ml; viremic controllers) range in the absence of antiretroviral therapy (ART) [14]. Some cross-sectional studies detected a relatively high frequency of dually subtype B infected HIC [15–17], suggesting the ability of these individuals to control replication of different viruses of the same subtype. A few longitudinal analyses, however, described a sharp increase in plasma viremia and concomitant loss of $CD4^+$ T cells after both intrasubtype and intersubtype superinfection in HIC [18–20], resembling the pattern observed in HIV noncontrollers [1–13].

Longitudinal studies in HIV noncontrollers individuals revealed that superinfection may have a high impact on HIV DNA reservoir reseeding and viral evolution, although the outcome of the original and superinfecting viruses seems to greatly vary across individuals. In some individuals, the secondary infecting strain is only transiently detected and declines rapidly, becoming undetectable few months after superinfection [13,21]. In other individuals, both original and superinfecting viruses cocirculate for several months after superinfection [22]. Finally, in other individuals, there is a complete turnover of the viral quasispecies within a few months after superinfection, and the initial HIV-1 strain is virtually replaced by the new infecting virus and/or by recombinants between initial and secondary strains [13,21–26]. To date, the impact of superinfection on cell-associated HIV DNA diversity in HIC has not yet been characterized.

HIV-1 Brazilian epidemic has been characterized by the circulation of different subtypes and recombinant forms, a scenario that favours the occurrence of intersubtype superinfection events [27]. In a recent cross-sectional study, we identified two Brazilian HIC dually infected with subtypes B and F1 viruses [28]. Here, we performed an extensive longitudinal analysis of these two cases of dual HIV-1 infection to distinguish between coinfection and superinfection. In addition, we evaluated the short and long-term impact of intersubtype HIV-1 dual infections on viremia, reservoir reseeding, viral evolution and disease progression. We showed that HIC analysed in this study were susceptible to intersubtype HIV-1 superinfection and that the event was associated to an extensive turnover of viral population in the plasma, whereas the impact of superinfection on cell-associated HIV DNA diversity varied highly across individuals. Furthermore, both HIC were able to maintain control of viremia and suppress evolution of both initial infecting (subtype B) and superinfecting (subtype F1) HIV-1 strains for several years after superinfection, without evidence of disease progression.

Materials and methods

Study participants

Individuals EEC09 and VC32 were followed-up at the Instituto Nacional de Infectologia Evandro Chagas (INI) from Rio de Janeiro (Brazil) and selected from a previous study [28], as they were dually infected with subtype B and F1 viruses. Both individuals were adults and provided written informed consent documents approved by the INI Institutional Review Board (Addendum 049/2010) and the Brazilian National Human Research Ethics Committee (CONEP 14430/2011). Individual EEC09 was a 48-year-old homosexual male who was diagnosed with HIV-1 infection in 2001 and remained treatment-naive until June 2015, when he initiated combined ART (cART) after the proposal of the clinicians following the recent recommendation of the Brazilian Ministry of Health. This determination follows the global strategy to control HIV/AIDS pandemic, in which all HIV-infected individuals should be treated, independent of the $CD4^+$ T-cell counts or viral load measurements. At enrolment in our cohort in February 2009, individual EEC09 was classified as an elite controller, as most (≥70%) of his plasma viral load determinations were below the limit of detection for the available assays (<50–80 copies/ml). VC32 was a 39-year-old homosexual male who received the diagnosis of HIV-1 infection in 2004 and had remained antiretroviral-naive until the present study (personal decision). At study entry in April 2012, individual VC32 was classified as a viremic controller, as most (≥70%) of his plasma RNA viral load determinations were between 80 and 2000 copies/ml. At each follow-up visit, samples of peripheral blood mononuclear cells (PBMC) were obtained using Histopaque-1077 (Sigma, St. Louis, Missouri, USA) by density gradient centrifugation and stored in liquid nitrogen until further use, and plasma samples were separated and stored at −80°C. PBMC and plasma samples of individuals EEC09 and VC32 collected over 7 (2009–2015) and 6 (2012–2016) years, respectively, were analysed. To avoid cross-sample contamination, nucleic acid extraction and amplification from each PBMC and/or plasma sample were performed in individual experiments as described in the following sections.

Quantification of total cell associated HIV DNA

Total DNA was extracted from PBMC samples (1×10^7 cells) using the QIAamp DNA Mini Kit (Qiagen, Hilden, North Rhine-Westphalia, Germany) on each visit. Cell-associated HIV-1 DNA was quantified using the Generic HIV DNA cell Kit (Biocentric, Bandol, Var, France), following the manufacturer's recommendations. Results were reported either as actual numbers of HIV DNA copies/10^6 cells or as the threshold value of detection.

Single genome amplification and sequencing of HIV DNA sequences

Single genome amplification (SGA) of PBMC-associated HIV DNA *env* sequences was performed by limiting dilution nested PCR using previously described conditions

[28]. The PCR products were sequenced using the ABI BigDye Terminator v.3.1 reaction Kit (Applied Biosystems, Foster City, California, USA) in an ABI PRISM 3100 automated sequencer (Applied Biosystems). Chromatograms were assembled into contigs using the SeqMan Pro 11 software (DNASTAR Inc., Madison, Wisconsin, USA). Sequences resulting from chromatograms with double peaks or showing APOBEC3G/F-mediated hypermutation as determined using Hypermut software [29] were discarded.

HIV RNA haplotype prediction using next-generation sequencing data

Total RNA from plasma samples was extracted using the QIAamp Viral RNA Mini Kit (Qiagen) if viral load was at least 200 copies/ml or the QIAamp UltraSens Virus Kit (Qiagen) if viral load was less than 200 copies/ml, according to the manufacturer's instructions. cDNA was obtained by RT-PCR using SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, California, USA) and subjected to nested PCR for *env* amplification as described above. cDNA library preparation was performed using the Nextera XT DNA Library Prep Kit (Illumina, San Diego, California, USA) and each sample was subjected to barcoding with a unique index from the Nextera XT Index Kit (Illumina). High-throughput sequencing was performed with MiSeq Reagent Nano Kit, v2 (250 nt paired-end reads). Demultiplexed reads were trimmed to remove adaptors, low-quality regions ($Q < 25$) and short reads (<100 bp). The remaining reads were mapped against SGA consensus sequences from each patient using Geneious software v.9.1.8 [30], ensuring high mapping quality (MAQ $\geq$30) and a minimum overlapping identity of 90%. Alignment regions with at least 500× coverage (Supplemental Fig. 1, http://links.lww.com/QAD/B405) were exported in BAM format and used for haplotype reconstruction using QuasiRecomb 1.2 [31] employing the flag '-conservative' to increase specificity while considering the recombination process. Only haplotypes with frequency of at least 1% were used for further analyses.

Analyses of *env* sequences and coreceptor tropism prediction

All SGA and NGS sequences were screened for potential cross-contamination using BLAST against both local and public repositories of HIV-1 sequences as previously described [32]. Viral *env* sequences obtained from SGA and NGS approaches for each patient's visit were aligned with HIV-1 subtype reference sequences using ClustalW and then manually edited, yielding a final alignment covering positions 7008–7650 relative to the HXB2 reference genome. Maximum-likelihood phylogenetic trees were reconstructed with the PhyML 3.0 program [33] as previously described [28]. The genetic complexity of cell-associated HIV DNA quasispecies was characterized by calculating the mean nucleotide diversity (π) using MEGA7 [34] as described previously [28], and the mean nucleotide divergence was determined by performing linear regression analysis of the root-to-tip distances against sampling time using the program TempEst [35]. 'Viral dating' was estimated from the nucleotide divergence of each individual virus to the most recent common ancestor of the HIV-1 subtype B pandemic as described previously [16,36,37]. The V3 region of *env* nucleotide sequences was translated using MEGA7 [34], and viral tropism was predicted using Geno2pheno (http://coreceptor.bioinf.mpisb.mpg.de/cgi-bin/coreceptor.pl) with a false positive rate (FPR) cutoff of 5% [38].

Statistical analysis

Linear regression analyses were performed using GraphPad v6 (Prism Software, La Jolla, California, USA) to verify evidence of clinical (decline in $CD4^+$ T-cell counts) progression after superinfection events. *P* value of 0.05 or less was considered significant.

Availability of data and material

HIV-1 sequences generated during the current study were deposited in GenBank under the accession numbers MH244562–MH244833. The data generated with NGS were made available in the SRA database under the accession number SRP139352.

Results

Longitudinal clinical monitoring of the patients

In a previous cross-sectional study, we described two HIC that were dually infected with subtype B and F1 variants [28]. Longitudinal analysis of plasma viremia revealed episodes of viral load increase during follow-up of both individuals. Individual EEC09 displayed undetectable plasma RNA viral load (<50–80 copies/ml) in most (21/24; 83%) measurements between HIV-1 diagnosis in January 2001–December 2012 (Fig. 1a), confirming the classification of this individual as an elite controller. After 10 years of follow-up, individual EEC09 presented six consecutive time points of detectable viral load (114–581 copies/ml), reaching a peak in July 2015. At this time, individual initiated cART following the recent recommendation of the Brazilian Ministry of Health to treat all HIV-infected individuals irrespective of viral load or $CD4^+$ T-cell counts. Despite the loss of elite control, patient EEC09 maintained the status of viremic controller and displayed stable $CD4^+$ T-cell counts within the normal range (595–2469 cells/μl) during follow-up (Fig. 1a). Individual VC32 displayed relatively stable plasma RNA viral load in the low range (>50–170 copies/ml) from HIV-1 diagnosis in July 2004 to April 2013 (Fig. 1b). After April 2013, plasma RNA viral load increased progressively up to 722 copies/ml in June 2015 and then gradually decreased, reaching undetectable levels in November 2017. Individual VC32 refuses to

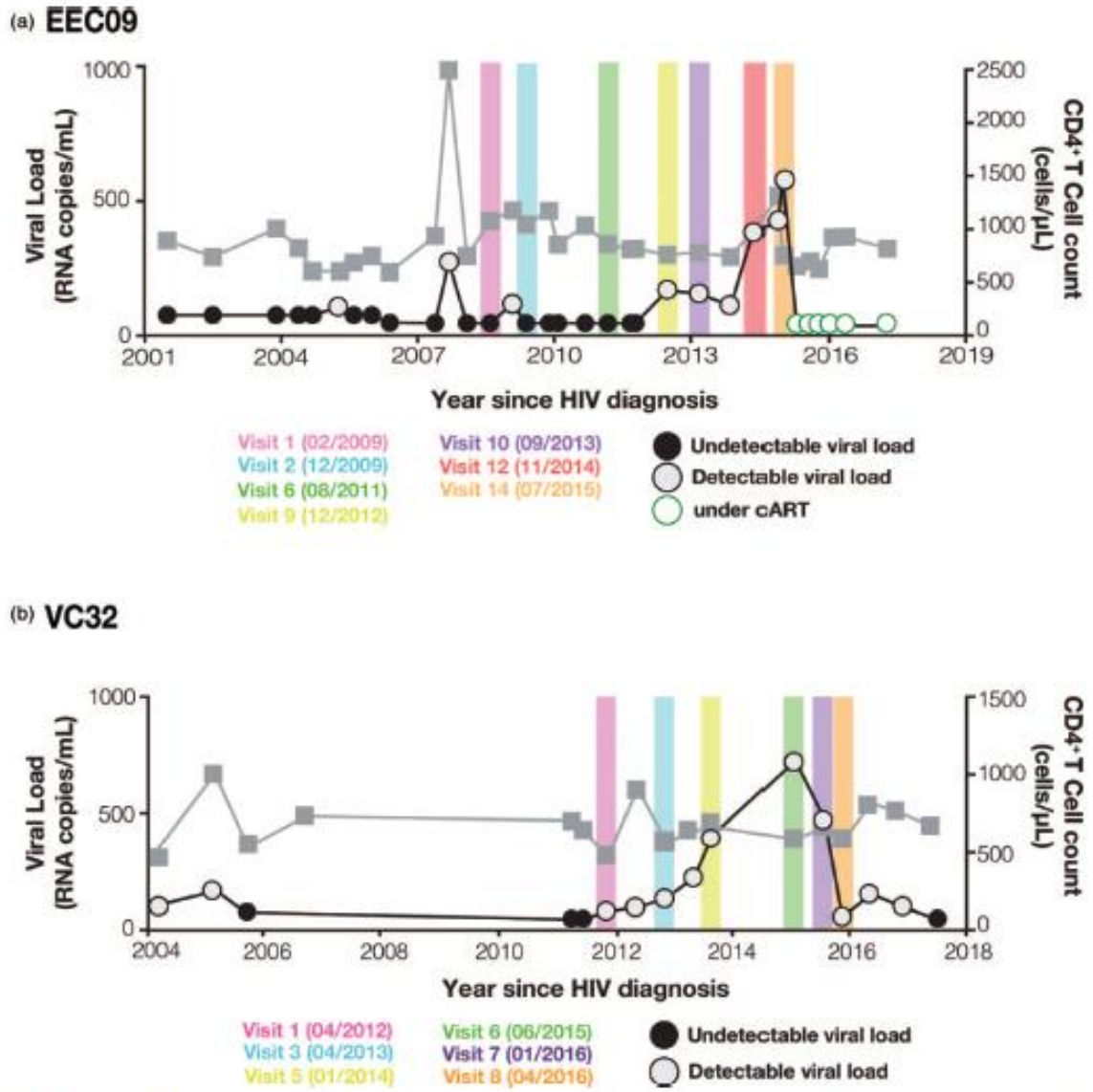

Fig. 1. Longitudinal clinical follow-up of HIC. Plasma RNA viral load (copies/ml, circles) and $CD4^+$ T-cell counts (cells/µl, squares) values since HIV diagnosis of individuals EEC09 (a) and VC32 (b) are shown on the left and the right Y-axis, respectively. Grey and black circles indicate detectable and undetectable viral loads, respectively. RNA viral load measurements after the start of combined antiretroviral therapy (cART) are in green. Coloured shaded areas indicate the time points (visit, month/year) selected for the DNA/RNA quasispecies analysis.

initiate cART and continuously fulfilled the viremic controller profile criteria and displayed stable $CD4^+$ T cell counts within the normal range (469–1005 cells/µl) at all time points (Fig. 1b). No clinical manifestation was reported in the medical records of both individuals that could be associated with viremia increase after several years of stable control. To better understand whether the increase in viremia was related to a superinfection event in the dually infected individuals EEC09 and VC32, we longitudinally assessed viral quasispecies diversity in the PBMC and plasma.

Evidence of intrasubtype and intersubtype HIV-1 superinfection in individual EEC09

We obtained 170 *env* sequences derived from samples collected at seven visits over a period of 6.5 years (February 2009–July 2015) before initiation of cART. ML phylogenetic analysis revealed that *env* sequences from individual EEC09 branched in three highly supported [approximate likelihood ratio test (aLRT) > 0.95] independent monophyletic clades, with two within-subtype B clade (hereafter called as B_1 and B_2 variants) and one within-subtype F1 clade (Fig. 2a). This

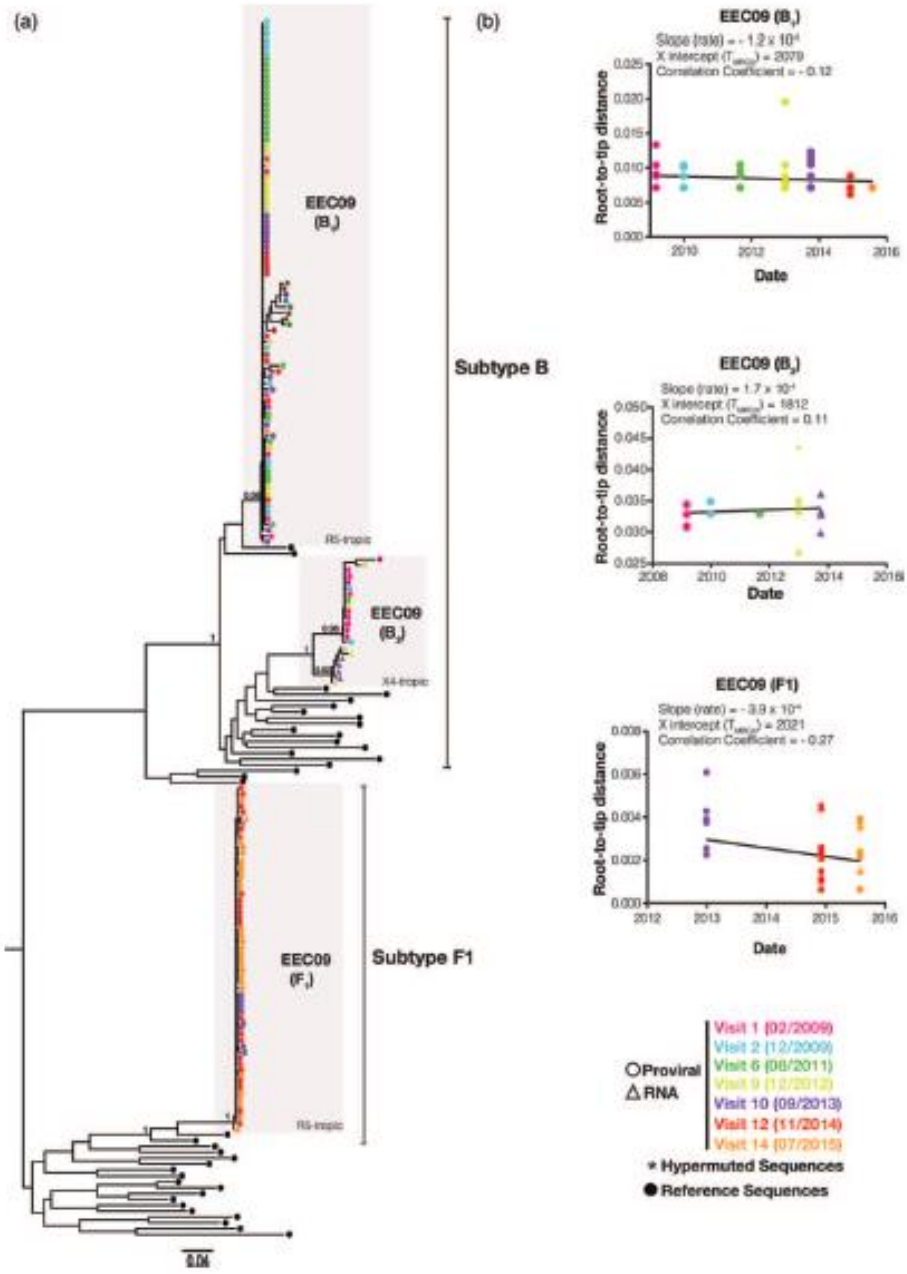

Fig. 2. Identification of a HIV-1 triple infection in individual EEC09. (a) Longitudinal analysis of HIV-1 PBMC-associated DNA (circles) and plasma RNA (triangle) *env* sequences obtained from individual EEC09 between 2009 and 2015. Circles in the tips of the ML phylogenetic tree are coloured according to the visit analysed as shown in the legend at bottom right. The shaded boxes highlight monophyletic clusters corresponding to each viral variant and its tropism is indicated at the bottom. Asterisks highlight the sequences with APOBEC3G/F-mediated G to A hypermutations. Black circles point to the reference sequences and subtype-specific clades (B and F1) are indicated by vertical lines. Horizontal branch lengths are proportional to the bar at the bottom indicating nucleotide substitutions per site. The aLRT support is shown for key nodes. (b) Plot of the root-to-tip distance against sequence sampling time of each viral variant. The colours of the circles (DNA) and triangles (RNA) represent the sampling time of viral sequences according to the legend at bottom right.

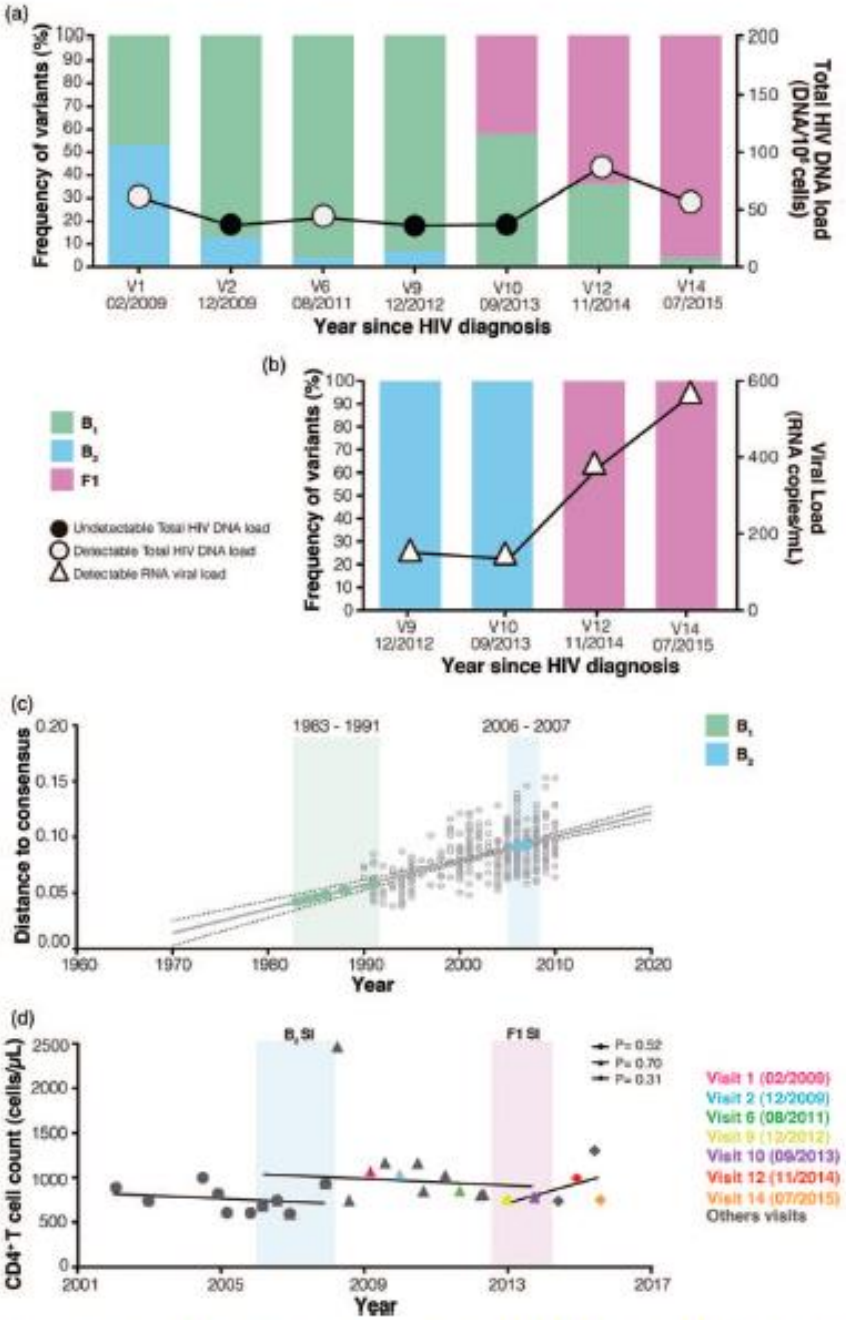

Fig. 3. Timing the HIV-1 SI events and their impact on immunologic control in individual EEC09. (a) Percentage of each viral variant at PBMC compartment and total HIV DNA load (HIV DNA/10^6 cells, circles) values over time (years) are shown on the left and right Y-axis respectively. DNA viral loads below or above the detection limit are coloured black and white, respectively. (b) Percentage of each viral variant at plasma compartment and plasma RNA viral load (HIV copies/ml, triangle) values over time (years) are shown on the left and right Y-axis respectively. RNA viral loads above of the detection limit are coloured white. The colours representing each viral variant are indicated at the bottom left. (c) Dating of the HIV-1 B_1 and B_2 variants *env* sequences (green and blue circles, respectively) employing a linear regression of the nucleotide divergence of reference HIV-1 subtype B pandemic variants (grey open circles) over time. The regression line (grey) and 95% confidence intervals (dotted line) are shown. (d) The longitudinal $CD4^+$ T-cell (y-axis) values obtained before the first SI even (circles), between the first and second SI events (triangles) and after the second SI event (diamonds), were plotted against time of sampling (years, x-axis). Time points corresponding to the visits analysed in this study were coloured as indicated in the legend at right.

finding together with the high mean *env* genetic distance between variants B_1 and B_2 (17.3%) indicated that this individual was actually triple infected. Variants B_2 and F1 were detected in both PBMC and plasma, whereas variant B_1 was only detected in PBMC (Fig. 2a). Prediction of viral tropism showed that variants B_1 and F1 were exclusively R5-tropic, whereas variant B_2 was entirely X4-tropic (Fig. 2a and Supplemental Table 1, http://links.lww.com/QAD/B405). The mean quasispecies genetic diversity (π) of each HIV-1 variant was very low in both PBMC (0.2–1.4%) and plasma (0.1–0.3%) (Supplemental Table 2, http://links.lww.com/QAD/B405), consistent with the low number of B_2 and F1 haplotypes reconstructed per visit in the plasma ($n = 2–4$) (Supplemental Fig. 2a, http://links.lww.com/QAD/B405). We did not detect a measurable increase in genetic divergence over time for any of the viral variants (Fig. 2b). Both subtype B variants were detected at similar frequencies (47% B_1 and 53% B_2) in PBMC in February 2009 (Visit 1; Fig. 3a). The frequency of variant B_2 in PBMC reservoir reduced progressively in subsequent visits but was the dominant variant in the plasma in December 2012 (Visit 9) and September 2013 (Visit 10) (Fig. 3b). Because this individual was already infected with both HIV-1 subtype B variants at enrolment in the cohort study, we estimated the probable timing of infections by performing viral dating of the HIV DNA sequences sampled between 2009 and 2015. Viral dating of B_1 sequences (1983–1991) resulted in much older dates than those of B_2 sequences (2006–2007) (Fig. 3c), thus, indicating that individual EEC09 was initially infected by the B_1 variant (probably in the 1980s) and superinfected by the B_2 variant (probably in the 2000s). We first detected the subtype F1 variant in PBMC in September 2013 at a high frequency (42%) (Fig. 3a), indicating that this individual was superinfected with the subtype F1 variant between December 2012 and September 2013. In subsequent visits, the new infecting subtype F1 virus virtually replaced the subtype B strains in both PBMC (Fig. 3a) and plasma (Fig. 3b). The estimated superinfection with the B_2 virus roughly coincided with detection of a plasma RNA viral load blip of 276 copies/ml in March 2008 (Fig. 1a), whereas superinfection with the subtype F1 variant coincided with the virologic breakthrough after 10 years of elite control of viremia (Fig. 1a) and with a transient increase in the total HIV DNA levels (Fig. 3a). By contrast, we did not observe a significant decline in $CD4^+$ T-cell counts after the estimated superinfection events and before the initiation of cART (Fig. 3d).

Evidence of intersubtype HIV-1 superinfection in individual VC32

We obtained 179 *env* sequences derived from samples collected at six visits over a period of 4 years (April 2012–April 2016). ML phylogenetic analysis revealed that *env* sequences from both PBMC and plasma branched in two highly supported (aLRT > 0.95) independent monophyletic clades within subtypes B and F1 (Fig. 4a), which confirm that individual VC32 was dually infected. Prediction of coreceptor usage showed that all subtype B and F1 *env* sequences derived from PBMC and plasma were R5-tropic (Fig. 4a and Supplemental Table 1, http://links.lww.com/QAD/B405). The mean π estimated from cell-associated HIV DNA quasispecies of HIV-1 subtype B (2.7–3.6%) was higher than the mean π of subtype F1 ($\leq$0.1%), which was consistent with an older infection by the subtype B variant (Table SII, http://links.lww.com/QAD/B405). The mean π of viral quasispecies in the plasma was very low (<0.5%; Supplemental Table 2, http://links.lww.com/QAD/B405), which was consistent with a low number of haplotypes per visit reconstructed for both subtype B ($n = 1–2$) and F1 ($n = 3–6$) populations (Supplemental Fig. 1b, http://links.lww.com/QAD/B405). No evidence of divergence over time was detected for subtype B or subtype F1 populations (Fig. 4b). Subtype B was the only variant detected between April 2012 and January 2014 in PBMC (Fig. 5a) or plasma (Fig. 5b). Subtype F1 was detected for the first time in both PBMC and plasma in June 2015 (Visit 6) and was the dominant variant (≥90%) in plasma in June 2015 and January 2016 (Visit 7). Subtype B, however, remained as the most prevalent clade (≥89%) in PBMC across all time points. These results clearly support that individual VC32 was infected with a subtype B variant before enrolment in the cohort study in April 2012 and superinfected with a subtype F1 variant between January 2014 and June 2015. The emergence of the subtype F1 variant coincided with a transient peak in plasma viremia (722 HIV-1 copies/ml; Fig. 1b), but it was not associated with a significant increase in total HIV DNA levels (Fig. 5a), nor with a significant decline in $CD4^+$ T-cell counts (Fig. 5c).

Discussion

In the present study, we report two cases of HIC that preserved virologic and immunologic control despite intrasubtype and/or intersubtype HIV-1 SI. Individual EEC09 was an elite controller who was probably sequentially infected with two subtype B strains (B_1 and B_2 variants) prior to his entry in our cohort in 2009 and then superinfected with a subtype F1 variant around 2012–2013. Individual VC32 was a viremic controller initially infected with a subtype B strain before 2004 (date of HIV diagnosis) and superinfected with a subtype F1 variant around 2014–2015. Previous studies have already identified some cases of dual infections in HIC [15–20,39,40] and of triple infection in HIV noncontrollers [41–46], but this is the first report of triple infection in an HIC.

HIV-1 superinfection typically has detrimental consequences for clinical outcome in both HIC [20] and noncontrollers [1–13]. Although individual EEC09 had lost his elite controller status after the second superinfection event and individual VC32 displayed a transient peak

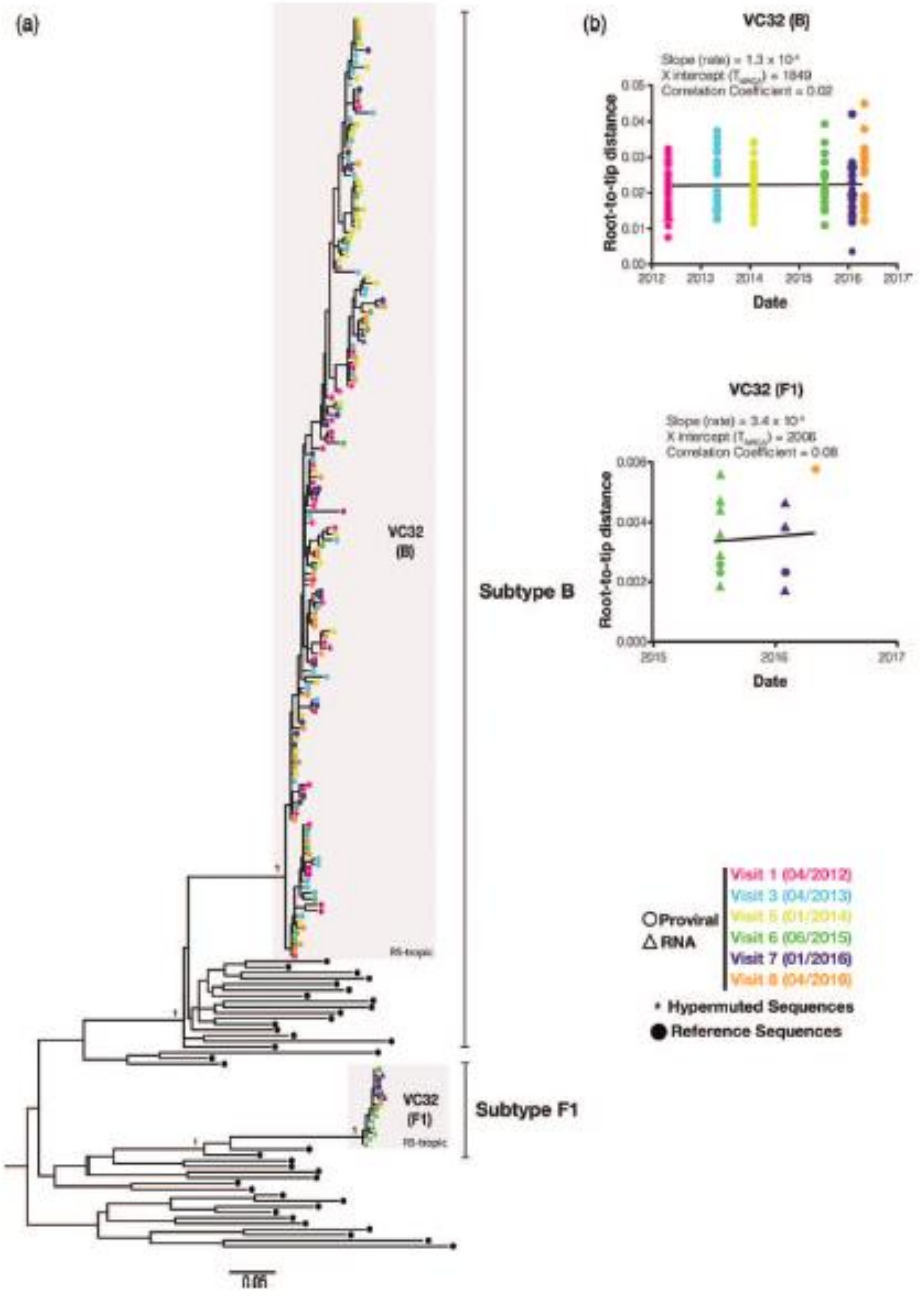

Fig. 4. Identification of a dual HIV-1 infection in individual VC32. (a) Longitudinal analysis of HIV-1 PBMC-associated DNA (circles) and plasma RNA (triangle) *env* sequences obtained from individual VC32 between 2012 and 2016. Circles in the tips of the ML phylogenetic tree are coloured according to the visit analysed as shown in the legend at bottom right. The shaded boxes highlight monophyletic clusters corresponding to each viral variant and its tropism is indicated at the bottom. Asterisks highlight the sequences with APOBEC3G/F-mediated G to A hypermutations. Black circles point to the reference sequences and subtype-specific clades (B and F1) are indicated by vertical lines. Horizontal branch lengths are proportional to the bar at the bottom indicating nucleotide substitutions per site. The aLRT support is shown for key nodes. (b) Plot of the root-to-tip distance against sequence sampling time of each viral variant. The colours of the circles (DNA) and triangles (RNA) represent the sampling time of viral sequences according to the legend at bottom right.

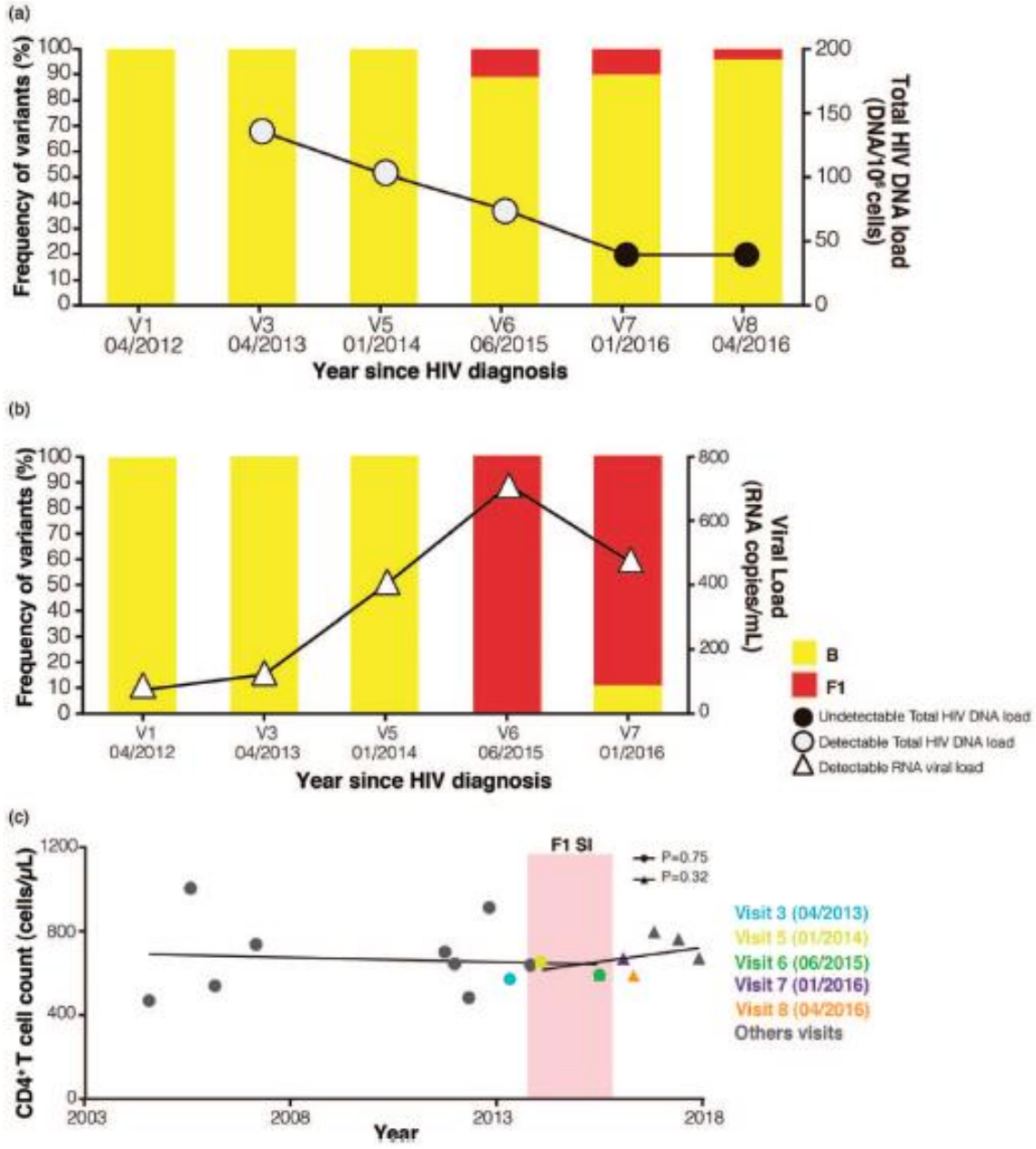

Fig. 5. Timing the HIV-1 SI event and its impact on immunologic control in individual VC32. (a) Percentage of each viral variant at PBMC compartment and total HIV DNA load (HIV DNA/10^6 cells, circles) values over time (years) are shown on the left and right Y-axis respectively. DNA viral loads below or above the detection limit are coloured black and white, respectively. (b) Percentage of each viral variant at plasma compartment and plasma RNA viral load (HIV copies/ml, triangle) values over time (years) are shown on the left and right Y-axis respectively. RNA viral loads above of the detection limit are coloured white. The colours representing each variant are indicated at the bottom left. (c) The longitudinal $CD4^+$ T-cell (y-axis) values obtained before (circles) and after (triangles) the SI event, were plotted against time of sampling (years, x-axis). Time points corresponding to the visits analysed in this study were coloured as indicated in the legend at right.

of viremia, both individuals maintained viremic control (<2000 copies/ml) of infecting viruses and presented no evidence of immunologic progression for at least 2 years after the intersubtype superinfection event. Individual EEC09 initiated cART around two years after the intersubtype superinfection event despite no signs of clinical progression, whereas plasma viremia in individual VC32 (who remained cART-free) gradually decreased after the peak and reached undetectable levels 2–3 years after superinfection. Furthermore, we found no measurable increase in viral divergence over time, which reinforces the extraordinary ability of these individuals to control the evolution of both initial and superinfecting viral strains. Although previous studies have already documented the ability of some HIC to maintain persistent virological and immunological control after dual infection [16,17,40] and superinfection [18,39] with HIV-1 subtype B viruses, this is the first report, to the best of our knowledge, of HIC who maintain sustained control of viral replication and evolution after intersubtype superinfection.

These results indicate that clinical consequences of superinfection in HIC could be different among individuals and may depend on the underlying mechanisms responsible for the natural control of viremia in each individual. Superinfection with a defective virus has been suggested as a factor associated with the low clinical impact of HIV-1 superinfection in one elite controller [39]. The increase in viremia and rapid turnover of viruses in the plasma following superinfection in individuals EEC09 and VC32, however, argue against superinfection with a defective subtype F1 virus. It is also remarkable that infection [47] or superinfection [9] with X4-tropic and dual-tropic subtype B variants has been associated with rapid disease progression. Our analyses, however, indicates that individual EEC09 was probably initially infected with the R5-tropic B_1 variant and maintained elite control after superinfected with the X4-tropic B_2 variant. Overall, these evidences support the relevance of host mechanisms in the natural control of viremia in individuals EEC09 and VC32, similar to that previously shown for other HIC [15,48,49].

Studies on HIV noncontrollers found that superinfection may have a variable impact on proviral reservoir composition [13,21–26], and our findings in HIC confirm these observations. In individual EEC09, the superinfecting B_2 strain co-circulated with the original B_1 strain between 2009 and 2012 but declined continuously in PBMC and was undetectable in 2013, whereas it was the dominant variant in the RNA plasma until 2013. Superinfection with the subtype F1 strain around 2013 was associated with a subsequent transient increase in total HIV DNA load and a complete turnover of the viral quasispecies in both PBMC and plasma. In contrast to individual EEC09, we did not detect significant changes in the size and composition of PBMC-associated HIV DNA reservoir in individual VC32. Although the superinfecting subtype F1 virus was the dominant variant in the plasma after superinfection, it failed to replenish the PBMC reservoir that was continuously dominated by the original subtype B virus. The divergent dynamics of PBMC-associated DNA and plasma RNA viral quasispecies corroborate that other reservoirs (probably lymph nodes and gut-associated lymphoid tissue), apart from PBMC, are a source of plasma viremia in HIC [50].

In summary, this study reports that some HICs are unable to prevent HIV-1 superinfection but seem to be able to repeatedly control viral replication and evolution of different infecting viral subtypes and further prevent disease progression for several years after HIV-1 superinfection. Identifying the host mechanisms associated with the natural control of HIV-1 replication and evolution following primary infection and whether these mechanisms are the same that lead to a sustained control after superinfection could offer important clues for the development of innovative therapeutic vaccines towards HIV remission.

Acknowledgements

The authors thank the patients, who participated in the study, as well as all the technical staff involved in the clinical follow-up of these patients. We thank the Plataforma Genômica de Sequenciamento de DNA – RPT01A-PDTIS/FIOCRUZ, Plataforma de Sequenciamento de Ácidos Nucléicos de Nova Geração – RPT01J-PDTIS/FIOCRUZ and the Programa de Pós-Graduação em Genética/Departamento de Genética, IB/UFRJ by the nucleotide sequencing, NGS Library preparation and sequencing run with Miseq, respectively. We also thank Ms Marilia Alves Figueira de Melo for the excellent technical support in NGS Library preparation. We thank Dr Caroline Passaes for the critical reading of the manuscript.

G.B. conceived and designed the study and supervised the experiments. S.S.D.A. conducted experiments and analysed the data. E.D. participated in the quantification of total cell associated HIV-1 DNA, N.G.S. experiments and its analysis. F.H.C. participated in sample processing and determination of $CD4^+$ T-cell counts. B.H., B.G. and V.G.V. conducted the patient recruitment and follow-up. T.M.L.S. and M.G.M. contributed to the study design and provided intellectual input. S.S.D.A. and G.B. wrote the first draft and all authors assisted with the writing and approved the final manuscript.

This work was supported by the Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro – FAPERJ (grant number E-26/110.123/2014) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico –

CNPq (Grant Number 401220/2016-8). S.S.D.A. was supported by funding from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro – FAPERJ. E.D. was supported by a Postdoctoral fellowship from the 'Programa Nacional de Pós-Doutorado (PNPD)'/CAPES-Brazil.

Conflicts of interest

There are no conflicts of interest.

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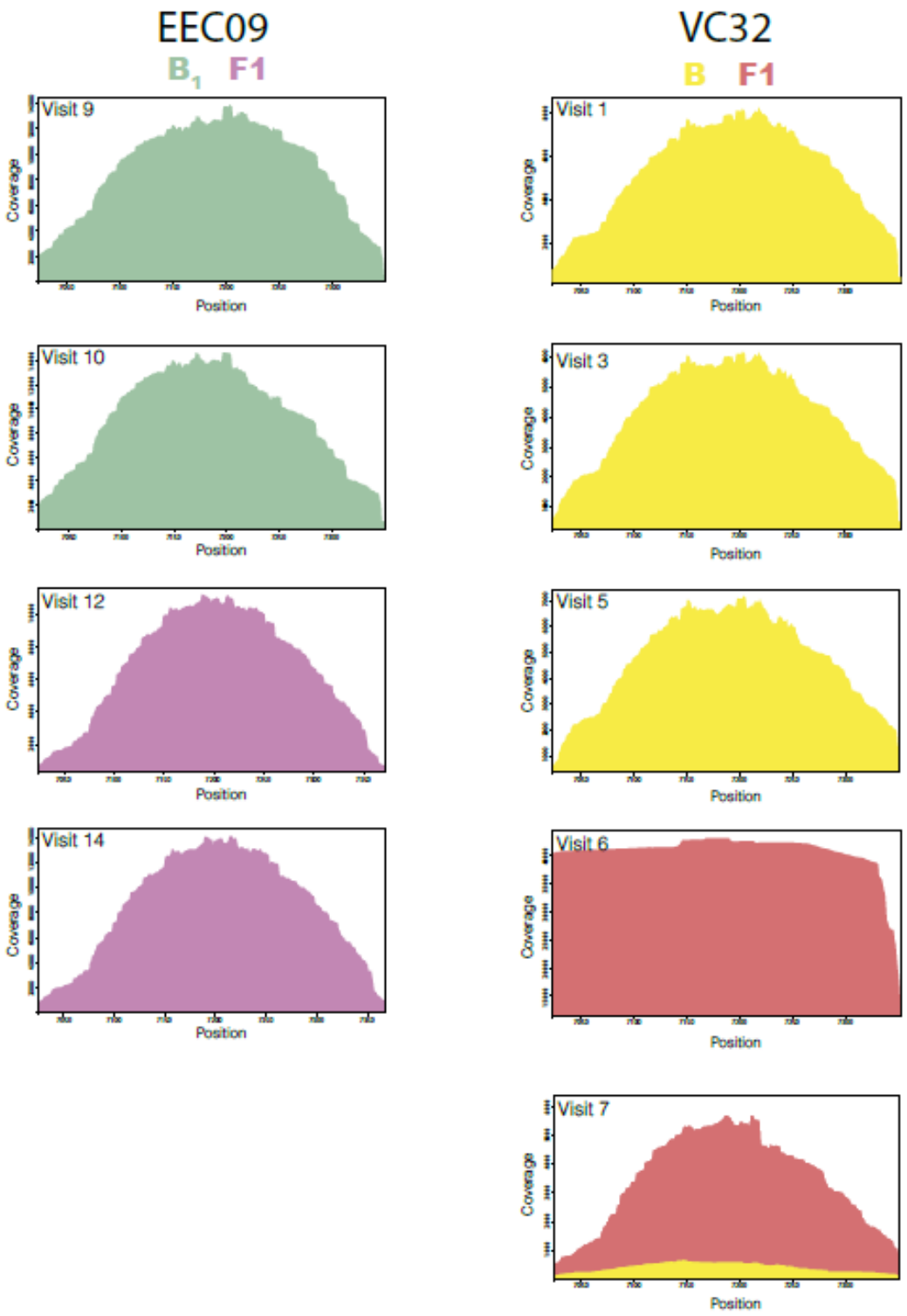
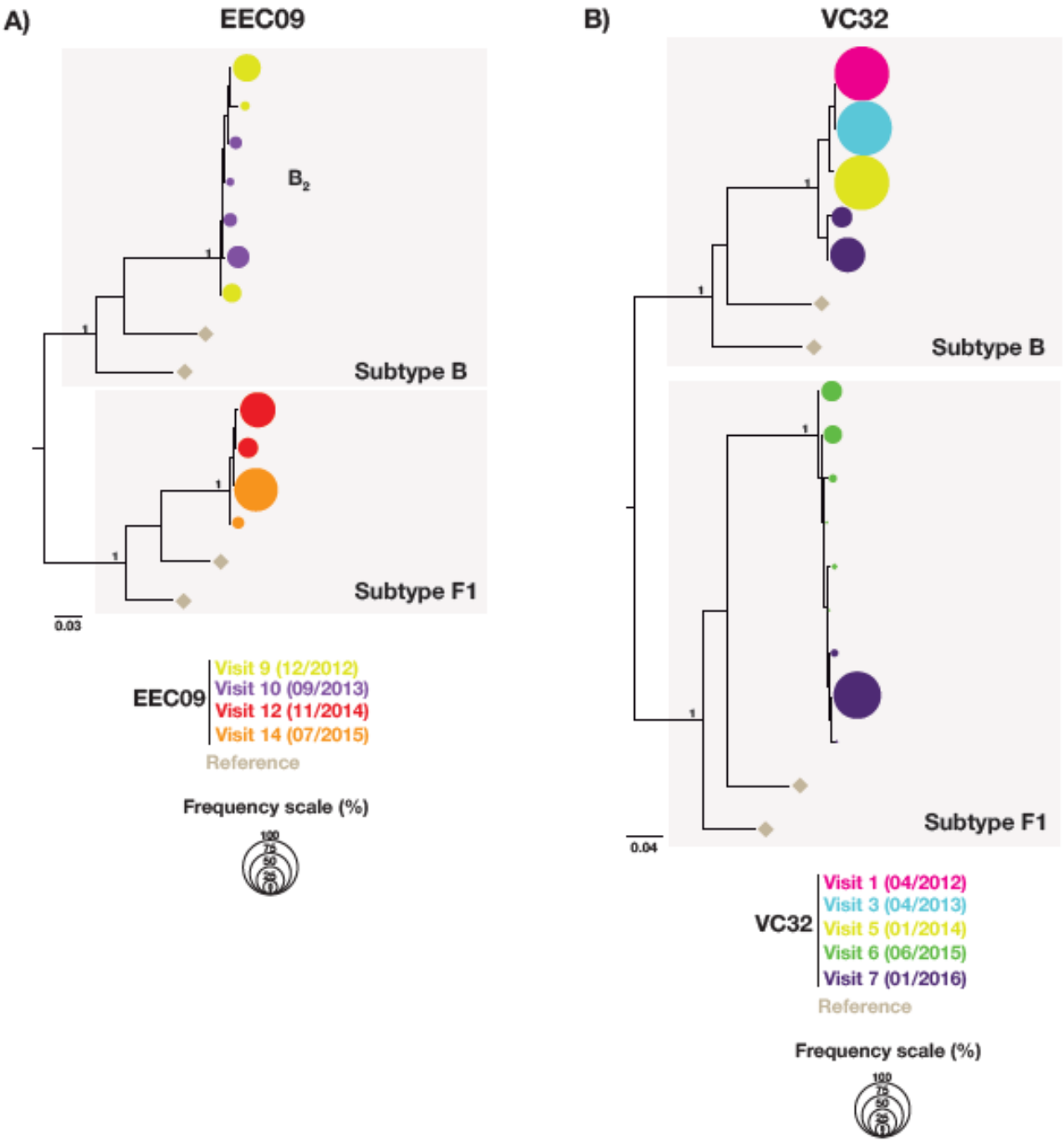


Supplemental Fig. 1. Sequencing coverage of the env amplicon from RNA from plasma samples. The depth of each base's coverage is indicated on the y-axis while the corresponding position in the HIV-1 genome is indicated on the x-axis (according to the HXB2 coordinates). Plots were colored according to the viral variant/s detect at each visit.



Supplemental Fig. 2. Frequency of viral haplotypes at different points in time found in the plasma compartment of the HIV controllers. ML phylogenetic tree of env sequences from subjects EEC09 (A) and VC32 (B) (circles) and HIV-1 subtype reference sequences (diamonds). The circles in the tips are colored according to the time point analyzed and their sizes are proportional to the haplotype frequency according to the scale at the right corner. Horizontal branch lengths are proportional to the bar at the bottom indicating nucleotide substitutions per site. The subtype of each cluster is indicated in the footer of the shaded boxes.

Supplemental Table 1. Coreceptor tropism prediction in quasispecies from PBMC and plasma compartments.

Patient ID	Variants	Visits	Source	V3 loop	Frequency of clones[a]	Predicted coreceptor use	Geno2pheno[b]
EEC09	B_1	V1-14	PBMC	CTRPSNNTRKGIHIGPGRAFYATGDIIGDIRQAHC	98	R5	51.8
		V6	PBMC	CTRPSNNTRKGIHIGPGRAFYATGDRIGDIRQAHC	1		21.4
		V10	PBMC	CTRPSNNTRKGIHIGPGRAFYATGDIIGDIR-A--	1		91.7
	B_2	V1-9	PBMC	CTRPNNNTRKGIHMGWGKALYATEKIIGDIRQAHC	100	X4	4
		V9,10	Plasma	CTRPNNNTRKGIHVGWGRALYATEKIIGDIRQAHC	100		2.6
	F1	V10,12,14	PBMC	CTRPNNNTRKSIPIGPGRAFYATGEIIGDIRKAHC	98	R5	59.2
		V12,14	Plasma		100		59.2
		V10	PBMC	CTRPNNNTRKSIPIGLGRAFYATGEIIGDIRKAHC	2		38
VC32	B	V1-8	PBMC	CTRPNNNTRKSINIGPGRAFYATGEIIGDIRQAHC	32	R5	64
		V1-8	PBMC	CTRPNNNTRKSIHIGPGRAFYATGEIIGDIRQAHC	22		42.6
		V1-8	PBMC	CTRPNNNTRKSINIGPGRAFYATEEIIGDIRQAHC	14		17
		V5	Plasma		100		
		V1-8	PBMC	CTRPNNNTRKSINIGPGRAFYATEGIIGDIRQAHC	13.5		13.5
		V1,3	Plasma		100		
		V1-8	PBMC	CTRPNNNTRKSINIGPGRAFYATGKIIGDIRQAHC	11.5		11.5
		V7	Plasma		100		
		V1,7,8	PBMC	CTRPNNNTRKSIHIGPGKAFYATGEIIGDIRQAHC	3		49.9
		V1,3	PBMC	CTRPNNNTRKSINIGPGRAFYATKGIIGDIRQAHC	2		30.1
		V1,5	PBMC	CTRPNNNTRKSINIGPGRAFYATGEVIGDIRQAHC	1		50.5
		V5	PBMC	CTRPNNNTRKSINIAPGRAFYATGEIIGDIRQAHC	0.5		88
		V8	PBMC	CTRPNNNTRKSIHIGPGRAFYATGEIIGDIRQAYC	0.5		38
	F1	V6-8	PBMC	CTRPNNNTRKGIHLGPGRTFYATGDIIGDIRQAQC	100		75.6
		V6,7	Plasma		100		

[a] Frequency represented in percentages (%); [b] Predicted using geno2pheno with a false positive rate (FPR) cutoff of 5%. Red shades represent changes related to the major haplotype of each variant. Grey shades highlight the crown of the V3 loop region.

Supplemental Table 2. Virological characteristics of HIV controllers EEC09 and VC32.

Patient ID	Visit (month/year)	RNA load (cp/mL)[a]	DNA load (cp/10^6cells)	Viral variant DNA-PBMCs			Viral variant RNA-Plasma	
EEC09	V1 (Feb 2009)	< 50	61	B_1=9 (47%) π[c] = 0.9%	B_2=10 (53%) π = 0.2%	-	-	-
	V2 (Dec 2009)	< 50	< 40	B_1=13 (87%) π = 0.2%	B_2=2 (13%) π = 1.2%	-	-	-
	V6 (Aug 2011)	< 50	43	B_1=27 (96%) π = 0.7%	B_2=1 (4%) π = ND	-	-	-
	V9 (Dec 2012)	170	< 40	B_1=13 (87%) π = 0.3%	B_2=2 (13%) π = 0.2%	-	B_2=100% π = 0.5%	-
	V10 (Sep 2013)	159	< 40	B_1=11 (58%) π = 1.4%	B_2=8 (42%) π = 0.2%	F1 = 8 (42%) π = 0.2%	B_2=100% π = 0.3%	-
	V12 (Nov 2014)	388	87	B_1=12 (36%) π = 0.5%		F1= 21 (64%) π = 0.2%	-	F1 = 100% π = 0.1%
	V14 (Jul 2015)	581	45	B_1=1 (4%) π = ND		F1= 26 (96%) π = 0.3%	-	F1 = 100% π = 0.1%
VC32	V1 (Apr 2012)	82	ND[b]	B = 33 (100%) π = 3.2%		-	B = 100% π =ND	-
	V3 (Apr 2013)	136	136	B = 30 (100%) π = 3.6%		-	B = 100% π = ND	-
	V5 (Jan 2014)	403	106	B = 34 (100%) π = 3.0%		-	B = 100% π = ND	-
	V6 (Jun 2015)	722	74	B = 17 (89%) π = 3.5%		F1 = 2 (11%) π = 0.1%	-	F1 = 100% π = 0.3%
	V7 (Jan 2016)	472	< 40	B = 19 (90%) π = 2.7%		F1 = 2 (10%) π = 0%	B = 10% π = 0.1%	F1 = 90% π = 0.1%
	V8 (Apr 2016)	58	< 40	B = 27 (96%) π = 3.3%		F1 = 1 (4%) π = ND	-	-

[a]cp, copies/ml; [b]ND, not determined; [c]π, mean nucleotide diversity.

3.3 ARTIGO 3

Proviral quasispecies diversity is not associated with virologic breakthrough, systemic activation or CD4$^+$ T cell loss in HIV-1 elite controllers.

Autores: Suwellen S. D. de Azevedo, Fernanda H. Côrtes, Edson Delatorre, Marcelo Ribeiro-Alves, Brenda Hoagland, Beatriz Grinsztejn, Valdilea G. Veloso e Gonzalo Bello.

Periódico: Frontiers in Microbiology, 2019.

Resumo:

Controladores de elite (EC) são capazes de controlar a replicação do HIV-1 a níveis extremamente baixos (<50 cópias de RNA do HIV-1/mL) na ausência de terapia antirretroviral. Alguns EC, no entanto, sofrem perda de células T CD4$^+$ e/ou perdem sua capacidade de controlar o HIV-1 durante o curso da infecção. Altos níveis de diversidade proviral do *env* do HIV-1, células T ativadas e citocinas pró-inflamatórias foram descritos como biomarcadores úteis para prever a progressão virológica e/ou imunológica em EC. O objetivo deste estudo foi avaliar a importância da diversidade proviral como marcador prognóstico da progressão virológica e/ou imunológica em EC. Para tanto, analisamos a viremia plasmática, o DNA do HIV total associado a célula, a dinâmica de células T, a ativação de células T CD8$^+$ (CD38$^+$HLA-DR$^+$) e citocinas pró-inflamatórias (IP-10, IL-18, RANTES, PDGF-AA e CTACK) em EC com baixos (EC_{LD} = 4) e altos (EC_{HD} = 6) níveis de diversidade proviral de HIV-1. Nenhum dos indivíduos EC_{LD} e EC_{HD} apresentou evidência de progressão imunológica (diminuição na contagem absoluta e percentagem de células T CD4$^+$), e apenas um indivíduo EC_{HD} apresentou progressão virológica (≥2 medições consecutivas de cargas virais acima do limite de detecção) 2-5 anos após a determinação da diversidade proviral. Apesar das diferenças na diversidade genética proviral, os subgrupos EC_{LD} e EC_{HD} apresentaram níveis comparáveis de DNA viral associado às células, células T CD8$^+$ ativadas e biomarcadores inflamatórios plasmáticos. Estes resultados indicam que a diversidade genética do reservatório proviral do HIV-1 não é um marcador substituto de replicação viral residual, ativação imune ou inflamação, nem um biomarcador preciso para a previsão de perda virológica ou perda de células T CD4$^+$ em EC.

frontiers in Microbiology

ORIGINAL RESEARCH
published: 02 April 2019
doi: 10.3389/fmicb.2019.00673

Proviral Quasispecies Diversity Is Not Associated With Virologic Breakthrough or CD4$^+$ T Cell Loss in HIV-1 Elite Controllers

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Elite controllers (EC) are able to control HIV-1 replication to extremely low levels (<50 HIV-1 RNA copies/mL) in the absence of antiretroviral therapy. However, some EC experience CD4$^+$ T cell loss and/or lose their ability to control HIV-1 over the course of infection. High levels of HIV-1 *env* proviral diversity, activated T cells and proinflammatory cytokines were pointed out as relevant biomarkers for detection of EC at risk of virologic/immunologic progression. The aim of this study was to assess the importance of proviral diversity as a prognostic marker of virologic and/or immunologic progression in EC. To this end, we analyzed plasma viremia, total HIV DNA levels, T cells dynamics, and activation/inflammatory biomarkers in EC with low (EC_{LD} = 4) and high (EC_{HD} = 6) HIV-1 *env* diversity. None of EC_{LD} and EC_{HD} subjects displayed evidence of immunologic progression (decrease in absolute and percentage of CD4$^+$ T cells) and only one EC_{HD} subject presented virologic progression (≥2 consecutive viral loads measurements above the detection limit) 2–5 years after determination of proviral *env* diversity. Despite differences in proviral genetic diversity, the EC_{LD} and EC_{HD} subgroups displayed comparable levels of total cell-associated HIV DNA, activated CD8$^+$ T (CD38$^+$HLA-DR$^+$) cells and plasmatic inflammatory biomarkers (IP-10, IL-18, RANTES, PDGF-AA, and CTACK). These results indicate that the genetic diversity of the HIV-1 proviral reservoir is not a surrogate marker of residual viral replication, immune activation or inflammation, nor an accurate biomarker for the prediction of virologic breakthrough or CD4$^+$ T cells loss in EC.

Keywords: elite controllers, HIV proviral diversity, plasma biomarkers, CD4$^+$ T cell loss, breakthrough viremia

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Edited by:
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Specialty section:
This article was submitted to Virology, a section of the journal Frontiers in Microbiology

Received: *14 February 2019*
Accepted: *18 March 2019*
Published: *02 April 2019*

Citation:
de Azevedo SSD, Côrtes FH, Delatorre E, Ribeiro-Alves M, Hoagland B, Grinsztejn B, Veloso VG, Morgado MG and Bello G (2019) Proviral Quasispecies Diversity Is Not Associated With Virologic Breakthrough or CD4$^+$ T Cell Loss in HIV-1 Elite Controllers. Front. Microbiol. 10:673. doi: 10.3389/fmicb.2019.00673

INTRODUCTION

A rare subset of HIV-1–infected individuals, termed elite controllers (EC), is able to naturally suppress viral replication to levels <50 HIV-1 RNA copies/mL during chronic infection in absence of antiretroviral therapy (ART) (Deeks and Walker, 2007). Despite the extraordinary ability to naturally suppress HIV-1 viremia, a proportion of EC exhibit a CD4$^+$ T cell counts decline (immunologic progression) and/or lose their ability to control HIV (virologic progression) over

the course of infection (Andrade et al., 2008; Okulicz et al., 2009; Sajadi et al., 2009; Boufassa et al., 2011; Yang et al., 2012; Olson et al., 2014; Leon et al., 2016; Chereau et al., 2017). Identify those EC at risk of $CD4^+$ T cell loss and/or of breakthrough viremia may help to guide the selection of individuals that may benefit from ART initiation.

Some EC subjects exhibit abnormally high levels of systemic T cell activation and inflammation that may contribute to both $CD4^+$ T cell counts loss and breakthrough viremia (Andrade et al., 2008; Hunt et al., 2008; Noel et al., 2014, 2015b; Pernas et al., 2017). Virologic progression has been also associated with the increase of intermittent viremia episodes, total HIV DNA and HIV proviral diversity in the blood (Noel et al., 2015b; Pernas et al., 2017). High levels of $CD8^+$ T cell activation and interferon gamma-induced protein 10 (IP-10) were pointed out as the most discriminant biomarkers for detection of EC at risk of immunologic progression (Noel et al., 2014, 2015b); while levels of HIV *env* diversity and plasma pro-inflammatory cytokines CCL5/RANTES (Pernas et al., 2017) and Galectin-3-binding protein (Rodríguez-Gallego et al., 2018) were described as useful baseline markers to predict virologic progression in EC 1 year before the loss of HIV control.

A recent study conducted by our group demonstrated two divergent patterns of intra-host proviral diversity in a cohort of Brazilian EC (de Azevedo et al., 2017). A subgroup of EC displayed highly homogeneous proviral quasispecies (mean *env* genetic diversity $< 2\%$), consistent with the maintenance of the viral reservoir by clonal expansion of long-lived HIV-infected memory $CD4^+$ T cells. The other subgroup of EC showed more diverse proviral populations (mean *env* genetic diversity $\geq 2\%$), consistent with residual evolution and continuous reseeding of the proviral reservoir. The precise association between these divergent patterns of intra-host *env* proviral diversity and the subsequent HIV-1 disease progression in our EC cohort was not addressed before.

Here, we evaluated the importance of HIV-1 *env* proviral diversity as a possible prognostic marker of immunologic and/or virologic progression in our EC cohort by analyzing the plasma viremia, total cell-associated HIV DNA levels, $CD4^+$ T cells dynamics, $CD8^+$ T cell activation and inflammatory biomarkers over infection course in EC harboring proviral quasispecies with low ($EC_{LD} = 4$) and high ($EC_{HD} = 6$) *env* diversity.

MATERIALS AND METHODS

Study Subjects

The EC was defined as HIV-1–infected subjects with the most ($\geq$70%) plasma viral load (VL) determinations under the detection limit (<50–80 copies/mL) in absence of ART as described previously (de Azevedo et al., 2017). Then, they were divided into two groups based on the proviral genetic diversity: (1) EC_{LD} for those harboring proviral quasispecies with a mean *env* genetic diversity $< 2\%$ ($n = 4$); and (2) EC_{HD} for those harboring proviral quasispecies with a mean *env* genetic diversity $\geq 2\%$ ($n = 6$). All EC subjects were followed at least by 2 years after HIV-1 genetic diversity evaluation. A group of viremic controllers (VC) that displayed most ($\geq$70%) VL determinations between 51 and 2,000 copies/mL ($n = 8$) was included as control. Immunologic progression was defined as a statistically significant decline in both absolute and percentage of $CD4^+$ T cells (Burcham et al., 1991; Hulgan et al., 2007). Virologic progression in EC was defined as ≥ 2 consecutive detectable VL within 1 year (Pernas et al., 2017).

$CD4^+$ T Cell Counts, Plasmatic Viral Load and Total Cell-Associated HIV-1 DNA Load Measurements

$CD4^+$ T cells counts and plasma HIV-1 RNA VL were determined as described previously (de Azevedo et al., 2017). Total DNA was extracted from PBMC (1×10^7 cells) using the QIAamp DNA Mini Kit (Qiagen, Germany) and total cell-associated HIV-1 DNA load was quantified using the Generic HIV®DNA cell Kit (Biocentric, France), following the manufacturer's recommendations. Results were reported either as actual numbers of HIV DNA copies/10^6 cells or as the threshold value of detection.

Analyses of Proviral Genetic Diversity

Proviral *env* sequences from PBMC were performed by single genome amplification (SGA) followed by analysis of viral diversity using conditions previously described (de Azevedo et al., 2017).

Markers of T Cell Activation and Inflammation

To quantify the expression of CD38 and HLA-DR on $CD8^+$ T cells, cryopreserved PBMC were thawed and stained with the following antibodies: anti-CD3 APC-H7, anti-CD4 PECF594, anti-CD8 APC, anti-CD38 BB515, and anti-HLA-DR PE (BD Biosciences, United States), and acquired using a BD FACSAria IIu Flow Cytometer (BD Biosciences, United States). The Fixable Viability Stain 450 (FVS 450-BD Biosciences, United States) was used to exclude non-viable cells. Flow cytometric analyses were performed with FlowJo v.10.0.7 (Tree Star Inc., Ashland, OR, United States). Plasmatic levels (pg/mL) of IP-10, IL-18, RANTES, CTACK, and PDGF-AA were measured using the human Magnetic Luminex Performance Assay (R&D systems, United States), following manufacturer's instruction and the analyses were performed on a Luminex 200 System (Luminex, United States).

Statistical Analysis

The Mann–Whitney *U*-test was used to compare data between EC_{LD} and EC_{HD} independent groups. The Wilcoxon signed-rank test was used to compare data between pre- and pos-diversity evaluation within either EC_{LD} or EC_{HD}. Correlations between markers cumulative measures and HIV-1 DNA diversity were assessed by the Spearman correlation coefficient. The slopes of subgroups of T cells were calculated for each one by linear regression analysis fitted by generalized least squares and with

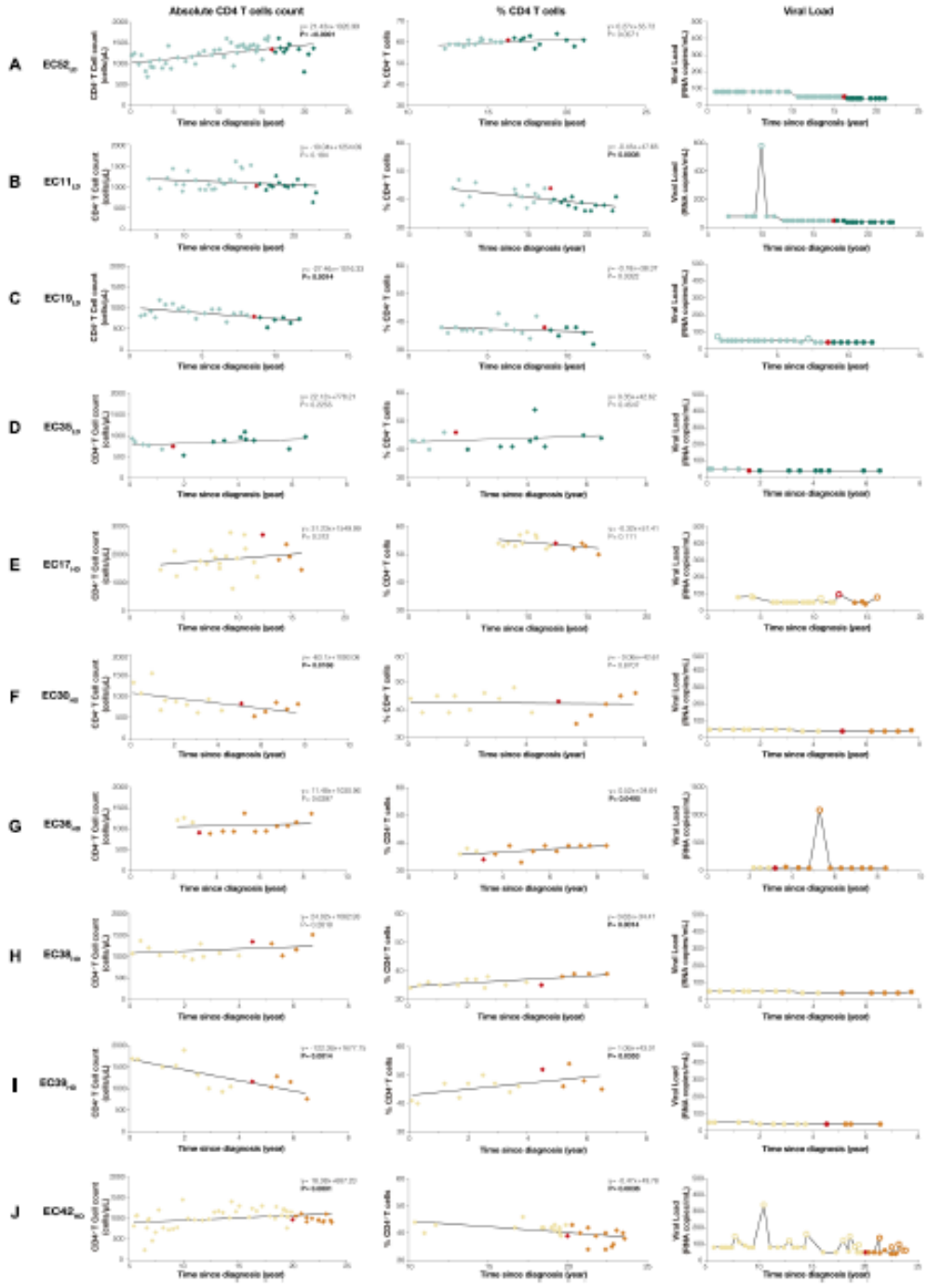

FIGURE 1 | Temporal dynamics of CD4$^+$ T cell counts and plasma viremia in EC_{LD} **(A–D)** and EC_{HD} **(E–J)** subgroups. Absolute CD4$^+$ T cells counts (cells/μL), percentages of CD4$^+$ T cell (%CD4) and plasma RNA viral load measurements (copies/mL) since HIV diagnosis are shown on the left, middle and right columns, respectively. The slope of CD4$^+$ T cells and %CD4 overtime, calculated by linear regression analysis fitted by generalized least squares and with an autocorrelation structure of first order in respect to the time after HIV diagnosis, are shown with their respective *P*-values in the upper right corner of each graph. The red symbols (diamonds or circles) indicate the time point selected for analysis of *env* proviral diversity, while light and dark colored symbols indicate pre- and post-period of *env* proviral diversity determination, respectively. RNA viral load measurements below and above the detection limit were represented by fill and empty circles, respectively. EC_{LD} and EC_{HD} subjects were represented by green and orange symbols, respectively.

an autocorrelation structure of first order in respect to the time after HIV diagnosis. Tests were two-sided, and $P \leq 0.05$ were considered as significant. Graphics and statistical analyses were performed using either GraphPad v6 (Prism Software, United States) or R (R Foundation for Statistical Computing, Austria) software.

RESULTS

There were no statistically significant differences between EC_{LD} and EC_{HD} groups in terms of epidemiological (age, sex, and HIV-1 transmission), immunologic (absolute $CD4^+$ and $CD8^+$ T cells counts, %CD4 and CD4/CD8 ratio), genetic (HLA-B*57/27 status) and virologic (plasma HIV-1 RNA, total HIV-1 DNA loads, and Hepatitis C status) characteristics at proviral diversity determination point or throughout the follow-up (**Supplementary Table S1**). Longitudinal analysis revealed no evidence of immunologic progression in our EC cohort (**Figure 1**). Subjects that showed a significant ($P < 0.05$) decrease in absolute $CD4^+$ T cells counts over time ($EC19_{LD}$, $EC30_{HD}$, and $EC39_{HD}$) maintained $CD4^+$ T cells counts > 500 cells/mm3 and stable percentage of $CD4^+$ T cells > 30%. Subjects that displayed significant ($P < 0.01$) decline in the percentage of $CD4^+$ T cells ($EC11_{LD}$ and $EC42_{HD}$) maintained stable or increasing numbers of $CD4^+$ T cells counts over time. All EC_{LD} and EC_{HD} subjects kept persistent virologic control for 2–5 years after determination of proviral *env* diversity, with exception of subject $EC42_{HD}$ that displayed ≥2 consecutive VL measurements above the detection limit (59–97 copies/mL) in a 1-year period and thus lost the elite virologic control profile during follow-up (**Figure 1** and **Supplementary Table S2**).

Longitudinal analyses of the cumulative mean $CD4^+$ T cell counts ($cCD4^+$ T cell) and VL (cVL) in the pre- and post-periods of the proviral diversity assay date revealed no statistically significant differences between EC_{LD} and EC_{HD} subgroups (**Figures 2A–D**) nor within each subgroup between time periods (**Supplementary Figure S1**). Similarly, cross-sectional analyses of total cell-associated HIV DNA levels, cellular

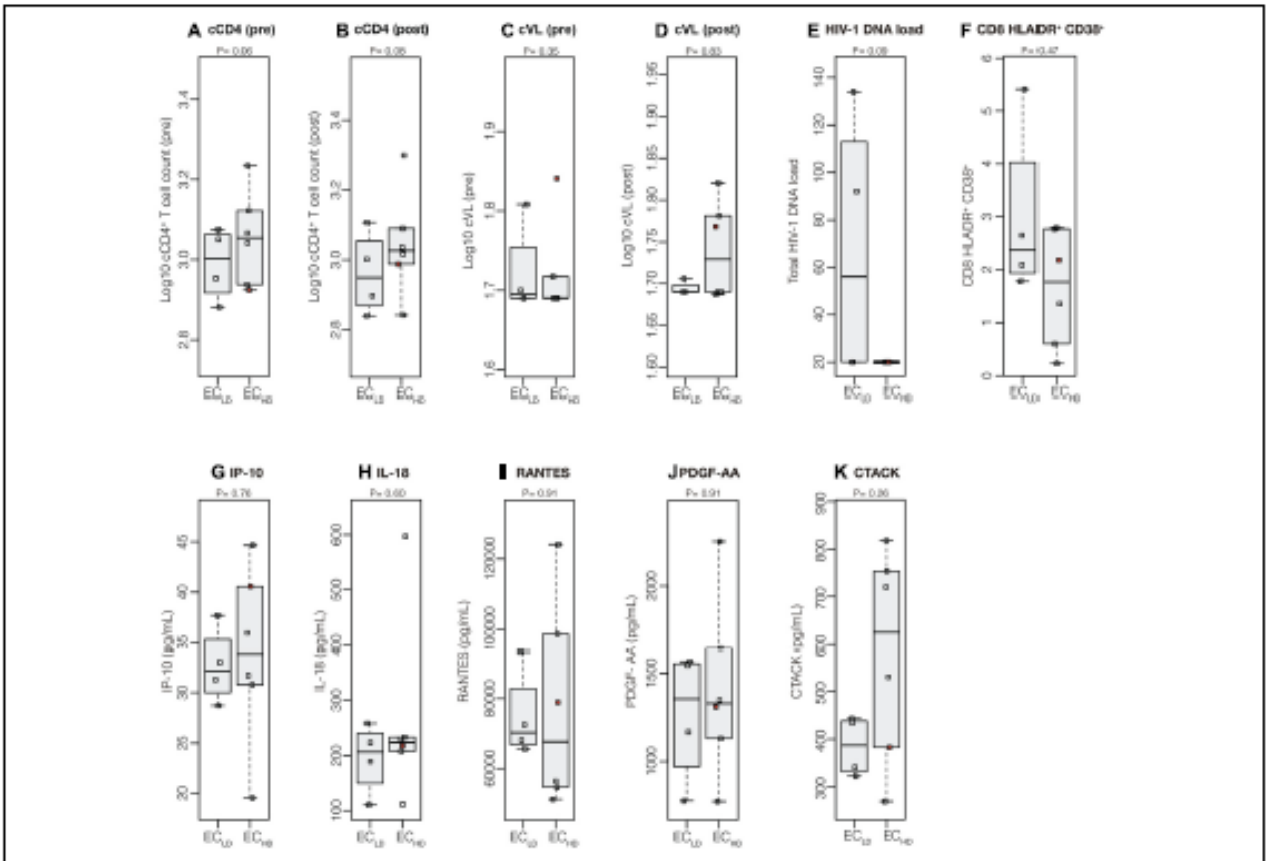

FIGURE 2 | Virologic and immunologic progression markers in EC_{LD} and EC_{HD} subgroups. Mean (log_{10}-transformed) cumulative measures of $CD4^+$ T cell counts **(A,B)** and VL **(C,D)** in the pre- and post-periods respect to the point of determination of *env* proviral diversity and the total cell-associated HIV-1 DNA load **(E)**, frequency of activated $CD8^+$ T cells **(F)**, and plasmatic levels of IP-10 **(G)**, IL-18 **(H)**, RANTES **(I)**, PDGF-AA **(J)**, and CTACK **(K)** at the *env* proviral diversity point were compared between EC subgroups. Central black solid bars and boxplots represent sample medians and interquartile, respectively. The red point in each graph indicate the values obtained for the subject who had putative virologic progression ($EC42_{HD}$). *P*-values were obtained with Wilcoxon signed-rank tests.

immune activation and plasma inflammatory biomarkers also did not distinguish both EC subgroups (**Figures 2E–K**). The $EC42_{HD}$ subject, who had putative virologic progression after *env* diversity assay, presented most of the parameters within the range of other EC, except for cVL (pre) for which he exhibited the highest value of the group (**Figure 2**). The *env* proviral diversity was not significantly correlated with any of the virologic or immunologic parameter evaluated when EC were analyzed separately (**Supplementary Figure S2**). However, $\%CD8^+CD38^+HLA\text{-}DR^+$ T cells ($P < 0.0001$), total HIV DNA load ($P = 0.003$), and IP-10 ($P = 0.03$), were significantly higher in VC compared with EC (**Supplementary Figure S3**) and significant positive correlations with *env* proviral diversity (cVL, IP-10, and IL-18; **Supplementary Figure S4A**) and cVL (total HIV DNA load, $\%CD8^+CD38^+HLA\text{-}DR^+$ T cells, IP-10 and IL-18; **Supplementary Figure S4B**) were detected when HIV controllers (EC and VC) were taken as a whole.

DISCUSSION

In a previous study, we identified two subgroups of EC (EC_{LD} and EC_{HD}) in a cohort of Brazilian subjects with divergent patterns of intra-host proviral diversity (de Azevedo et al., 2017). Our results comparing the clinical and epidemiologic aspects of the EC subgroups revealed no difference between them. Moreover, biomarkers previously associated with the risk of immunologic and/or virologic progression ($CD4^+$ and $CD8^+$ T cells counts, %CD4, $CD8^+$ T cell activation, inflammation, HIV-1 RNA and DNA loads) in EC were not significantly different either before or after the proviral diversity assay among EC subsets.

The combined use of absolute and percentage $CD4^+$ T cells trends over time reinforce the absence of true immunologic damage in our EC cohort. Subjects with a decrease in absolute $CD4^+$ T cell counts showed stable ($EC19_{LD}$ and $EC30_{HD}$) or increasing ($EC39_{HD}$) $\%CD4^+$ T cells, indicating that reduction was not specific to $CD4^+$ T cells (**Supplementary Figure S5**). Subjects with a significant decrease in the $\%CD4^+$ T cells displayed stable ($EC11_{LD}$) or even increasing ($EC42_{HD}$) absolute $CD4^+$ T cells counts. Furthermore, all EC_{LD} and EC_{HD} subjects maintained $CD4^+$ T cell counts > 500 cells/mm^3 and percentage of $CD4^+$ T cells > 30% during follow-up, supporting a low risk for disease progression (Burcham et al., 1991; Hulgan et al., 2007). The only subject with a virologic breakthrough ($EC42_{HD}$) maintained VL in the very low range (59–97 copies/mL) and it could be argued that he did not display a true virologic progression (>2,000 copies/mL) (Noel et al., 2015b; Chereau et al., 2017). These data show that immunologic and/or virologic progression was rare in both EC_{LD} and EC_{HD} groups and reinforce the need to unify criteria for the definition of true progression in EC.

A recent study described higher levels of HIV *env* diversity and proinflammatory cytokines (RANTES, PDGF-AA, and CTACK) in EC that lost virologic control 1 year later, compared with EC that maintained persistent virologic control (Pernas et al., 2017). Our analyses did not reveal significant differences in the levels of RANTES, PDGF-AA, and CTACK between EC_{LD} and EC_{HD} subgroups. Similarly, levels of $CD8^+$ T cell activation and IP-10, previously pointed out as biomarkers for detection of EC at risk of immunologic progression (Noel et al., 2014; Côrtes et al., 2018), were also not significantly different between the EC_{LD} and EC_{HD} groups. Noteworthy, the only EC subject who had allegedly virologic progression in our cohort ($EC42_{HD}$) displayed similar levels of immune activation and inflammatory biomarkers than subjects with persistent elite virologic control.

These results support that some EC are able to maintain stable $CD4^+$ T cells and persistent control of viral replication for several years (>2–5 years) in the setting of high proviral diversity. Although HIV-1 diversity and other surrogate markers of viral replication (total HIV-1 DNA load, $CD8^+$ T cell activation, and IP-10) were positively correlated among each other and with the cVL when EC and VC were taken as a whole, consistent with previous findings (Bello et al., 2005; Sajadi et al., 2007; Groves et al., 2012; Côrtes et al., 2015; Noel et al., 2015a; Platten et al., 2016; Canouï et al., 2017; Tarancon-Diez et al., 2018); we did not detect any significant association between the *env* proviral diversity and those biomarkers when EC were analyzed separately. Hence, the genetic diversity of the HIV-1 proviral reservoir in many EC is probably not driven by continuous residual viral replication (de Azevedo et al., 2017), which may explain why EC_{HD} does not seem to display higher risk of immunologic or virologic progression in compared with EC_{LD} in our cohort. Those EC for which diversity of HIV-1 proviral reservoir truly reflects continuous viral replication and persistent inflammation are probably the only ones at risk of immunologic or virologic progression.

The main limitation of this study was the small number of EC individuals analyzed, which may have resulted in the apparent absence of significant differences in various markers evaluated between EC subgroups. This limitation was partially counterbalanced by the well recorded long period of follow-up of these extremely rare group of patients and the very rigorous classification criteria used to ensure that only EC individuals with a long-lasting HIV-1 control profile were included in our cohort (Walker and Yu, 2013). Further studies comprising a larger number of individuals are needed to complement our findings and to define the most suitable combination of biomarkers necessary to predict immunologic and/or virologic progression in long-term EC.

CONCLUSION

Our data suggest that genetic diversity of the HIV-1 proviral reservoir is not a surrogate marker of residual viral replication, immune activation or inflammation, nor an accurate biomarker for the prediction of virologic breakthrough or $CD4^+$ T cells loss in EC. Most EC in our cohort maintained a persistent control of viremia and stable $CD4^+$ T cells for up to 5 years after determination of HIV-1 quasispecies composition, irrespective of proviral genetic diversity. Understand the mechanisms leading to the divergent patterns of intra-host viral diversity in EC is of

paramount importance to determine the potential impact of such divergent patterns on the long-term natural control of HIV-1 infection and their relevance for clinical management of EC.

DATA AVAILABILITY

All datasets generated for this study are included in the manuscript and/or the **Supplementary Files**.

ETHICS STATEMENT

All participants provided written informed consent and the ethical committee of Instituto Nacional de Infectologia Evandro Chagas (INI-Fiocruz) approved the study (CAAE 1717.0.000.009-07).

AUTHOR CONTRIBUTIONS

GB conceived and designed the study and supervised the experiments. SA and FC conducted the experiments and analyzed the data. ED performed the quantification of total cell-associated HIV-1 DNA, its analysis, and provided intellectual input. MR-A analyzed the data. BH, BG, and VV conducted the patient recruitment and follow-up. MM contributed to the study design and provided intellectual input. SA, FC, and GB wrote the first draft. All authors assisted with the writing and approved the final manuscript.

FUNDING

This work was supported by the Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro – FAPERJ (Grant No. E-26/110.123/2014) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq (Grant No. 401220/2016-8). SA was supported by funding from Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro – FAPERJ. ED was financed by a Postdoctoral fellowship from the "Programa Nacional de Pós-Doutorado (PNPD)" by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – Brasil (CAPES) – Finance Code 001. GB was supported by fundings from CNPq (Grant No. 302317/2017-1) and FAPERJ (Grant No. E-26/202.896/2018).

ACKNOWLEDGMENTS

We thank the patients, who participated in the study, as well as all the technical staff involved in the clinical follow-up of these patients. We also thank Leila Botelho Rodrigues da Silva and Marcelle Bral de Mello from Laboratório Tecnologia e Diagnóstico – Biomanguinhos/FIOCRUZ for sharing the Luminex 200 System facility.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2019.00673/full#supplementary-material

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Supplementary Material

Proviral quasispecies diversity is not associated with virologic breakthrough or $CD4^+$ T cell loss in HIV-1 elite controllers

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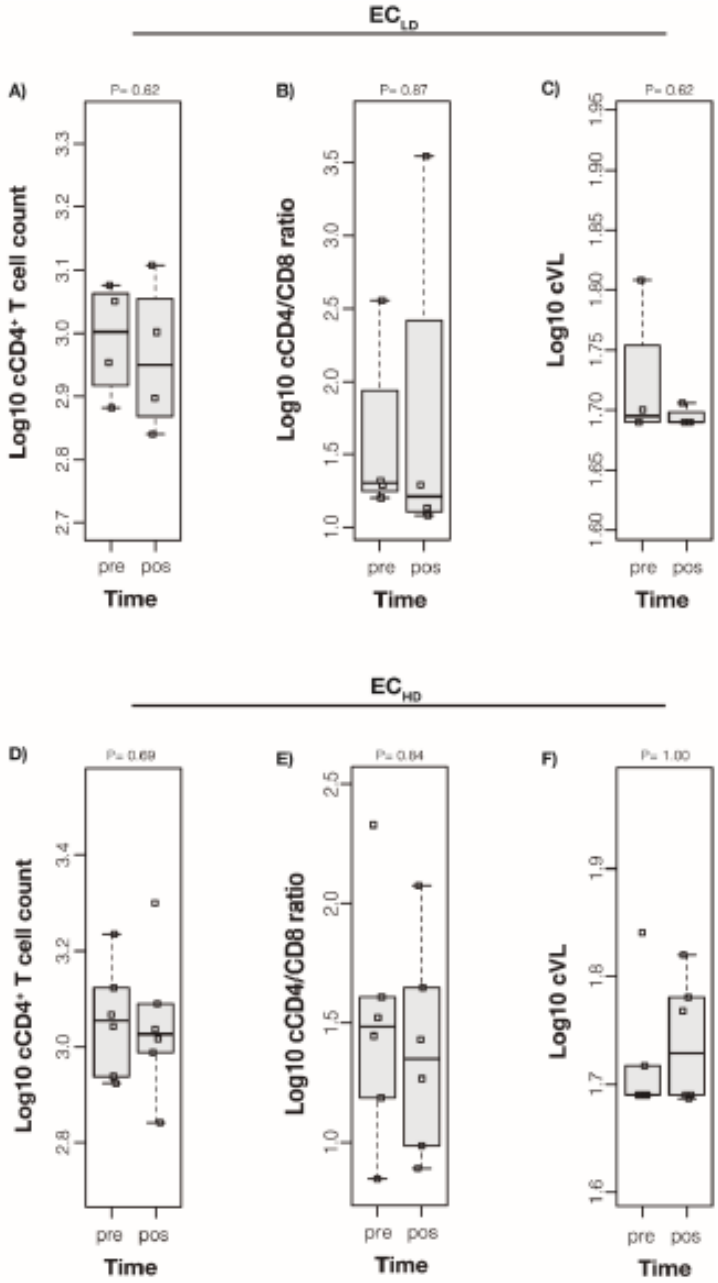
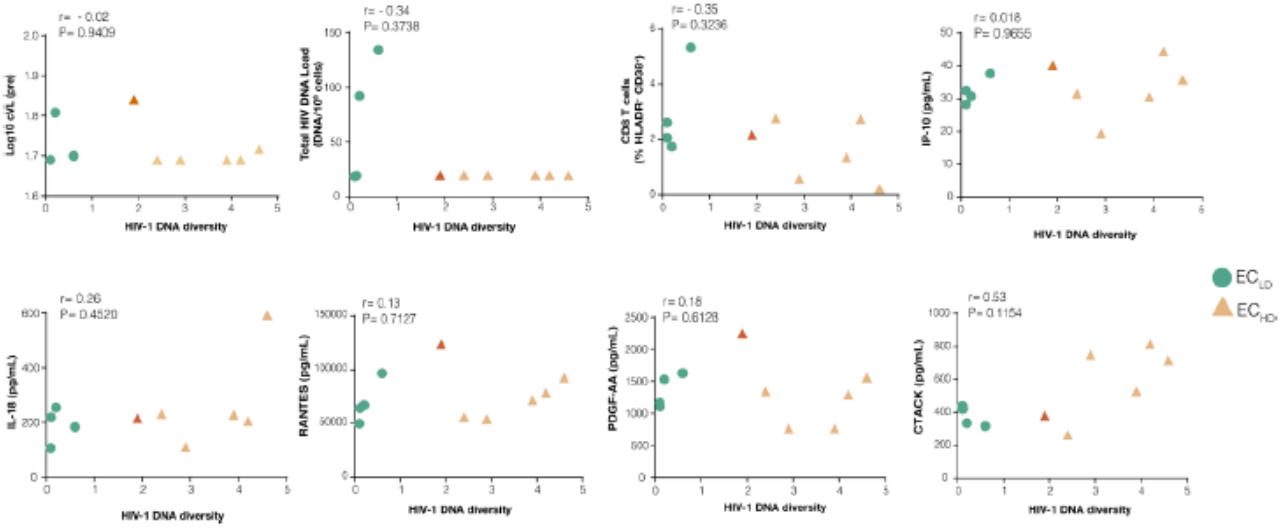
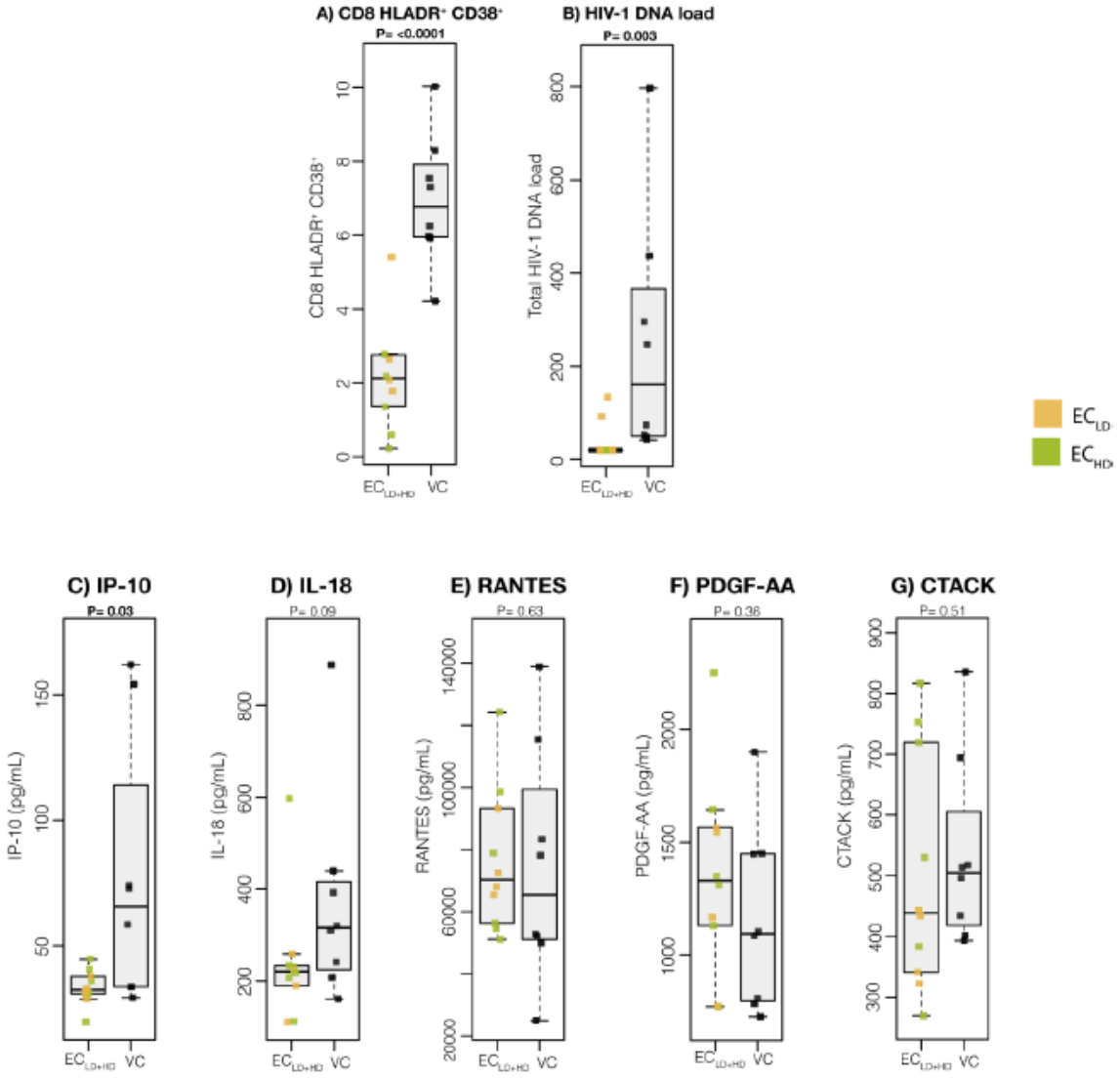
Supplementary Figures



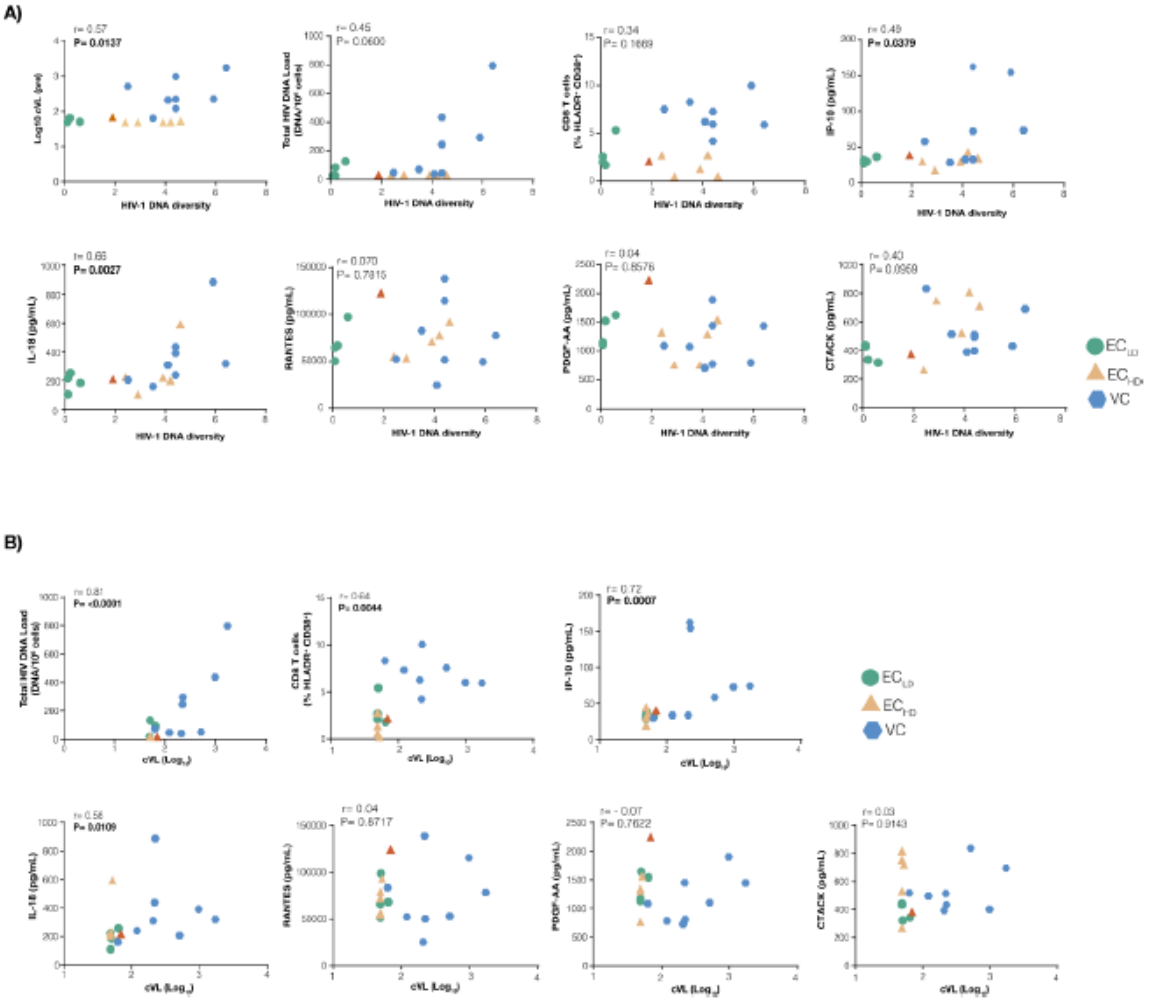
Supplementary Figure S1. Immunologic and virologic characteristics in pre- and post-period of determination of *env* diversity in EC_{LD} and EC_{HD}. Cumulative measurements (log_{10} – transformed) of the $CD4^+$ T cell count (A, D), CD4/CD8 ratio (B, E) and viral load (C, F) between pre- and post-period of determination of *env* diversity in EC subgroups. Central black solid bars and boxplots represent sample medians and interquartile, respectively. *P*-values were obtained with Wilcoxon signed rank tests.



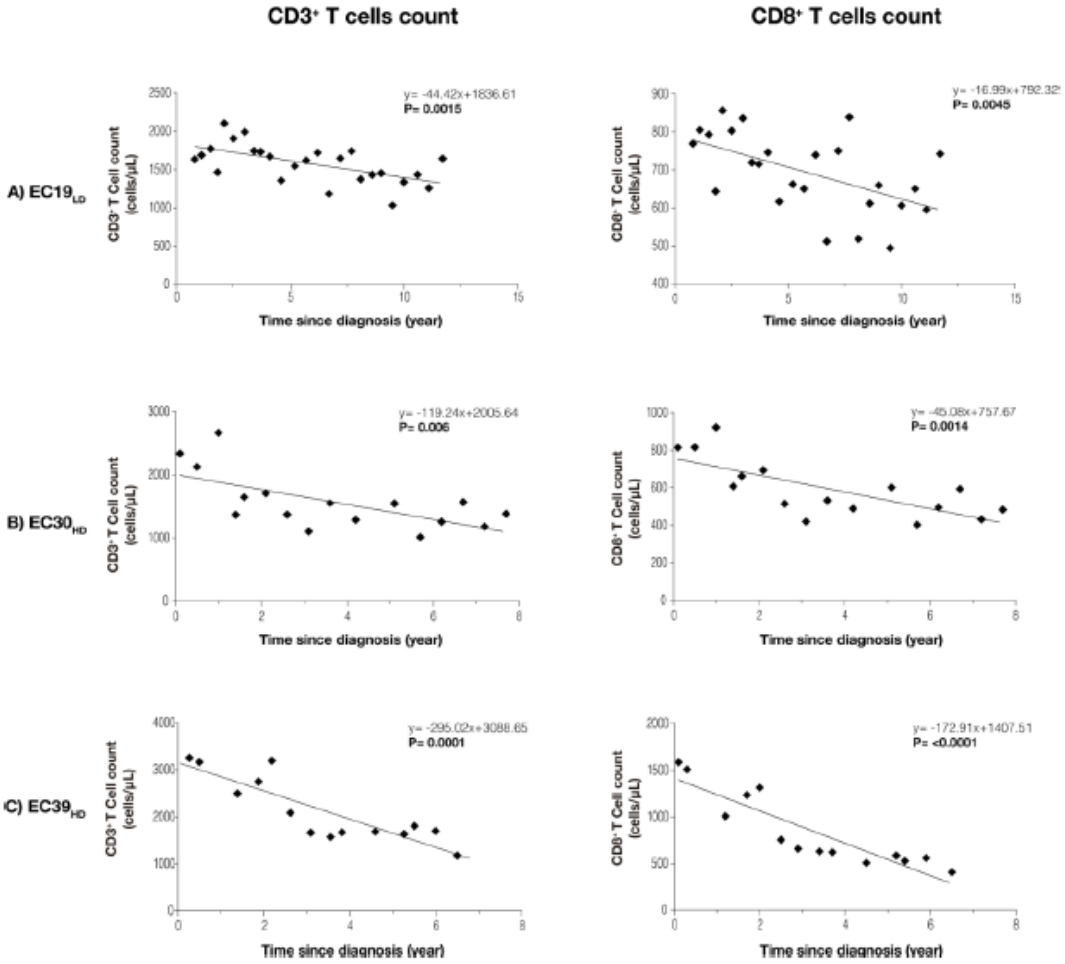
Supplementary Figure S2. Relationships between *env* proviral diversity and virologic/immunologic parameters in EC. Spearman's Rank Correlations Coefficient analysis was performed between *env* proviral diversity and cumulative measurements of the viral load (cVL, $\log_{10}$ – transformed), total HIV-1 DNA load, proportion of HLA-DR$^+$CD38$^+$ CD8$^+$ T cells, and levels of the IP-10, IL-18, RANTES, PDGF-AA and CTACK. The coefficient of correlation (r) and their respective *P*-values are shown in the upper left corner of each graph. The green circles and light orange triangles in each graph represent the EC_{LD} and EC_{HD} subjects, respectively. The dark orange triangles in each graph represent the subject with putative loss of virologic control ($EC42_{HD}$).



Supplementary Figure S3. Comparison of virologic and immunologic characteristics between the EC and VC. $CD8^+$ T cell activation (A), total HIV-1 DNA load (B) and plasmatic markers of inflammation/immune activation (C-H). The orange and green points in each graph indicate the EC_{LD} and EC_{HD} subjects, respectively. Central black solid bars and boxplots represent sample medians and interquartile, respectively. *P*-values were obtained with Mann–Whitney U tests.



Supplementary Figure S4. Relationships between *env* proviral diversity (A) or cVL (B) with virologic/immunologic parameters in HIV controllers (EC and VC). Spearman's Rank Correlations Coefficient analysis was performed between *env* proviral diversity and cVL, total HIV-1 DNA load, proportion of HLA-DR^{+}CD38^{+} CD8^{+} T cells, and levels of the IP-10, IL-18, RANTES, PDGF-AA and CTACK. The coefficient of correlation (r) and their respective *P*-values are shown in the upper left corner of each graph. The green circles and light orange triangles in each graph represent the EC_{LD} and EC_{HD} subjects, respectively. The dark orange triangles in each graph represent the subject with putative loss of virologic control ($EC42_{HD}$).



Supplementary Figure S5. $CD3^+$ and $CD8^+$ T cell dynamics in selected EC subjects. Slopes of absolute $CD3^+$ and $CD8^+$ T cells counts since HIV diagnosis are shown on the Y-axis of left and right columns, respectively. The slopes and their respective *P*-values are shown in the upper right corner of each graph and were calculated by linear regression analysis fitted by generalized least squares and with an autocorrelation structure of first order in respect to the time after HIV diagnosis.

Supplementary Tables

Supplementary Table S1. Clinical and epidemiologic characteristics EC_{LD} and EC_{HD} subgroups.

Parameter	EC_{LD} (n=4)	EC_{HD} (n=6)	*P* value*
Age (years)**	43.5 (35.0 – 46.5)	48.5 (35.5 – 65.0)	0.70
Sex (female), no. (%)	3 (75%)	5 (83%)	> 0.99
Heterosexual transmission, no. (%)	4 (100%)	5 (83%)***	0.67
HLA-B*57/27 presence, no. (%)	2 (50%)	1 (17%)	0.50
Hepatitis C coinfection, no. (%)	0	1 (17%)	> 0.99
Study point			
Time since of HIV-1 diagnosis (year)	12.4 (3.4 – 16.7)	4.8 (4.2 – 14.0)	0.72
$CD4^+$ T cell count (cells/mm^3)	912 (763 – 1267)	1048 (891 – 1688)	0.33
% CD4	45.0 (39.5 – 57.2)	41.0 (34.8 – 52.5)	0.47
$CD8^+$ T cell count (cells/mm^3)	544 (432 – 742)	856 (578 – 1597)	0.11
CD4/CD8 ratio	1.44 (1.3 – 2.8)	1.24 (0.98 – 1.87)	0.47
Plasma HIV RNA load (copies/ml)	49 (12.25 – 49)	49 (49 – 60.75)	0.53
Total HIV DNA load (DNA/10^6 cells)	56 (20 – 123)	20 (20 – 20)	0.13
Follow-up			
Time maintaining EC status (years)	16 (8.18 – 21.85)	8 (6.65 – 17.90)	0.47
$CD4^+$ T cell count (cells/mm^3)	981 (845 – 1215)	1123 (929 – 1424)	0.33
% CD4	43.0 (39.0 – 56.0)	41.5 (35.5 – 48.0)	0.52
$CD8^+$ T cell count (cells/mm^3)	682 (519 – 889)	851 (724 – 1006)	0.33
CD4/CD8 ratio	1.25 (1.2 – 2.4)	1.4 (1.2 – 1.8)	0.63
$CD4^+$ T cell slope (cells/mm^3/year)	5.5 (-23.08 – 22.08)	11.90 (-77 – 26.15)	> 0.99
%CD4 slope	0.34 (-0.45 – 0.35)	0.22 (-0.40-0.72)	0.48
cVL (log10–transformed)	1.7 (1.7-1.8)	1.7 (1.7-1.7)	0.92

* Statistical analyses were performed using the Mann–Whitney test. **Age at study point. ***A female subject had unknown HIV mode of transmission. cVL: cumulative viral load.

Supplementary Table S2. Virologic characteristics of EC_{LD} and EC_{HD} subgroups.

Patient	Subtype	HIV DNA π	HIV DNA load (cp/10^6 cells)	VL Blip Frequency	VL Blip Amplitude (cp/mL)	Virologic breakthrough
$EC_{LD}52$	B	0.1%	20	0/10	-	No
$EC_{LD}11$	B	0.2%	92	0/14	-	No
$EC_{LD}19$	B	0.6%	134	0/5	-	No
$EC_{LD}35$	F1	0.1%	20	0/9	-	No
Mean	-	**0.3%**	**67**	**0/38**	-	**0/4**
$EC_{HD}17$	B	4.6%	20	2/4	51-96	No
$EC_{HD}30$	B	2.9%	20	0/6	-	No
$EC_{HD}36$	B	2.4%	20	2/10	61-1,086	No
$EC_{HD}38$	A	3.9%	20	0/5	-	No
$EC_{HD}39$	B	4.2%	20	0/5	-	No
$EC_{HD}42$	B	1.9%	20	6/13	59-97	Yes
Mean	-	**3.3%**	**20**	**10/50**	-	**1/6**

VL: viral load.

3.4 ARTIGO 4

Increased expression of MCPIP1 in HIV-1 controllers is correlated with overexpression of p21

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Periódico: Preprint BIORXIV

Resumo:

Algumas proteínas celulares multifuncionais, como a proteína 1 induzida por proteína quimiotáxica de monócito (MCPIP1) e o inibidor de quinase dependente de ciclina p21, também mostraram ser capazes de modular a susceptibilidade celular ao vírus da imunodeficiência humana do tipo 1 (HIV-1). Vários estudos descreveram uma alta expressão *ex vivo* de p21 em células de indivíduos que controlam naturalmente a replicação do HIV-1 (HIVc). O nível de expressão de MCPIP1 em HIVc nunca foi anteriormente descrito, mas um estudo recente em um modelo de células de carcinoma renal mostrou que a superexpressão de MCPIP1 foi associada com um aumento tanto dos transcritos quanto da proteína p21. Aqui, exploramos as possíveis associações entre a expressão de MCPIP1 e p21, bem como sua relação com a ativação celular em HIVc, sustentando cargas virais indetectáveis (controladores elite - EC) ou baixas (controladores virêmicos - VC). Encontramos uma regulação positiva seletiva dos níveis de RNAm de MCPIP1 e p21 em PBMC de HIVc em comparação com ambos os grupos controles de indivíduos suprimidos pela terapia e HIV-negativos ($P \leq 0,02$) e uma forte correlação positiva ($r \geq 0,57$; $P \leq 0,014$) entre as expressões de ambos os transcritos independente da carga viral, condição de tratamento e status do HIV-1. Os níveis de RNAm de p21, mas não de MCPIP1, foram positivamente correlacionados com níveis de células T $CD4^+$ ativadas em HIVc e EC ($r \geq 0,53$; $P \leq 0,017$). Em relação a ativação de monócitos, os níveis de RNAm de ambos p21 ($r = 0,74$; $P = 0,005$) e MCPIP1 ($r = 0,58$; $P = 0,040$) foram positivamente correlacionados com os níveis plasmáticos de sCD14 somente em EC. Análise multivariada confirmou a associação entre os níveis de RNAm de MCPIP1 e p21, e entre estes últimos com a frequência de células T $CD4^+$ ativadas. Estes dados mostram pela primeira vez a superexpressão concomitante e a correlação positiva dos transcritos de MCPIP1 e

p21 no cenário da supressão natural da replicação do HIV-1 *in vivo*. A correlação positiva entre os transcritos de MCPIP1 e p21 indicam uma via regulatória comum conectando esses fatores multifuncionais do hospedeiro e um possível efeito sinérgico no controle do HIV-1. A manipulação farmacológica dessas proteínas celulares pode abrir novas perspectivas terapêuticas para prevenir a replicação do HIV-1 e a progressão da doença.

Increased expression of MCPIP1 in HIV-1 controllers is correlated with overexpression of p21

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Keywords: HIV-1 controllers, restriction factors, MCPIP1, p21, immune activation

Words count abstract: 343

Words count text: 3.442

Figures: 3

Table: 1

Abstract

Some multifunctional cellular proteins, as the monocyte chemotactic protein-induced protein 1 (MCPIP1) and the cyclin-dependent kinase inhibitor p21, have also shown to be able to modulate the cellular susceptibility to the human immunodeficiency virus type 1 (HIV-1). Several studies described that p21 is expressed at high levels *ex vivo* in cells from individuals who naturally control HIV-1 replication (HIC). The expression level of MCPIP1 in HIC was never described before, but a recent study in a model of renal carcinoma cells showed that MCPIP1 overexpression was associated with an increase of both p21 transcripts and proteins levels. Here, we explored the potential associations between MCPIP1 and p21 expression, as well as with cellular activation in HIC, sustaining undetectable (elite controllers – EC) or low (viremic controllers – VC) viral loads. We found a selective upregulation of MCPIP1 and p21 mRNA levels in PBMC from HIC compared with both ART–suppressed and HIV–negative control groups ($P \leq 0.02$) and a strong positive correlation ($r \geq 0.57$; $P \leq 0.014$) between expressions of both transcripts independently of the VL, treatment condition and HIV status. The mRNA levels of p21, but not of MCPIP1, were positively correlated with activated $CD4^+$ T cells levels in HIC and EC ($r \geq 0.53$; $P \leq 0.017$). In relation to the monocyte activation, the mRNA levels of both p21 ($r = 0.74$; $P = 0.005$) and MCPIP1 ($r = 0.58$; $P = 0.040$) were positively correlated with plasmatic levels of sCD14 only in EC. Multivariate analysis confirmed the association between MCPIP1 and p21 mRNA levels, and between the latter with the frequency of activated $CD4^+$ T cells. These data show for the first time the simultaneous overexpression and positive correlation of MCPIP1 and p21 transcripts in the setting of natural suppression of HIV-1 replication *in vivo*. The positive correlation between MCPIP1 and p21 transcripts supports a common regulatory pathway connecting these multifunctional host factors and a possible synergistic effect on HIV-1 replication control. Pharmacological manipulation of these cellular proteins may open novel therapeutic perspectives to prevent HIV-1 replication and disease progression.

1 Introduction

Among the individuals infected by the human immunodeficiency virus type 1 (HIV-1), a rare group called HIV controllers (HIC) suppress viral replication in absence of antiretroviral therapy, maintaining RNA viral loads (VL) below the limit of detection (LOD) (elite controllers, EC) or at low levels (> LOD and < 2,000 copies/ml; viremic controllers, VC). Natural control of HIV-1 replication is probably a multifactorial feature that involves different combinations of host and/or viral factors (1).

Some intrinsic host proteins, termed restriction factors (RF), are components of the innate immune response (2,3) that have the ability to cause a significant reduction in viral infectivity by interacting directly with the pathogen and are generally induced by interferon (IFN), hence being known as IFN-stimulated genes (ISGs) (4). Several RF has been shown to limit HIV replication *in vitro* at different stages of its life cycle (3), including some classical RF such the Apolipoprotein B mRNA-Editing enzyme, Catalytic polypeptide-like (APOBEC3G), the Bone Stromal Tumor protein 2 (BST2)/Tetherin, and the Sterile Alpha Motif domain and HD domain-containing protein 1 (SAMHD1) (2), and others more recently characterized like the Myxovirus resistance protein 2 (Mx2), the Interferon-inducible transmembrane family proteins (IFITM1-3 members) and Schlafen 11 (SLFN11) (3). The mRNA levels of some RF including SAMHD1, Theterin, IFITM1, Mx2 and SLFN11 have been described to be elevated in peripheral blood mononuclear cells (PBMC) or $CD4^+$ T cells of HIC compared to antiretroviral (ART)-suppressed and/or HIV-uninfected individuals (5–9), although with contrasting findings across different HIC cohorts.

Others host multifunctional proteins, not recognized as classical RF, are also able to modulate the cellular susceptibility to HIV-1 infection. The cyclin-dependent kinase (CDK) inhibitor p21, encoded by the CDKN1A gene, modulates multiple relevant processes of the immune system, including proliferation of activated/memory T cells, macrophage activation and inflammation (10–17). This protein also indirectly limits the HIV-1 replication *in vitro* in various cellular systems by blocking the biosynthesis of dNTPs required for viral reverse transcription and by inhibiting the CDK9 activity required for HIV-1 mRNA transcription (18–23). Several studies described that p21 is expressed at high levels *ex vivo* in $CD4^+$ T cells from HICs (21,24–26) and that p21 mRNA levels correlated with $CD4^+$ T cell activation in EC, but not in other HIV-infected groups (5). These evidences suggest that the inducibility of p21 to immune activation is a singular characteristic of EC and may contribute to the natural control of HIV-1 replication *in vivo*.

The monocyte chemotactic protein–induced protein 1 (MCPIP1), encoded by ZC3H12A gene, is another newly discovered host multifunctional modulator of immune response with antiviral activity (27). MCPIP1 plays a critical role in the regulation of the inflammatory response and immune homeostasis and also blocks HIV-1 replication *in vitro* by promoting the viral mRNA degradation through its RNase activity, particularly in quiescent $CD4^+$ T cells (27,28). In activated $CD4^+$ T cells, MCPIP1 is rapidly degraded (28) after its cleavage by the mucosa-associated lymphoid-tissue lymphoma-translocation 1 (MALT1) protein (29,30). In activated macrophage cells, by contrast, MCPIP1 transcripts are induced by TLR ligands and pro-inflammatory cytokines (mainly, TNF-α, IL-1β and CCL2/MCP-1), and its expression stimulate a negative feedback loop that attenuates the inflammatory state by decreasing its fundamental mediators (27,31).

The expression level of MCPIP1 in HIC was never described before. Interestingly, a recent study in renal carcinoma cells (Caki-1 cells) revealed that MCPIP1 overexpression reduces the cellular growth by increasing the levels of p21 transcripts, along with other proteins involved in cell cycle progression/arrest, supporting a coordinate regulation of MCPIP1 and p21 transcripts in that cell-line

(32). This evidence prompted us to ask whether the expression of MCPIP1 could be elevated and positively correlated with p21 in the setting of natural control of HIV-1 infection. To test this hypothesis, we quantified the *in vivo* expression of MCPIP1, p21 and several antiviral host RF mRNAs in PBMC from HIC, ART-suppressed and HIV-uninfected individuals. We further explored the potential relationship between MCPIP1/p21 expression and levels of systemic cellular activation in HIC.

2 Methods

2.1 Study Subjects

We analyzed a cohort of 21 HIC subjects followed-up at the Instituto Nacional de Infectologia Evandro Chagas (INI) in Rio de Janeiro, Brazil. All HIC maintained RNA VL of < 2,000 copies/ml without antiretroviral therapy for at least five years and were subdivided in two sub-groups: EC (n = 13) when most (≥ 70%) plasma VL determinations were below the limit of detection (LOD), and VC (n = 8) when most (≥ 70%) VL determinations were > LOD and < 2,000 copies/ml. The limit of detection of plasma VL determinations varied over the follow-up period in according to the Brazilian Ministry of Health guidelines, with methodologies being updated overtime to improve sensitivity: Nuclisens HIV-1 RNA QT assay (Organon Teknika, Durham, NC, limit of detection: 80 copies/mL) from 1999 to 2007; the Versant HIV-1 3.0 RNA assay (bDNA 3.0, Siemens, Tarrytown, NY, limit of detection: 50 copies/mL) from 2007 to 2013; and the Abbott RealTime HIV-1 assay (Abbott Laboratories, Wiesbaden, Germany, limit of detection: 40 copies/mL) from 2013 to until today. Virological and immunological characteristics of these subjects were described in detail in previous studies (33,34). Two groups of ART-suppressed subjects (ART, n = 8) and healthy HIV-1-uninfected subjects (NEG, n = 10) were used as controls.

2.2 mRNA gene-expression analysis

Total RNA was extracted from 1 x 10^7 PBMC using RNeasy mini kit (Qiagen, Hilden, North Rhine-Westphalia, Germany) in which buffer RLT was supplemented with β-mercaptoethanol and displaced on-column DNase treatment using a Qiagen RNase-Free DNase Set (Qiagen, Hilden, North Rhine-Westphalia, Germany) according to manufacturer's instruction. Total RNA yield and quality were determined using NanoDrop® 8000 spectrophotometer and an Agilent® 2100 Bioanalyzer. Only samples with an RNA integrity number (RIN) greater than 8.0 were used. Purified RNA (1 µg) was reverse-transcribed to cDNA using RT^2 First Strand Kit (Qiagen, Hilden, North Rhine-Westphalia, Germany). The cDNA was mixed with RT^2SYBR Green/ROX qPCR Master Mix (Qiagen, Hilden, North Rhine-Westphalia, Germany) and the mixture was added into customized RT^2RNA PCR Array (Qiagen, Hilden, North Rhine-Westphalia, Germany) to measure the mRNA expression of 10 cellular target genes (APOBEC3G, SAMHD1, Tetherin, Mx1, Mx2, SLFN11, IFITM1, IFITM3, MCPIP1, and p21) besides three housekeeping genes (GAPDH, β-actin, and RNase-P), according to manufacturer's instructions. Values of the crossing point at the maximum of the second derivative of the four-parameters fitted sigmoid curve second derivative, Cp, was determined for each sample. The efficiency of each amplification reaction was calculated as the ratio between the fluorescence of the cycle of quantification and fluorescence of the cycle immediately preceding that. Genes used in the normalization among samples were selected by the geNorm method (35). Data were expressed as fold-changes in mRNA abundance calculated as the normalized gene expression in any test sample divided by the mean normalized gene expression in the control HIV-negative group.

2.3 T cell and monocyte activation analyses

We used data of T cell and monocyte activation obtained in a previous study conducted by our group including these patients (34), in which plasma levels of soluble CD14 (sCD14) were determined by ELISA-sCD14 Quantikine assay (R&D Systems Minneapolis, MN) according to the manufacturer's protocol and surface expression of combined HLA-DR and CD38 on $CD4^+$ and $CD8^+$ T cells was analyzed by flow cytometry.

2.4 Data analyses

The comparisons of mean log-fold changes in mRNA abundance were performed by either t-tests or one-way ANOVA nonparametric permutation tests (B = 1,000 permutations), followed by pair-wise comparisons with Holm-Bonferroni adjustment (36), for two or more groups respectively. Spearman coefficient was used for correlation analyses. A first-order log-Normal multiple regression analysis was fitted to model p21 gene expression as a function of MCPIP1 gene expression, $CD4^+$ T cell activation ($HLA\text{-}DR^+CD38^+$), and HIC groups (EC and VC). The threshold for statistical significance was set to $P < 0.05$. Data were analyzed with R software (version 3.5.2) (37).

3 Results

Twenty-nine HIV-1 positive (21 HIC and 8 ART-suppressed) and 10 HIV-negative individuals were included in this cross-sectional study. Most HIV-positive (59%) and HIV-negative (60%) individuals were females and all individuals displayed $CD4^+$ T cells counts above 500 cells/µl (Table 1). Although the EC subgroup shows a higher proportion of females (77%), the difference was not significant (Supplementary Table 1).

Analysis of the expression of multifunctional genes revealed a significant upregulation of both MCPIP1 and p21 transcripts in PBMC from HIC (Figure 1). The MCPIP1 mRNA was upregulated in PBMC from HIC compared to cells from both ART-suppressed (1.68-fold increase; P = 0.003) and HIV-negative (1.37-fold increase; P = 0.02) individuals (Figure 1A). A similar overexpression of the p21 mRNA was observed in PBMC from HIC compared to ART-suppressed (1.63-fold increase; P = 0.003) and HIV-negative (1.55-fold increase; P = 0.003) individuals (Figure 1B). In contrast, we found no significant differences in the mRNA levels of antiretroviral RF between the HIC and control groups, with the only exception of IFITM1 that was significantly elevated (1.15-fold increase; P = 0.03) in HIC in comparison to the HIV-negative group (Supplementary Figure S1).

We observed a significant positive correlation between the mRNA expression of MCPIP1 and p21 ($r \geq 0.57$; $P \leq 0.014$) in our cohort independently of the VL, treatment condition and HIV status (Figure 2). This positive correlation was maintained when individuals were subdivided by sex (Supplementary Figure S2). No significant correlations were observed between the mRNA expression of multifunctional genes MCPIP1/p21 and RF, with the only exception of a significant, negative correlation between MCPIP1/p21 and APOBEC3G in HIC (Supplementary Figure S3) and EC (Supplementary Figure S4).

To explore the potential relationship of p21 or MCPIP1 expression with immune activation, we measured the frequency of phenotype $HLA\text{-}DR^+CD38^+$ on $CD4^+$ and $CD8^+$ T cells (T cell activation) and plasma levels of sCD14 (monocyte activation) in our cohort. Frequencies of activated $CD4^+$ T cell populations in VC and ART-suppressed subjects were higher than in EC ($P < 0.0001$) and HIV-negative ($P = 0.0002$) individuals (Supplementary Figure S5A). The VC subgroup also had significantly higher frequencies of activated $CD8^+$ T cell than EC ($P = 0.0007$) and control groups ($P \leq 0.0009$) (Supplementary Figure S5B). The median concentration of sCD14 in plasma was not significantly different across the groups (Supplementary Figure S5C). No significant correlations between mRNA levels of MCPIP1 and $CD4^+$ T cell (Figure 3A) or $CD8^+$ T cell (data not shown)

activation were observed for HIC or EC subsets. The mRNA levels of p21 were positively associated with activated $CD4^+$ T cells levels in HIC ($r = 0.53$; $P = 0.016$) and EC ($r = 0.68$; $P = 0.017$) (Figure 3B); but not with activated $CD8^+$ T cell levels (data not shown). Levels of sCD14 were positively correlated with both MCPIP1 ($r = 0.58$; $P = 0.04$) and p21 ($r = 0.74$; $P = 0.005$) mRNA levels only in the EC subset (Figure 3C and D). No significant correlations between mRNA levels of MCPIP1/p21 and $CD4^+$/$CD8^+$ T cell activation or sCD14 levels were observed when ART-suppressed and HIV-negative individuals were included (Supplementary Figures S6). Multivariate analysis showed that the upregulation of MCPIP1 was positively associated with the increase of p21 expression in HIC (1.44-fold increase; $P = 0.0035$) (Supplementary Figure S7A). The frequency of activated $CD4^+$ T cells also was positively associated with the increase of p21 expression in both EC and VC (1.48-fold increase; $P = 0.0116$), although this increase of the p21 expression was down-regulated by the increase of activated $CD4^+$ T cells in VC when compared to EC (1.30-fold decrease by an increase of 1% $CD4^+HLA\text{-}DR^+CD38^+$ T cells; $P = 0.0284$) (Supplementary Figure S7B). Overall, the model was highly significant ($P = 0.003$) and could explain as much as 70% ($R^2 = 0.492$) of p21 expression.

4 Discussion

In this study, we observed that MCPIP1 and p21 mRNA expression were significantly increased in PBMC of HIC compared to cells of HIV-negative and -positive/ART-suppressed individuals. While elevated expression of p21 in PBMC of HIC had already been previously described (5,21,24–26), this is the first study to show overexpression of MCPIP1 alongside with p21 in these individuals.

The mRNA levels of MCPIP1 and p21 were positively correlated in HIC as well as in HIV–positive and –negative individuals. This supports a coordinated expression of these cellular genes in different settings, consistent with what has been shown for a renal carcinoma cell line (32). According to this study, MCPIP1 expression triggers the activation of p21 by two mechanisms: 1) down-modulation of damage-specific DNA binding protein 1 (DDB1) which regulates degradation of p21; and 2) upregulation of the mRNA levels of chromatin licensing and DNA replication factor 1 (CDT1) which activates p21 (32). In addition, following HIV-1 infection, the cellular let-7c miRNA is upregulated and it downregulate p21, resulting in higher copy number of viral genome transcripts in infected cells (38). MCPIP1 acts as a broad suppressor of the biogenesis pathway of both cellular (39) and viral miRNA (40). The involvement of the MCPIP1 in the degradation of another precursor of let-7 family (pre-let-7g) was already described (41), reinforcing the hypothesis that MCPIP1 might enhance the antiviral responses triggered by HIV-1 entry and infection by downregulating the miRNAs that target p21.

Increased expression of some host RF, which are also ISGs (4), has been previously observed in $CD4^+$ T cells (i.e., SAMHD1, SLFN11 and IFITM1) (5,7,8) and PBMC (i.e., Mx1, Mx2, Tetherin and SLFN11) from HIC (6,9). With the only exception of IFITM1, no other RF analyzed here were upregulated in PBMC from our HIC cohort. In the chronic phase of HIV−1 infection in viremic untreated patients, most ISGs are upregulated in $CD4^+$ T cells (42–44) and their expression is positively correlated with the percentage of activated T cells and negatively correlated with $CD4^+$ T cell counts (42–46). This suggests that residual or low-level viremia observed in our HIC might not be enough to induce a generalized upregulation of ISGs during chronic infection (44). In addition, MCPIP1 (47,48) and p21 (16) negatively regulate the NF-κB cascade and their overexpression may also contribute to limit the chronic overexpression of ISGs in HIC. While most RF are mainly induced by IFN type I, IFITM1 can also be induced by IFN type II (49), indicating that another pathway may have stimulated its expression in our HIC cohort.

Although we have failed to detect an overall up-regulation of host RF in our HIC cohort, it is interesting to note that a few individuals displayed mRNA levels of SAMHD1 and/or SLFN11 well above the normal range (Supplementary Figure S1). These observations suggest that there might not be a unique host RF expression signature common to all HIC, but that different combinations of host RF could be associated with natural control of HIV-1 replication in distinct individuals. Thus, the particular set of increased host RF may vary across different HIC cohorts and this might explain the apparently contrasting findings across studies (5–9,50). Additionally, even though we were able to identify statistically significant differences in expression levels of MCPIP1 and p21 in PBMC between HIC and control groups, these findings warrant validation using larger cohorts.

Our results confirm previous observations that levels of p21 mRNA are positively correlated with $CD4^+$ T cell activation in EC and HIC groups (5) and further support a positive correlation with sCD14, a marker of monocyte activation, in EC. These correlations are fully consistent with the critical role of p21 as a negative regulator of the proliferation of activated/memory T cells (10,13,14) and of macrophage-mediated inflammatory responses (15–17). Although MCPIP1 expression is also essential for suppressing peripheral T cell (51) and macrophage (52,53) activation, we only found a positive correlation of MCPIP1 mRNA with sCD14 in EC. While induction of MCPIP1 mRNA *in vitro* in response to TLR as well as IL-1β stimulation in macrophages is rapid and long-lasting ($\geq$ 24h) (52–54), the corresponding induction upon T cell receptor stimulation in $CD4^+$ T cell is more ephemeral ($<$ 12 hours) (55), which could have hindered the observation of a direct correlation between these two parameters. Notably, increased expression of MCPIP1/p21 associated with T cell and/or monocyte activation seems to be a unique characteristic of HIC/EC, because similar correlations were not observed in our study for other HIV-infected or HIV-negative subjects and previous studies have shown that viremic progressors display reduced levels of p21 even though exhibit high levels of cellular activation and inflammation (21). These results suggest that MCPIP1/p21 overexpression may be a distinctive homoeostatic innate response of HIC to limit the deleterious effects of aberrant chronic immune activation and inflammation driven by HIV-1 infection.

Transcript levels of RF here analyzed were not significantly correlated with T cell activation or sCD14, with the only exception of a negative correlation between APOBEC3G mRNA and sCD14 levels in EC ($r = -0.73$. $P = 0.006$; data not shown). Surprisingly, transcripts levels of APOBEC3G were also negatively correlated with MCPIP1 and p21 mRNA levels in both HIC and EC. One possible explanation for these negative correlations lies in the interaction of APOBEC3G, MCPIP1, and p21 with the product of an important monocyte differentiation gene, the Kruppel-like factor 4 (KLF4). The expression of KLF4 in human macrophages is induced after IFN-γ, LPS, or TNF-α stimulus (56), mediating the proinflammatory signaling and the direct transcriptional regulation of CD14 *in vitro* (57). Interestingly, KLF4 is also able to induce expression of both MCPIP1 (58) and p21 (59,60), whereas APOBEC3G binds to the 3'-UTR of KLF4 mRNA and results in the reduction of its expression (61). Thus, lower levels of APOBEC3G mRNA may be associated with an upregulation of KLF4 that in turn induce higher levels of sCD14 and MCPIP1/p21 mRNA.

Selective upregulation of MCPIP1 and p21 in $CD4^+$ T, macrophages and/or dendritic cells may directly limit HIV-1 replication by 1) reducing the reverse transcription and chromosomal integration of HIV-1 in quiescent cells and thus limiting the size of the latent proviral reservoir (18–20,62–64); 2) restricting HIV-1 LTR transcription (47,48,65,66); and, 3) degrading viral mRNA and miRNA (28,39,40,67). Upregulation of p21 and MCPIP1 may also indirectly limit HIV-1 replication and further prevent $CD4^+$ T cells loss by reducing chronic IFN-I signaling, generalized inflammation and over-activation of the immune system (10,14–17,52,53,68–70), without affecting the activation of antiviral cellular responses. Although the enhanced antiviral and anti-inflammatory state may not be

enough to fully restrict HIV-1 replication (71), it could act in concert with other innate and adaptive immune mechanisms to control HIV replication in HIC.

The enhanced expression of a few select host genes, including p21, was strongly associated with reduced $CD4^+$ T cell-associated HIV RNA during ART, indicating that the p21 may contribute to the control of viral expression and ongoing replication during ART (72). Another study demonstrates that atorvastatin, a lipid-lowering medication, exert a broad spectrum of anti-inflammatory functions and further reduced HIV infection in both rested and activated $CD4^+$ T cells *in vitro* via p21 upregulation (22). Interestingly, atorvastatin was found to up-regulates p21 through a p53 independent pathway, which is consistent with a potential role of MCPIP1 in that antiviral mechanism. These observations suggest that pharmacological manipulation of p21 and MCPIP1 may open novel therapeutic perspectives to prevent HIV-1 replication and to attenuate HIV-associated inflammation and immune activation during ART.

An important limitation of our study is the impossibility of assigning which cell(s) population(s) has increased expression of p21 and MCPIP1 in HIC. The expression profile of many RF and ISGs may be different between $CD4^+$ T cells and monocytes (8), suggesting that the individualization of these cell types might better decipher the mechanisms of host factors regulation in the setting of natural control of HIV-1 infection. Another potential limitation is that only mRNA levels were analyzed. Previous studies showed that p21 mRNA levels mirror p21 protein levels in $CD4^+$ T cells from HIC (21) and that MCPIP1 mRNA levels reflect MCPIP1 protein levels in HCV-infected hepatoma cells (73). Although this evidence indicates a close match between transcripts and protein expression levels, measuring the levels/activity of p21 and MCPIP1 proteins in cells from HIC should also help to elucidate the relevance of these RF for HIV control.

In summary, our data confirm the high levels of p21 mRNA expression and shows for the first-time the concurrent overexpression of MCPIP1 mRNA in HIC. Moreover, we found a positive correlation between p21 and MCPIP1 transcripts in HIC, indicating a possible synergistic effect of both innate host RF on natural suppression of HIV-1 replication *in vivo*. Further studies are needed to better understand the role of p21 and MCPIP1 in the natural control of HIV-1 replication and disease progression in HIC. These findings may also have important implications for the development of new immune-based therapeutic strategies for a functional cure of HIV-1 infection.

5 Figure legends

Figure 1. MCPIP1 and p21 mRNA levels are upregulated in PBMC from HIC. Boxplots represent the interquartile and sample median (central solid black line) of the relative changes (fold-change values relative to the mean of HIV-1-uninfected (NEG) subjects) of MCPIP1 (A) and p21 (B) expression comparing NEG and ART-suppressed subjects (ART) with HIV controllers (HIC). P-values < 0.05 were considered statistically significant.

Figure 2. p21 and MCPIP1 mRNA levels in PBMC from HIC are positively correlated. The p21 and MCPIP1 normalized expression correlations were calculated considering all groups (A), HIV-infected (B), HIC (C), and EC (D). The points' colors indicate the patient group, accordingly to the legend. Correlation coefficients (Spearman's ρ) are shown in the upper right corner of each graph. P-values < 0.05 were considered statistically significant.

Figure 3. p21 transcripts are positively correlated with $CD4^+$ T cell and monocyte activation while MCPIP1 transcripts are positively correlated only with monocyte activation in EC. The correlations were made evaluating the relationship between activated $CD4^+$ T cells (A and B) or sCD14 levels (C and D) with the normalized expression of p21 and MCPIP1 for EC and HIC groups. The points' colors present in each graph indicate the groups present according to the legend. Correlations coefficient (Spearman's ρ) are shown in the upper left corner of each graph.

6 Ethics Statement

This study was carried out in accordance with the recommendations of the ethical committee of Instituto Nacional de Infectologia Evandro Chagas (INI-Fiocruz) that approved the study protocol (CAAE 1717.0.000.009-07). All subjects gave written informed consent in accordance with the Declaration of Helsinki.

7 Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

8 Author Contributions

GB and TMLS conceived and designed the study and supervised the experiments. SSDA conducted experiments and analyzed the data together with MR-A and GB. FH performed the $CD4^+$ T cell and monocyte activation assays. ED collaborated with mRNA gene-expression analysis. BH, BG, and VGV conducted patient recruitment and follow-up. FH, ED and MGM provided intellectual input for results interpretations. SSDA, GB and MR-A wrote the first draft and all authors assisted with the writing and approved the final manuscript.

9 Acknowledgments

The authors thank the patients, who participated in the study, as well as all the technical staff involved in the clinical follow-up of these patients. We also thank Ms Marilia Alves Figueira de Melo for the excellent technical support in RNA quantification and integrity analyses and the Plataforma de PCR em Tempo Real – RJ (RPT09A) – FIOCRUZ and Plataforma de Sequenciamento de Ácidos Nucléicos de Nova Geração – RJ (RPT01J) – FIOCRUZ.

10 Funding

This work was supported by the Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro – FAPERJ (grant number E-26/110.123/2014) and the Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq (Grant Number 401220/2016-8). SSDA was supported by funding from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado do Rio de Janeiro – FAPERJ. ED was financed by a Postdoctoral fellowship from the "Programa Nacional de Pós-Doutorado (PNPD)" by the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior - Brasil (CAPES) - Finance Code 001.

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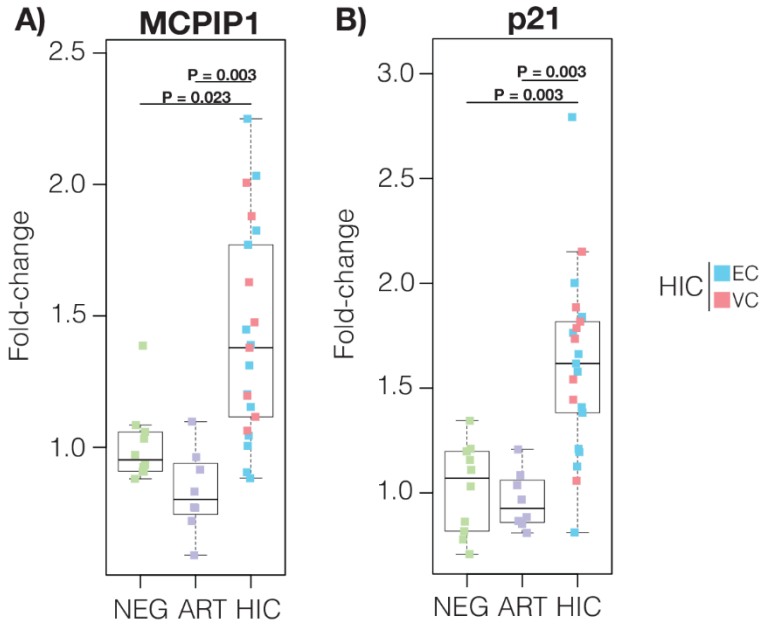
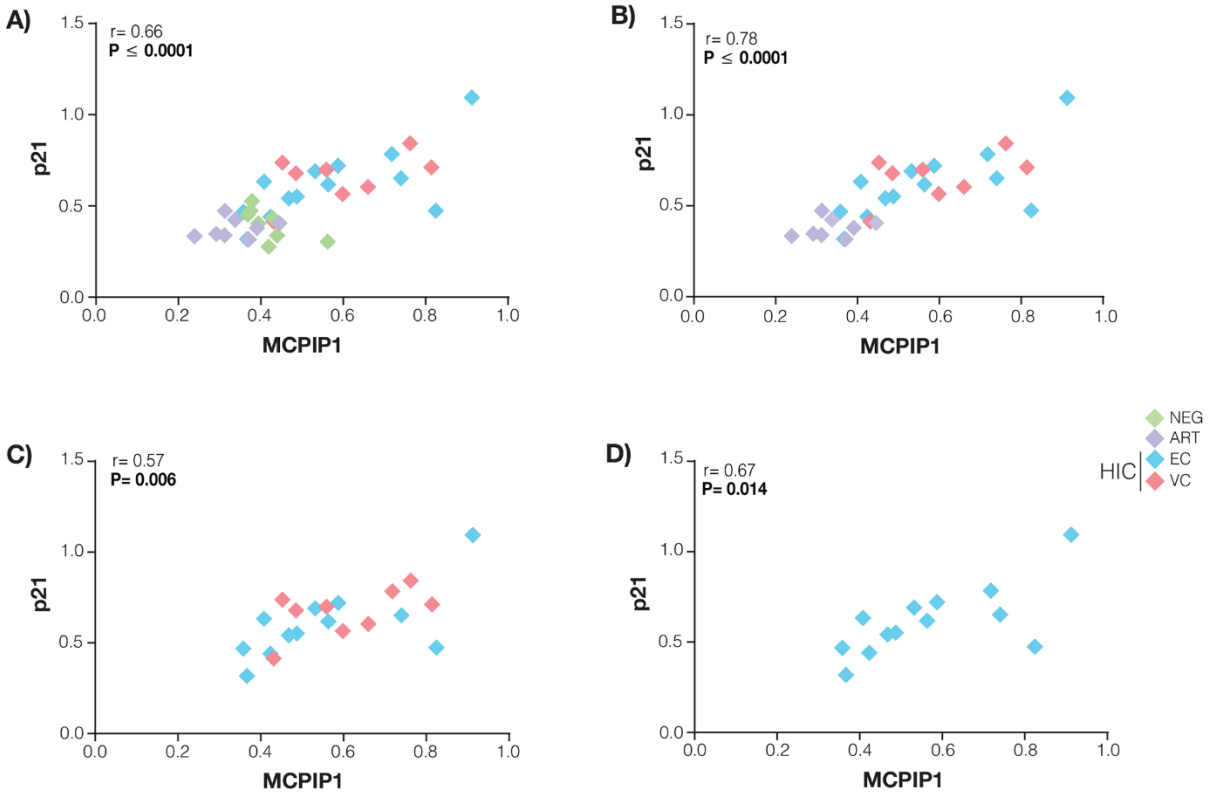
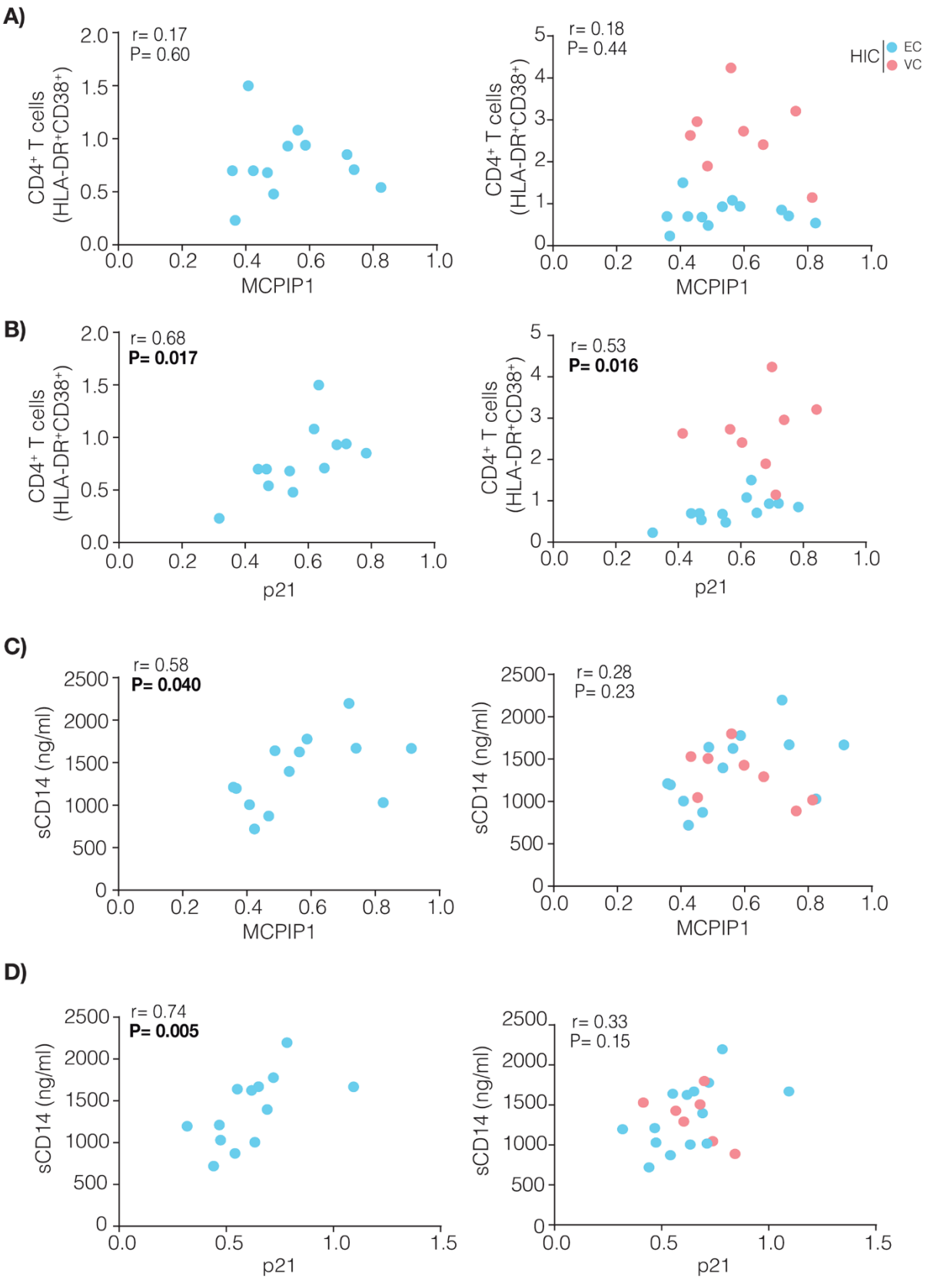
Table 1. Main clinical and epidemiologic characteristics of individuals of this study.

Characteristics	HIC (n = 21)		ART-suppressed (n = 8)	HIV-1 negative (n = 10)
	EC (n = 13)	VC (n = 8)		
Sex, no. (%)				
Female	10 (77)	3 (38)	4 (50)	6 (60)
Male	3 (23)	5 (62)	4 (50)	4 (40)
Age (years)*	45 (39-60)	43.5 (39-47)	47 (38-53)	47 (36-51)
Study point				
Time since HIV-1 diagnosis (years)	9 (5.5-15)	12.5 (7-16)	NA	-
CD4+ T cell (cells/μl)	1027 (834-1255)	664 (563-1228)	889 (678-1097)	1043 (784-1581)
Plasma HIV RNA (copies/ml)	<50	641 (327-915)	<40	-
CD4/CD8 ratio	1.33 (1.24-1.61)	0.91 (0.67-1.23)	1.06 (0.73-1.5)	1.69 (1.62-2.03)

* Age at study point; Interquartile ranges are shown in parenthesis. HIC, HIV controllers; ART, antiretroviral therapy; EC, elite controllers; VC, viremic controllers. NA, not available.


A)
MCPIP1
P = 0.003
P = 0.023
Fold-change
NEG ART HIC
B)
p21
P = 0.003
P = 0.003
Fold-change
NEG ART HIC
HIC
EC
VC


A)
r= 0.66
P ≤ 0.0001
p21
MCPIP1
B)
r= 0.78
P ≤ 0.0001
p21
MCPIP1
C)
r= 0.57
P= 0.006
p21
MCPIP1
D)
r= 0.67
P= 0.014
p21
MCPIP1
NEG
ART
HIC
EC
VC


A)
r= 0.17
P= 0.60
CD4⁺ T cells (HLA-DR⁺CD38⁺)
MCPIP1
r= 0.18
P= 0.44
HIC
EC
VC
B)
r= 0.68
P= 0.017
p21
r= 0.53
P= 0.016
C)
r= 0.58
P= 0.040
sCD14 (ng/ml)
r= 0.28
P= 0.23
D)
r= 0.74
P= 0.005
r= 0.33
P= 0.15

Supplementary Material

Increased expression of MCPIP1 in HIV-1 controllers is correlated with overexpression of p21

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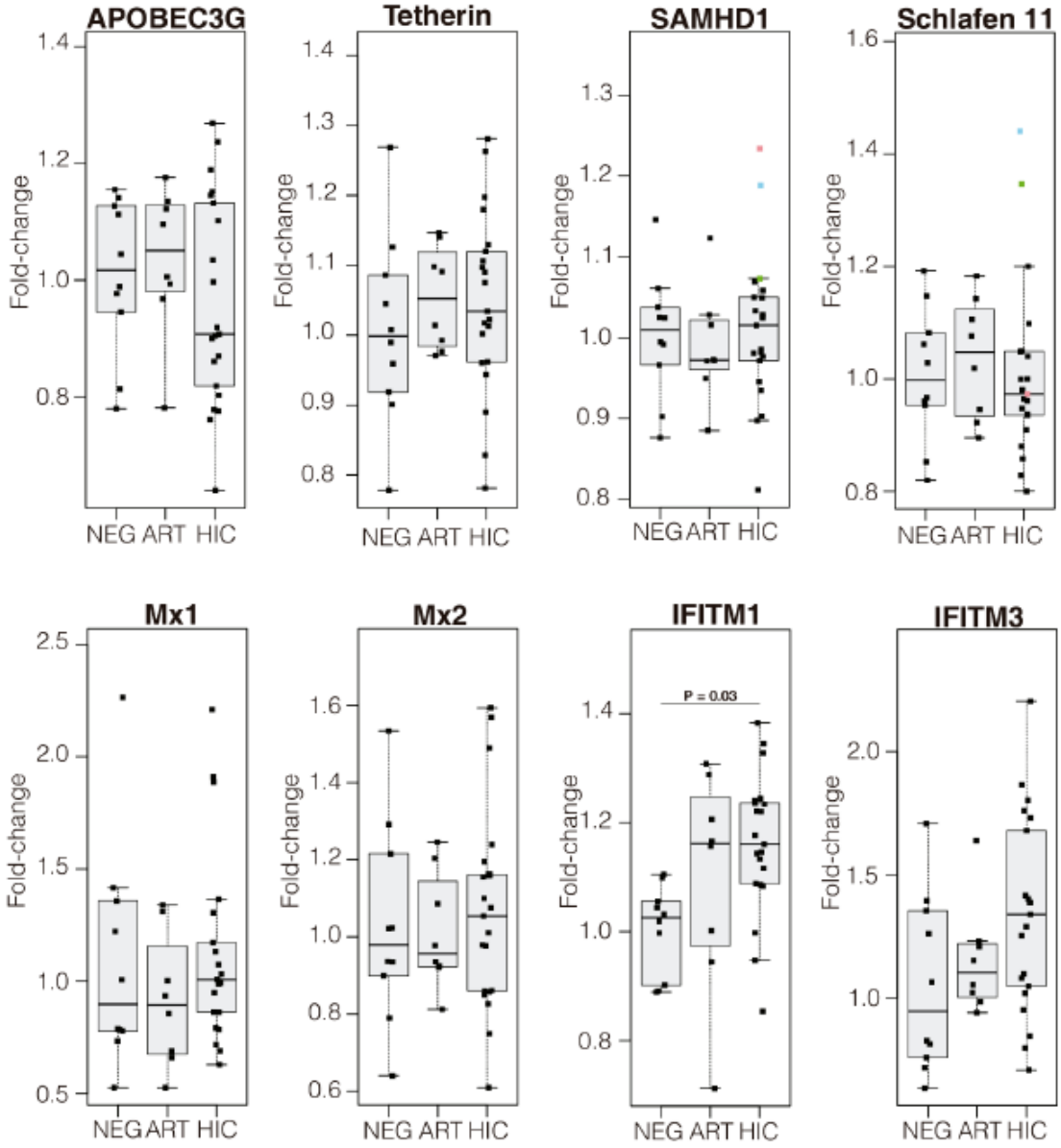
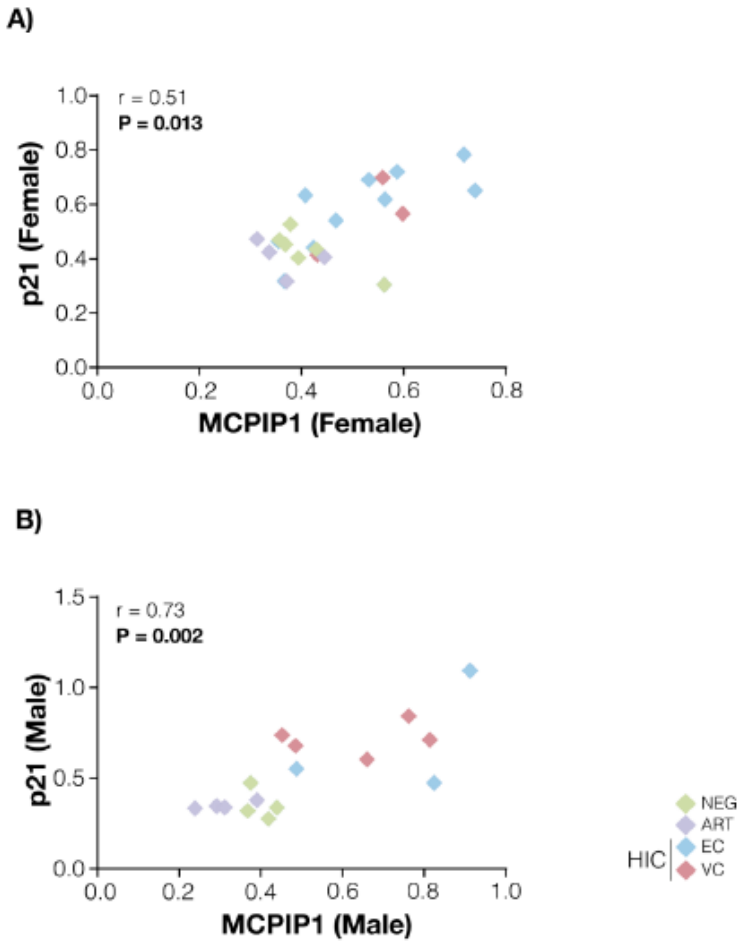
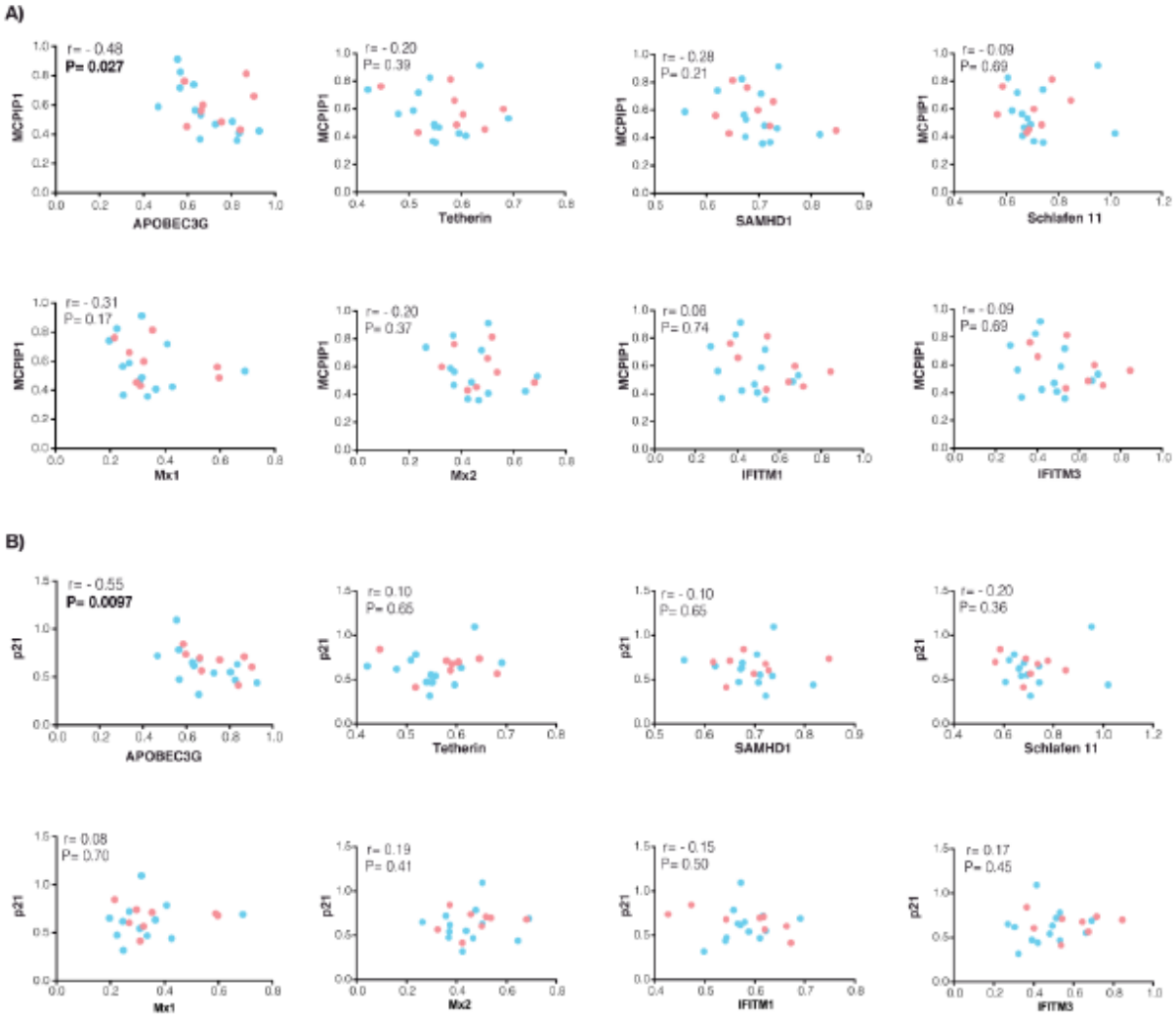
Supplementary Figures



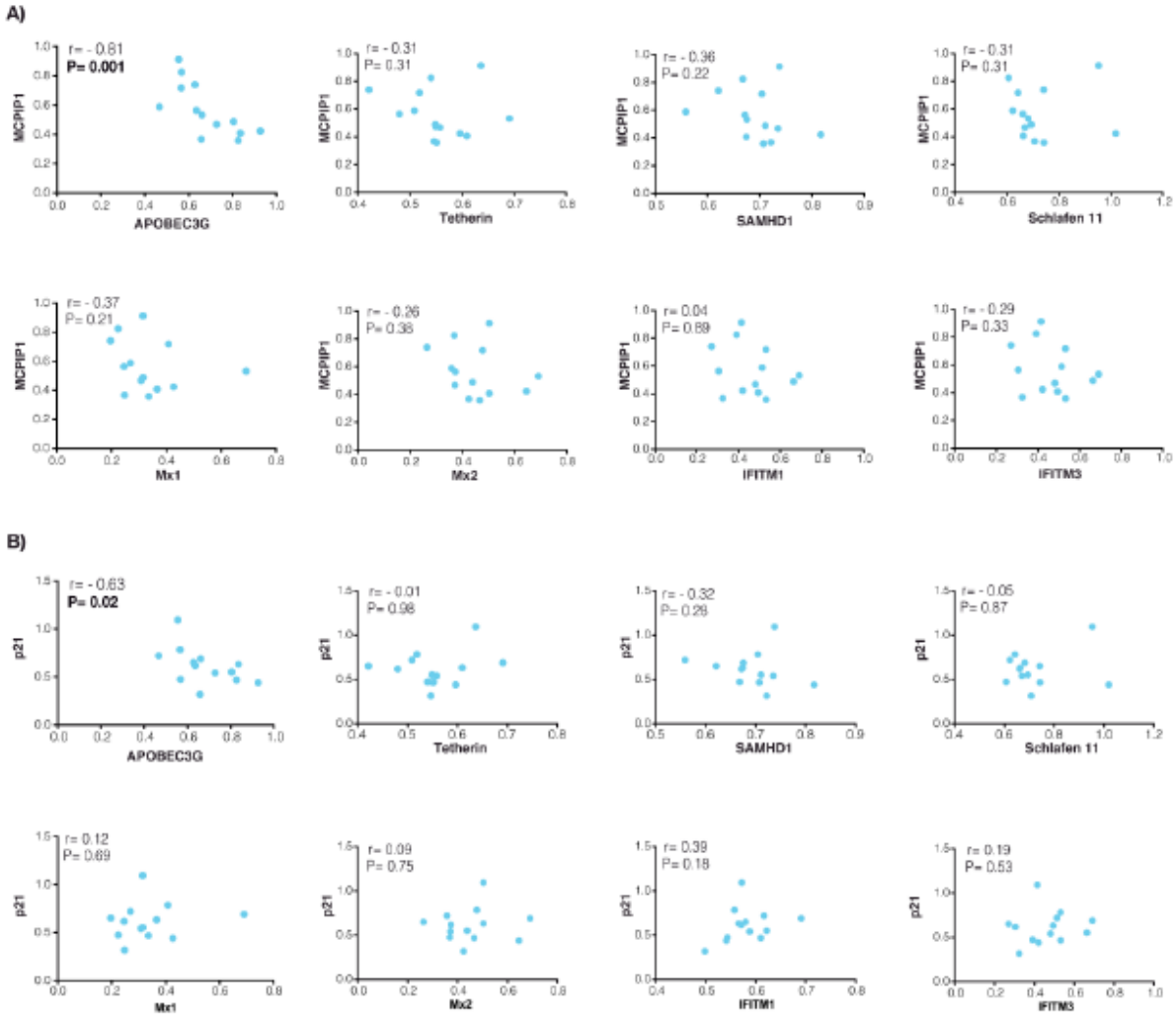
Supplementary Figure S1. Boxplots represent the interquartile and sample median (central solid black line) of the relative changes (fold-change values relative to the mean of HIV-1-uninfected (NEG) subjects) of different restriction factor comparing NEG and ART-suppressed subjects (ART) with HIV controllers (HIC). The RF's names used in the analysis are indicated above each graph. HIC exhibited statistically significant differences (P-values < 0.05) with respect to NEG group only for IFITM1. The colored squares (one per individual) in SAMHD1 and Schlafen 11 from HIC represent individuals with mRNA levels well above the normal range in one or both RF.



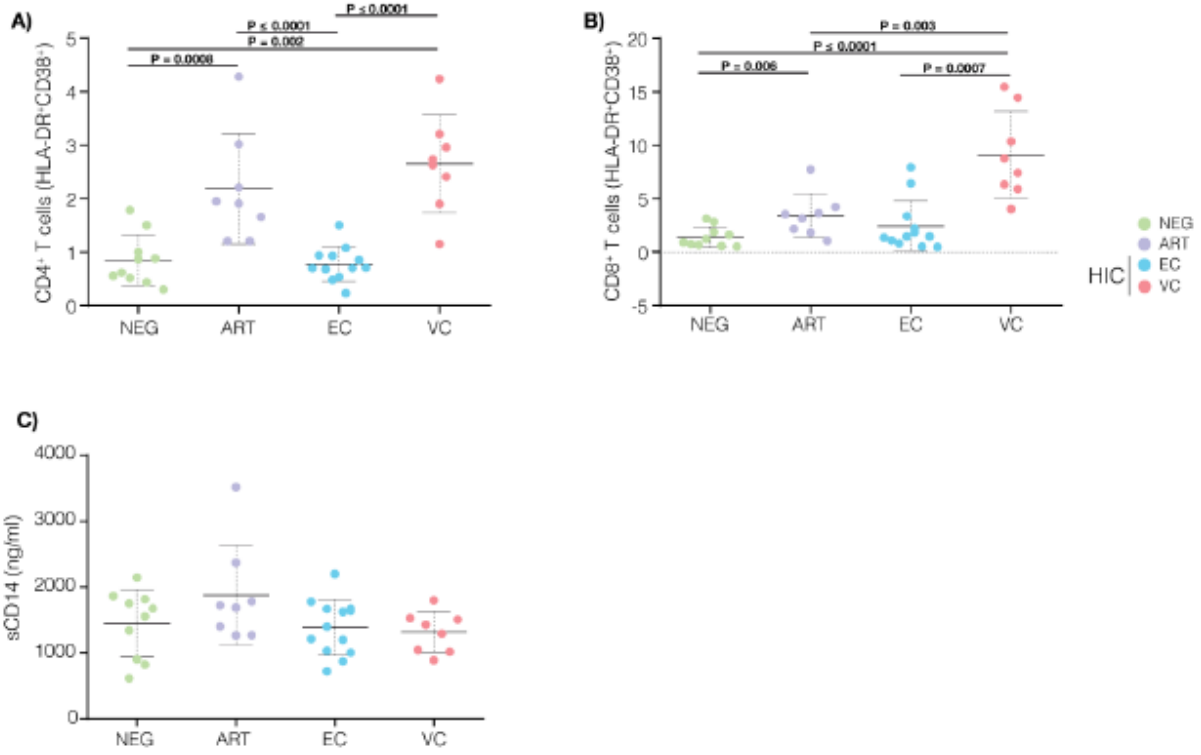
Supplementary Figure S2. MCPIP1 and p21 mRNA levels in PBMC are positively correlated, regardless of sex. The MCPIP1 and p21 normalized expression correlations were calculated considering all groups. The points' colors indicate the patient group, accordingly to the legend. Correlation coefficients (Spearman's ρ) are shown in the upper left corner of each graph. P-values < 0.05 were considered statistically significant.



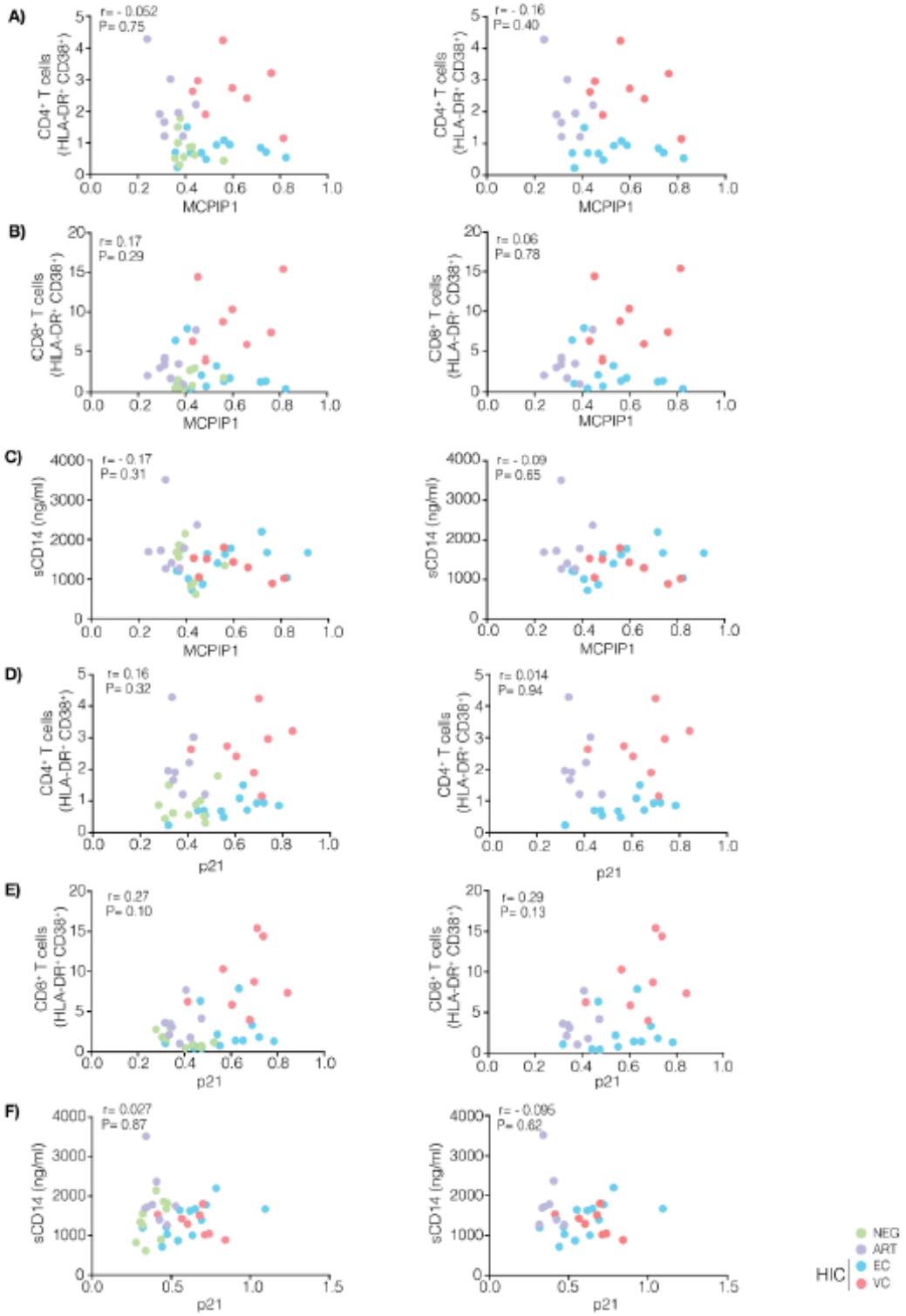
Supplementary Figure S3. Correlations between normalized expression levels of MCPIP1 (A) and p21 (B) with several anti-HIV-1 restriction factors (RF) in HIC. Blue points represent values form elite controllers while the red ones represent values from viremic controllers. The RF's names used in the correlation are indicated on the x-axis and the corresponding correlation coefficient (Spearman's rho) are shown in each graph.



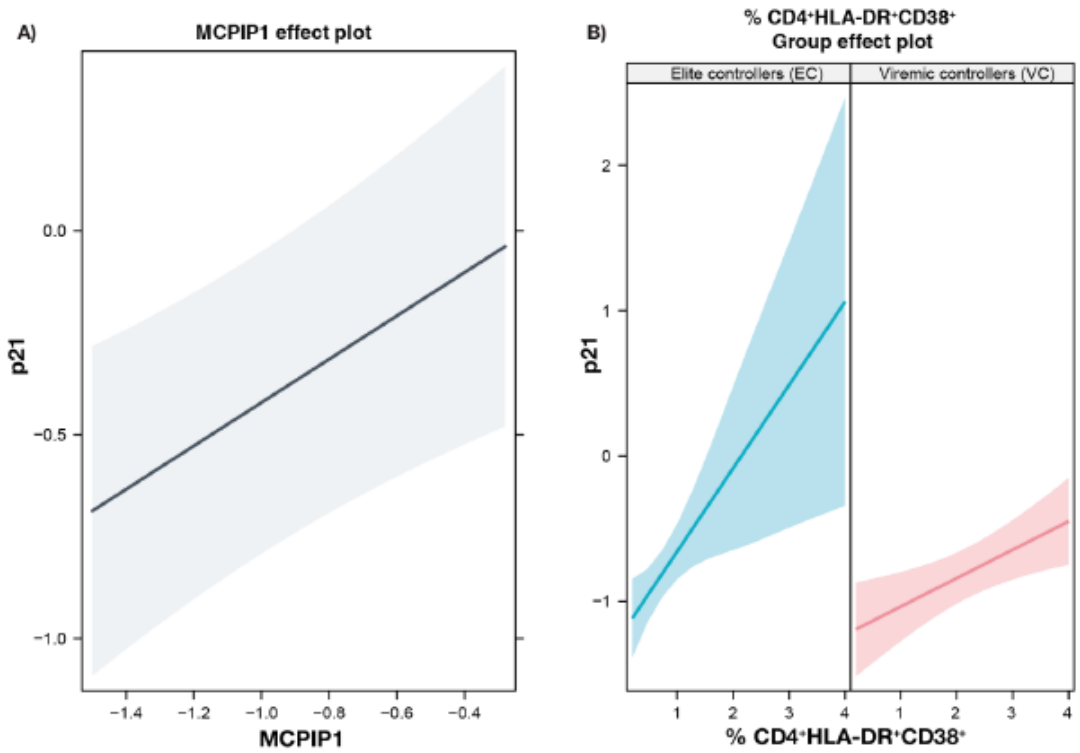
Supplementary Figure S4. Correlations between normalized expression levels of MCPIP1 (A) and p21 (B) with several anti-HIV-1 restriction factors (RF) in EC. Blue points represent values form elite controllers while the red ones represent values from viremic controllers. The RF's names used in the correlation are indicated on the x-axis and the corresponding correlation coefficient (Spearman's rho) are shown in each graph.



Supplementary Figure S5. The mean of activated $CD4^+$ T cells ($HLA\text{-}DR^+CD38^+$) counts (A), activated $CD8^+$ T cells ($HLA\text{-}DR^+CD38^+$) counts (B), and soluble CD14 (sCD14) in plasma (C) were compared for each group. The color of each dot represents the group as indicated in the legend at right. P-values < 0.05 were considered statistically significant.



Supplementary Figure S6. MCPIP1 and p21 are not correlated with $CD4^+$/$CD8^+$ T cell, and monocyte activation in all groups and in HIV-1 infected individuals. Correlations were made evaluating the relationship between activated $CD4^+$, $CD8^+$ T cells or sCD14 levels with the normalized expression of MCPIP1 (A, B, and C, respectively) and p21 (D, E and F, respectively) for different combinations of groups. The points' colors present in each graph indicate the groups present according to the legend. The correlation coefficient (Spearman's rho) are shown in the left corner.



Supplementary Figure S7. MCPIP1 and the frequency of $CD4^+HLA\text{-}DR^+CD38^+$ T cells up-regulate p21 mRNA levels in PBMC from HIC. Effects plots demonstrating (A) the up-regulation of MCPIP1 is positively associated with the increase of the expression of p21 in PBMC from HIC; while in (B) the frequency of $CD4^+HLA\text{-}DR^+CD38^+$ T cells is positively associated with the increase of the expression of p21 in PBMC from both elite (EC) and viremic (VC) controllers, and this increase was of the p21 expression was down-regulated by the increase of $CD4^+HLA\text{-}DR^+CD38^+$ T cells in VC when compared to EC individuals. P-values < 0.05 were considered statistically significant.

Supplementary Table 1. Distribution of individuals in different groups according to sex.

Group	n (%)	Sex (n [%])		*P- value**
		Female	**Male**	
NEG	10 (25.6)	6 (15.4)	4 (10.3)	0.9083
ART	8 (20.5)	4 (10.3)	4 (10.3)	
HIC	21 (53.8)	13 (33.3)	8 (20.5)	
NEG	10 (25.6)	6 (15.4)	4 (10.3)	0.3273
ART	8 (20.5)	4 (10.3)	4 (10.3)	
EC	13 (33.3)	10 (25.6)	3 (7.7)	
VC	8 (20.5)	3 (7.7)	5 (12.8)	

* Statistical analyses were performed using the Fisher exact test.

4 DISCUSSÃO

Após mais de 30 anos desde a identificação do HIV como agente causador da aids, cerca de 80 milhões de pessoas já foram infectadas e quase metade dessas morreram de doenças relacionadas à aids no mundo. Desde então, importantes avanços têm sido feitos na prevenção de novas infecções e no controle da replicação do vírus, sobretudo através da implementação da ART. Apesar do tratamento com a ART ser eficaz na supressão da replicação do HIV, um dos principais desafios para a erradicação da doença é a manutenção do vírus na sua forma latente (provírus), principalmente em células T $CD4^+$ de memória tanto do sangue periférico quanto em linfonodos (Chun et al. 1998; Finzi et al. 1999).

A formação do reservatório proviral acontece desde a fase aguda da infecção pelo HIV-1 (Chun et al. 1998). Durante essa fase, a resposta imune é ativada em decorrência do aumento da viremia plasmática, com a proliferação extensiva de células T e B específicas para o HIV. A ativação da resposta imune promove a morte de grande parte das células T $CD4^+$ específicas para o HIV, enquanto outras se tornam células T $CD4^+$ de memória, que facilitam o estabelecimento do reservatório latente viral. As células T $CD4^+$ de memória apresentam um estado metabólico reduzido, com menor presença de fatores de transcrição (NF-kB, NFAT e SP1) disponíveis, fazendo com que o HIV permaneça integrado no genoma do hospedeiro, transcricionalmente silencioso e mantendo-se inacessível tanto para a resposta imune do hospedeiro quanto para a ART (Pankrac et al. 2017). As células T $CD4^+$ de memória também apresentam uma meia-vida prolongada, podendo nesse período serem reativadas e em decorrência disso, reestabelecer a replicação de vírus competentes.

Na maioria dos indivíduos infectados pelo HIV-1 (PT), o principal mecanismo de manutenção do reservatório proviral durante a fase crônica da doença é a replicação viral contínua que promove a renovação das quasispécies provirais, que se diversificam aproximadamente 1% ao ano no gene *env* (Shankarappa et al. 1999). Diversos estudos já demonstraram que indivíduos HIVc avaliados durante a fase crônica, apresentam um reservatório proviral reduzido, extremamente homogêneo (< 2% diversidade *env*) e sem divergência (acúmulo de substituições nucleotídicas em relação ao ancestral) mensurável ao longo do tempo (Wang et al. 2003b; Bello et al. 2005, 2007b, Bailey et al. 2006b, 2006a; Sandonís et al. 2009; O'Connell et al. 2010; Salgado et al. 2010; Boritz et al. 2016). Estes estudos apontam que o reservatório

proviral nos HIVc é composto principalmente por variantes ancestrais, provavelmente integradas no genoma celular durante a infecção primária e mantidas pela expansão clonal homeostática de células T $CD4^+$ de memória, latentemente infectadas. Entretanto, muitos desses estudos observaram sequências virais mais divergentes no plasma de indivíduos HIVc, sugerindo que mesmo em indivíduos com carga viral indetectável, o HIV-1 é capaz de replicar e evoluir, embora em uma escala significativamente menor que a observada em PT (Bailey et al. 2006b; Miura et al. 2009a; Mens et al. 2010; O'Connell et al. 2010, 2011; Salgado et al. 2010; Boritz et al. 2016).

Para tentar comprovar estes dois mecanismos de manutenção do reservatório proviral em HIVc, avaliamos a diversidade das populações provirais em três HIVc (dois indivíduos EC e um VC) ao longo de um extenso período de acompanhamento (≥ 12 anos) (**ARTIGO 1**). Os resultados desta análise longitudinal revelaram que as populações provirais no indivíduo EC, com carga viral indetectável ao longo do acompanhamento (PEC52), foram extremamente homogêneas e sem evidências de evolução. Isto é consistente com a manutenção do reservatório proviral através da expansão clonal de células T $CD4^+$, provavelmente infectadas pelo HIV-1 durante a fase aguda da infecção, quando normalmente se observa uma população viral altamente homogênea (Sagar et al. 2004, 2006; Gottlieb et al. 2008; Abrahams et al. 2009; Kearney et al. 2009; Bar et al. 2010; Li et al. 2010; Novitsky et al. 2011). Em contraste, o indivíduo EC, que ao longo do acompanhamento apresentou episódios esporádicos de viremia de baixo nível (EEC42), exibiu uma população proviral mais diversa e com sinais de evolução, similar ao indivíduo VC05 que apresentou cargas virais detectáveis de baixo nível na maior parte do acompanhamento. Nesses dois indivíduos, foi observada uma renovação parcial/completa das populações provirais ao longo do tempo, provavelmente sustentada por uma replicação viral de baixo nível, consistente com a detecção de sequências plasmáticas divergentes em alguns HIVc (Bailey et al. 2006b; Miura et al. 2009a; Mens et al. 2010; O'Connell et al. 2010, 2011; Salgado et al. 2010; Boritz et al. 2016).

A análise transversal de 23 HIVc, demonstrou a presença de um reservatório proviral homogêneo (<2%) em cinco EC (2 PEC e 3 EEC) e de um reservatório proviral diverso (>2%) em todos os VC e oito EC (4 PEC e 4 EEC) (**ARTIGO 1**). Este resultado sugere que na maioria dos HIVc o reservatório proviral seria mantido pela contínua ressemeadura das populações provirais ao longo do tempo. Não podemos descartar, no entanto, que alguns indivíduos EC tenham sido infectados por múltiplas variantes

virais (Bailey et al. 2006b; Miura et al. 2009b, 2009a; Mens et al. 2010; O'Connell et al. 2010, 2011; Salgado et al. 2010; Boritz et al. 2016) e que a subsequente expansão homeostática das células T CD4$^+$, latentemente infectadas, promove a manutenção de um reservatório proviral diverso. O acompanhamento longitudinal da diversidade proviral nesses indivíduos permitirá comprovar se a diversidade observada é resultado da expansão clonal de células T CD4$^+$ infectadas por uma população diversa ou de uma lenta, porém continua, ressemeadura das populações provirais.

Foi observado que EC perdem o controle virológico e/ou apresentam um declínio nas contagens de linfócitos T CD4$^+$ após alguns anos de acompanhamento, mas as causas associadas a essa progressão virológica e/ou imunológica ainda não foram esclarecidas (Andrade et al. 2008; Okulicz et al. 2009; Sajadi et al. 2009; Boufassa et al. 2011; Yang et al. 2012; Olson et al. 2014; Leon et al. 2016; Chereau et al. 2017). Alguns marcadores como a ativação de células T CD8$^+$, o nível plasmático de IP-10 e os níveis de RNA e DNA viral estariam associados a um risco de progressão virológica e/ou imunológica (Noel et al. 2014, 2015). Recentemente, um estudo de Pernas e colaboradores (2017) (Pernas et al. 2017) apontou que a diversidade proviral elevada nos EC reflete em uma contínua ressemeadura das populações provirais, e seria um importante marcador prognóstico da perda do controle virológico nessa população. Os autores desse estudo observaram que EC que perderam o controle da replicação viral apresentaram um ano antes da perda do controle uma maior diversidade proviral no gene *env* (>2%), além de níveis aumentados de citocinas pró-inflamatórias (RANTES, CTACK e PDG-F-AA), quando comparados a EC que mantiveram um controle persistente da replicação viral. Essa observação é de grande relevância na prática clínica, porque permitiria distinguir aqueles EC que eventualmente podem perder o status de controle e que se beneficiariam pelo uso precoce da ART.

Os resultados da análise transversal mostraram a existência de dois subgrupos de EC na nossa coorte: um subgrupo que apresentou uma baixa diversidade proviral em *env* (EC_{LD}, <2%) e outro subgrupo que apresentou uma diversidade proviral mais alta (EC_{HD}, <2%). Para entender a relevância da diversidade genética proviral no risco de progressão imunológica e virológica na nossa coorte de EC, avaliamos a carga viral e proviral, a dinâmica das células T CD4$^+$, e os níveis de ativação (T CD8$^+$ HLA-DR$^+$ CD38$^+$) e inflamação (IP-10, IL-18 RANTES, CTACK e PDGF-AA) sistêmica nos subgrupos EC_{LD} e EC_{HD} (**ARTIGO 3**). Os resultados obtidos não mostraram diferenças significativas na carga viral/proviral, contagens de linfócitos T CD4$^+$, frequência de

células T CD8$^+$ ativadas, ou níveis de biomarcadores pró-inflamatórios entre EC_{LD} e EC_{HD}. Nós também não observamos nenhuma associação entre esses marcadores com a diversidade proviral nos EC. A maioria dos EC_{LD} e EC_{HD} foi capaz de manter o controle virológico e imunológico por longos períodos (2-5 anos) após a determinação da diversidade proviral, contrapondo a hipótese de Pernas e colaboradores (2017) (Pernas et al. 2017). Estes resultados reforçam a hipótese de que a diversidade proviral em alguns EC_{HD} pode refletir a manutenção de um reservatório formado por múltiplas variantes virais desde a primoinfecção, e que por tanto, a diversidade proviral nos EC não seria um marcador prognostico inequívoco de replicação residual contínua, nem de perda do controle virológico nessa população.

A caracterização do reservatório proviral em HIVc apontou a presença de dois indivíduos (EEC09 e um VC32) infectados por pelo menos duas variantes do HIV-1 de distintos subtipos (uma do subtipo B e outra do subtipo F1), evidenciando a presença de duplas infecções (DI) na nossa coorte. Eventos de DI em HIVc foram associados a diferentes desfechos clínicos. Estudos transversais, mostraram ausência de progressão clínica em HIVc duplamente infectados por vírus do subtipo B, indicando a habilidade de alguns HIVc de controlar a replicação de mais de uma variante viral (Casado et al. 2007; Lamine et al. 2007; Pernas et al. 2013). Entretanto, estudos longitudinais mostram um cenário mais complexo em que a superinfecção (SI) em HIVc está associada ao alto risco de progressão virológica e imunológica (Rachinger et al. 2008; Braibant et al. 2010; Clerc et al. 2010), similar ao observado em indivíduos não-controladores. Estes resultados questionam a capacidade dos HIVc de manterem o controle natural após a SI, particularmente com uma variante do HIV-1 de um subtipo diferente ao da variante inicial (SI intersubtipo).

Nós avaliamos a possibilidade de SI e de progressão imunológica e/ou virológica nos dois HIVc identificados com DI (**ARTIGO 2**). As análises longitudinais do reservatório viral confirmaram que os dois indivíduos foram inicialmente infectados por variantes do HIV-1 do subtipo B e superinfectados por variantes do subtipo F1 durante o acompanhamento. Embora foi observado um aumento da carga viral plasmática após a SI em ambos indivíduos, os dois HIVc foram capazes de manter um controle virológico (< 2000 cópias/mL) dos dois vírus e não demonstraram sinais de progressão imunológica por pelo menos dois anos após a SI. Além disso, nós não encontramos aumento mensurável da diversidade/divergência proviral ao longo do tempo de acompanhamento, demonstrando a capacidade desses indivíduos de controlarem a evolução tanto da população viral inicial quanto da superinfectante. Em

conjunto, essas análises demonstraram pela primeira vez a capacidade de alguns HIVc de controlar infecções sucessivas de diferentes subtipos do HIV-1, e suportam claramente a importância de fatores do hospedeiro no controle da replicação viral nesses indivíduos. Identificar os mecanismos do hospedeiro associados ao controle natural da replicação do HIV-1 após a infecção primária e/ou após a SI poder oferecer pistas importantes, para o desenvolvimento de vacinas terapêuticas afim de conseguir a remissão (cura funcional) do HIV.

Embora a infecção pelo HIV-1 seja uma doença crônica, a primeira resposta gerada na tentativa de controle da infecção pelo HIV-1, principalmente durante a fase aguda da infecção, é através de uma resposta rápida e de curta duração fornecida pelo sistema imune inato. Durante essa fase da infecção pelo HIV-1, se observa um aumento da expressão de IFN-α e outras citocinas pro-inflamatórias que contribuem para o controle da replicação viral (Doyle et al. 2015; Colomer-Lluch et al. 2018). Essas citocinas atuam recrutando células dendríticas, macrófagos e natural killer para o sítio de entrada, onde orquestram a ativação das células da resposta imune adaptativa, como linfócitos T $CD4^+$, T $CD8^+$ e células B. Por outra parte, a resposta imune inata mediada por IFN-α estimula a expressão de diversos genes responsáveis pela produção de proteínas celulares com potente atividade antirretroviral comprovada em experimentos *in vitro*, que são denominados fatores de restrição (FR) (Colomer-Lluch et al. 2018).

Estudos prévios *in vivo* mostraram a expressão elevada de RNAm de alguns FR induzidos ou não por IFNs em HIVc/EC em comparação com outros indivíduos infectados pelo HIV (em supressão da viremia pela ART e/ou indivíduos não-controladores) e/ou não-infectados pelo HIV-1. O aumento na expressão de FR induzidos por IFN (principalmente IFN-I) como SFLN11 (Abdel-Mohsen et al. 2013), IFITM1 (Canoui et al. 2017), Mx1 (Krishnan et al. 2014), SAMHD1 (Riveira-Muñoz et al. 2014) e de outros fatores do hospedeiro não induzidos por IFN como p21 (Chen et al. 2011; Madlala et al. 2018; Moosa et al. 2018) em linfócitos T $CD4^+$ e/ou PBMC desses indivíduos, sugere que os FR podem apresentar um papel importante no controle natural da infecção pelo HIV-1. Dessa forma, nós propomos investigar o papel de FR na manutenção do controle da replicação do HIV-1 em 21 HIVc da nossa coorte (**ARTIGO 4**). Nós selecionamos os FR previamente observados aumentados em HIVc (SFLN11, IFITMI, Mx1, SAMHD1 e p21), como também outros FR (APOBEC3G, BST2, Mx2, IFITM3 e MCPIP1) descritos previamente apresentarem ação anti-HIV *in vitro* e *in vivo* (Liu et al. 2013; Doyle et al. 2015; Colomer-Lluch et al. 2018). Nossas

análises revelaram níveis elevados de expressão de p21 e MCPIP1 em PBMC de HIVc, quando comparados com indivíduos infectados pelo HIV-1 em supressão pela ART e indivíduos não-infectados pelo HIV-1. Além da expressão aumentada desses dois FR, nós observamos que p21 e MCPIP1 correlacionam-se positivamente independente da carga viral, uso de ART e status HIV.

A capacidade de MCPIP1 em restringir a infecção pelo HIV-1 *in vitro* foi observada no estudo desenvolvido por Liu e colaboradores (2013) (Liu et al. 2013) em células T $CD4^+$ em repouso, em que os níveis de RNAm virais foram reduzidos através da atividade RNase desenvolvida por MCPIP1. Nosso estudo demonstra pela primeira vez o protagonismo de MCPIP1 no contexto do controle natural da infecção pelo HIV-1 *in vivo*. Nós também descrevemos pela primeira vez uma correlação positiva entre os níveis de expressão de p21 e MCPIP1 no contexto da infecção pelo HIV-1. Essa associação foi recentemente observada em um estudo com linhagem celular (Caki-1) de carcinoma de células renais, nesse trabalho níveis aumentados de MCPIP1 foram capazes de inibir a proliferação da linhagem Caki-1, através do aumento da expressão de p21 (Lichawska-Cieslar et al. 2018). A expressão aumentada de p21 em Caki-1 foi relacionada com a expressão reduzida dos transcritos da proteína ligante ao dano de DNA (DDB1, *do inglês, DNA damage-binding protein 1*), responsável por regular a degradação de p21, e com o aumento da expressão dos transcritos do fator de replicação do DNA (CDT1, *do inglês, chromatin licensing and DNA replication factor 1*) que ativa p21. Além disso, MCPIP1 também atua como um supressor amplo das vias de biogênese de miRNAs (Suzuki et al. 2011) e é capaz de degradar pre-miRNAs da família Let-7 (Suzuki et al. 2015). Na infecção pelo HIV-1, um membro dessa família, o miRNA let-7c, encontra-se em níveis aumentados e é responsável por regular negativamente proteínas celulares, como p21 (Farberov et al. 2015). Dessa forma, o aumento da expressão de MCPIP1 poderia induzir uma degradação do miRNA let-7c que por sua vez degrada p21, favorecendo a manutenção de p21 na célula.

Em contraste com os estudos que observaram níveis aumentados de transcritos de FR induzidos por IFN em HIVc, a maioria dos FR aqui estudados não apresentaram níveis aumentados de RNAm nesse grupo (Abdel-Mohsen et al. 2013; Krishnan et al. 2014; Riveira-Muñoz et al. 2014), exceto IFITM1, como observado por Canoui e colaboradores (Canoui et al. 2017). O fato de IFITM1 também ser induzido por IFN-II (Diamond and Farzan 2013), indica que uma outra via pode ser responsável por estimular o aumento da expressão de IFITM1 e não a via induzida por IFN-I. Além

disso, uma vez que a expressão de muitos desses genes induzidos por IFN (ISGs), sobretudo IFN-I, estão associados com o aumento da replicação viral, a ausência de níveis mais expressivos desses ISGs nos indivíduos da nossa coorte, pode ser relacionada a uma insuficiência da viremia residual ou de baixo nível em estimular a expressão desses ISGs (Rotger et al. 2010; Murira and Lamarre 2016).

Por outro lado, MCPIP1 tem um papel fundamental na modulação da inflamação celular, regulando negativamente a atividade de algumas citocinas pró-inflamatórias através da degradação dos seus RNAm (Matsushita et al. 2009; Liang et al. 2011; Fu and Blackshear 2017) e da regulação negativa da atividade do fator de transcrição NF-κB (Skalniak et al. 2009; Liang et al. 2010). A cascata de NF-κB é uma das vias responsáveis por induzir a expressão de citocinas pró-inflamatórias, IFN-I e ISGs (Chan and Greene 2012) e o fato de transcritos de MCPIP1 terem sido observados em níveis aumentados nos indivíduos do nosso estudo, ressalta uma outra possibilidade para a limitação da expressão de ISGs na nossa coorte.

Nós também observamos que os níveis aumentados de transcritos de p21 em HIVc e em EC foram positivamente correlacionada com a ativação de células T $CD4^+$, como observado em um estudo anterior (Chen et al. 2011). Uma correlação positiva também foi observada entre os níveis aumentados de RNAm de p21 e MCPIP1 e a ativação de monócitos, medida através dos níveis plasmáticos de sCD14 em EC. A análise multivariada reforçou a existência de interação entre os transcritos de p21, MCPIP1.e a ativação de células T $CD4^+$ em HIVc. Os altos níveis de transcritos de p21/MCPIP1 associados com a ativação dessas duas populações celulares pode representar um mecanismo homeostático para limitar a hiperproliferação dessas células e a inflamação crônica normalmente induzida pela infecção do HIV-1 (Balomenos et al. 2000).

A regulação positiva dos transcritos de p21 e MCPIP1 em células T $CD4^+$, macrófagos e/ou células dendríticas pode limitar diretamente a replicação do HIV-1 através de 3 mecanismos distintos: 1) reduzindo a transcrição reversa e a integração cromossômica do HIV-1 em células quiescentes contribuindo diretamente com um reservatório proviral latente mais limitado (Zhang et al. 2007; Bergamaschi et al. 2009; Allouch et al. 2013; Leng et al. 2014; Pauls et al. 2014; Valle-Casuso et al. 2017); 2) restringindo a transcrição do HIV-1 (Wang et al. 2001; Skalniak et al. 2009; Liang et al. 2010; Kumari et al. 2014); e 3) degradando o RNAm e o miRNA viral (Suzuki et al. 2011; Lin et al. 2013; Liu et al. 2013; Happel et al. 2016).

Essa regulação positiva entre os transcritos de p21 e MCPIP1 podem também atuar indiretamente restringindo a replicação viral e na prevenção na depleção de células T $CD4^+$ ao reduzir a sinalização crônica de IFN-I, inflamação generalizada e ativação exacerbada do sistema imunológico (Santiago-Raber et al. 2001; Khanna et al. 2005; Arias et al. 2007; Lloberas and Celada 2009; Matsushita et al. 2009; Scatizzi et al. 2009; Trakala et al. 2009; Li et al. 2017; Uehata and Takeuchi 2017), sem afetar a ativação de respostas celulares antivirais. Embora o aumento do estado antiviral e anti-inflamatório possa não ser suficiente para restringir totalmente a replicação do HIV-1 (de Pablo et al. 2016), ele pode atuar em conjunto com outros mecanismos dos sistemas imunes inato e adaptativo, controlando a replicação do HIV em HIVc.

Nesse estudo, através do acompanhamento longitudinal e do estudo aprofundado dos padrões evolutivos intra-hospedeiro, fatores da imunidade inata e ativação sistêmica, observamos que essas diferentes facetas virológicas e imunológicas podem estar envolvidas no perfil de controle observado nos indivíduos dessa coorte. Elucidar os principais mecanismos que promovem o controle natural de longa duração da replicação do HIV e da progressão para aids pode fornecer novos caminhos para alcançar um estado de remissão a longo prazo ou de novas estratégias de cura funcional em pacientes infectados pelo HIV-1.

5 CONCLUSÕES

- Diferentes padrões de diversidade proviral intra-hospedeiro podem ser detectados no cenário de supressão natural da infecção pelo HIV-1, sugerindo que a proliferação homeostática de células T $CD4^+$ de memória infectadas e/ou a continua ressemeadura do reservatório proviral são mecanismos importantes na persistência do HIV nos EC.
- A diversidade genética do reservatório proviral não pode ser considerada um marcador indireto da replicação viral residual, ativação imune ou inflamação, nem um biomarcador preditivo do risco de progressão virológica e/ou imunológica nos indivíduos EC.
- Apesar de não serem capazes de prevenir a superinfecção pelo HIV-1, fatores do hospedeiro em alguns HIVc podem controlar a replicação viral e prevenir a progressão da infecção por vários anos após o evento de superinfecção, mesmo que por um subtipo viral distinto ao da primoinfecção.
- Células mononucleares do sangue periférico de HIVc apresentam uma expressão aumentada dos FR MCPIP1 e p21. Estes FR poderiam atuar de forma sinérgica limitando a replicação do HIV-1 nos HIVc, e se tornar alvos terapêuticos promissores para controlar tanto a replicação viral quanto a hiperativação imune e a inflamação induzida pelo HIV-1.

6 PERSPECTIVAS

- Verificar o papel dos FR através de análises longitudinais no âmbito da superinfecção pelo HIV-1, para entender o papel desse mecanismo imune inato na manutenção do controle observada nessa coorte de HIVc.
- Avaliar o perfil de expressão gênica global através da análise do transcriptoma de linfócitos T $CD4^+$ no intuito de identificar potenciais biomarcadores associados ao fenótipo de controle e também para confirmar a associação da expressão de MCPIP1 e p21 e tentar elucidar potenciais vias de interação.

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8 APÊNDICES

8.1 APÊNDICE 1

Tracing the origin of a singular HIV-1 CRF45_cpx clade identified in Brazil

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Jornal: Infection, Genetics and Evolution, 2016.

Infection, Genetics and Evolution 46 (2016) 223–232

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Tracing the origin of a singular HIV-1 CRF45_cpx clade identified in Brazil

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ARTICLE INFO

Article history:
Received 20 February 2016
Received in revised form 26 May 2016
Accepted 30 May 2016
Available online 31 May 2016

Keywords:
HIV-1
CRF45_cpx
NFLG
Phylogeny
Brazil

ABSTRACT

The HIV-1 epidemiology has changed over the past decade toward a marked increase in the circulation of strains previously restricted to local epidemics. Recent molecular epidemiological surveys identified some HIV-1 strains of probable African origin circulating in Brazil, including the Circulating Recombinant Form (CRF) 45_cpx, a complex A1/K/U recombinant that circulates in Central Africa. Here, we characterize partial genomic sequences and reconstruct the evolutionary history of HIV-1 CRF45_cpx-related recombinant samples identified in independent studies carried out with HIV + individuals in Brazil. The sequences were obtained by overlapping PCR amplifications followed by direct sequencing. Recombination profiles were determined by phylogenetic and bootscaning analyses. The evolutionary history was estimated by a Bayesian coalescent-based method using datasets representing the *gag*, *pol* and *env* gene fragments. Six of the 10 samples isolated in Rio de Janeiro showed a CRF45_cpx-like pattern throughout the sequenced genome. The remaining were classified as second-generation recombinants, showing the mosaic patterns: CRF45_cpx/B/D/F1/U, CRF45_cpx/B/F1/U, CRF45_cpx/B/U and CRF45_cpx/F1. All Brazilian CRF45_cpx sequences, except one, formed a monophyletic clade (CRF45-BR), which seems to be the result of a single introduction event that has spread to the Rio de Janeiro, São Paulo and Minas Gerais states and is related to sequences from Argentina, Italy and Belgium. The Bayesian analyses pointed out quite consistent onset dates for CRF45-BR clade (~1984: 1976–1996) in the three gene datasets. These results indicate that the CRF45-BR clade has been circulating in the Southeastern Brazilian region for about 30 years, although its presence was not detected until recently due to its very low prevalence. This reinforces the relevance of large-scale molecular surveillance data to identify the emergence of new HIV variants and their impact on local epidemics.

1. Introduction

The human immunodeficiency virus type 1 (HIV-1) originated from multiple zoonotic transmission events of simian immunodeficiency virus from non-human primates to humans in Western-Central Africa in the first decades of the twentieth century (Hemelaar, 2012). One of these transmission events originated the M group of HIV-1, that shortly thereafter diversified into genetic subtypes (named A–D, F–H and J–K) that disseminated worldwide, and are currently the responsible for the HIV-1 pandemic (Worobey et al., 2008). The distinct chances of spread and establishment of lineages originated at the HIV-1 epicenter to other geographical regions resulted in the current global HIV-1 subtype distribution (Faria et al., 2014). Even though this distribution has been broadly stable over the 2000–2007 period, there were dynamic changes in some regions, possibly due to several factors including population growth, increasing migrations and founder effects (Hemelaar et al., 2011).

In some regions, the epidemiology of HIV-1 has changed over the past decade toward a marked increase in the circulation of non-B strains, such as Western Europe, for example, that although having a characteristic epidemic marked by the predominance of HIV-1 subtype B, increasing in the prevalence of other subtypes has been found, such as the subtype G and CRF02_AG in Spain (Lospitao et al., 2005), the subtype D and CRF01_AE in France (Brand et al., 2012) and subtype F1 in Italy (Lai et al., 2010). This pattern is also observed in Brazil, where, despite the HIV-1 epidemic being dominated by subtypes B, C, F1 and recombinants between them, HIV-1 clades A, D and CRF02_AG were sporadically found (Alencar et al., 2013; Caride et al., 2000; Delatorre et al., 2012; Eyer-Silva and Morgado, 2007; Ferreira et al., 2013; Inocencio et al., 2009; Machado et al., 2009; Morgado et al., 1998; Pimentel et al., 2013). In recent years, additional isolations of HIV-1 strains containing subtype K and/or unclassified (U) segments in the

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http://dx.doi.org/10.1016/j.meegid.2016.05.040
1567-1348/

8.2 APÊNDICE 2

The Neuropeptides Vasoactive Intestinal Peptide and Pituitary Adenylate Cyclase-Activating Polypeptide Control HIV-1 Infection in Macrophages through Activation of Protein Kinases A and C

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Jornal: Frontiers in Immunology, 2018.

frontiers in Immunology

ORIGINAL RESEARCH
published: 12 June 2018
doi: 10.3389/fimmu.2018.01336

The Neuropeptides Vasoactive Intestinal Peptide and Pituitary Adenylate Cyclase-Activating Polypeptide Control HIV-1 Infection in Macrophages Through Activation of Protein Kinases A and C

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OPEN ACCESS

Edited by:
Aurelio Cafaro, Istituto Superiore di Sanità, Italy

Reviewed by:
Lucia Lopalco, San Raffaele Hospital (IRCCS), Italy
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Specialty section:
This article was submitted to Viral Immunology, a section of the journal Frontiers in Immunology

Received: *15 December 2017*
Accepted: *29 May 2018*
Published: *12 June 2018*

Citation:
Temerozo JR, de Azevedo SSD, Insuela DBR, Vieira RC, Ferreira PLC, Carvalho VF, Bello G and Bou-Habib DC (2018) The Neuropeptides Vasoactive Intestinal Peptide and Pituitary Adenylate Cyclase-Activating Polypeptide Control HIV-1 Infection in Macrophages Through Activation of Protein Kinases A and C. Front. Immunol. 9:1336. doi: 10.3389/fimmu.2018.01336

Vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase-activating polypeptide (PACAP) are highly similar neuropeptides present in several tissues, endowed with immunoregulatory functions and other systemic effects. We previously reported that both neuropeptides reduce viral production in HIV-1-infected primary macrophages, with the participation of β-chemokines and IL-10, and now we describe molecular mechanisms engaged in this activity. Macrophages exposed to VIP or PACAP before HIV-1 infection showed resistance to viral replication, comparable to that observed when the cells were treated after infection. Also, multiple treatments with a suboptimal dose of VIP or PACAP after macrophage infection resulted in a decline of virus production similar to the inhibition promoted by a single exposure to the optimal inhibitory concentration. Cellular signaling pathways involving cAMP production and activation of protein kinases A and C were critical components of the VIP and PACAP anti-HIV-1 effects. Analysis of the transcription factors and the transcriptional/cell cycle regulators showed that VIP and PACAP induced cAMP response element-binding protein activation, inhibited NF-kB, and reduced Cyclin D1 levels in HIV-1-infected cells. Remarkably, VIP and PACAP promoted G-to-A mutations in the HIV-1 provirus, matching those derived from the activity of the APOBEC family of viral restriction factors, and reduced viral infectivity. In conclusion, our findings strengthen the antiretroviral potential of VIP and PACAP and point to new therapeutic approaches to control the progression of HIV-1 infection.

Keywords: HIV-1, vasoactive intestinal peptide, pituitary adenylate cyclase-activating polypeptide, neuropeptides, macrophages, protein kinase C, protein kinase A

INTRODUCTION

The neuropeptides vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide (VIP and PACAP, respectively) are members of the secretin/glucagon family of peptides and are distributed systemically; VIP and PACAP act through three G-protein coupled receptors that are expressed in several cell types, namely, VPAC1, VPAC2, and PAC1 (1, 2). Both neuropeptides have

8.3 APÊNDICE 3

Plasmatic Levels of IL-18, IP-10, and Activated CD8+ T Cells Are Potential Biomarkers to Identify HIV-1 Elite Controllers with a True Functional Cure Profile

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Jornal: Frontiers in Immunology, 2018.

frontiers in Immunology

ORIGINAL RESEARCH
published: 11 July 2018
doi: 10.3389/fimmu.2018.01576

Plasmatic Levels of IL-18, IP-10, and Activated CD8+ T Cells Are Potential Biomarkers to Identify HIV-1 Elite Controllers With a True Functional Cure Profile

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OPEN ACCESS

Edited by:
Aurelio Cafaro, Istituto Superiore di Sanità, Italy

Reviewed by:
Claudia Cicala, National Institute of Allergy and Infectious Diseases (NIAID), United States
Anthony Jaworowski, RMIT University, Australia

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Specialty section:
This article was submitted to Viral Immunology, a section of the journal Frontiers in Immunology

Received: *23 February 2018*
Accepted: *25 June 2018*
Published: *11 July 2018*

Citation:
Côrtes FH, de Paula HHS, Bello G, Ribeiro-Alves M, de Azevedo SSD, Caetano DG, Teixeira SLM, Hoagland B, Grinsztejn B, Veloso VG, Guimarães ML and Morgado MG (2018) Plasmatic Levels of IL-18, IP-10, and Activated CD8+ T Cells Are Potential Biomarkers to Identify HIV-1 Elite Controllers With a True Functional Cure Profile. Front. Immunol. 9:1576. doi: 10.3389/fimmu.2018.01576

Elite controllers (ECs) are rare individuals able to naturally control HIV-1 replication below the detection limit of viral load (VL) commercial assays. It is unclear, however, whether ECs might be considered a natural model of a functional cure because some studies have noted CD4+ T cell depletion and disease progression associated with abnormally high levels of immune activation and/or inflammation in this group. Here, we propose the use of immunological parameters to identify HIV-1 ECs that could represent the best model of a functional cure. We compared plasma levels of six inflammatory biomarkers (IP-10, IL-18, sCD163, sCD14, CRP, and IL-6) and percentages of activated CD8+ T cells (CD38+HLA-DR+) between 15 ECs [8 with persistent undetectable viremia (persistent elite controllers) and 7 with occasional viral blips (ebbing elite controllers)], 13 viremic controllers (VCs—plasma VL between 51 and 2,000 RNA copies/mL), and 18 HIV-1 infected patients in combined antiretroviral therapy, with suppressed viremia, and 18 HIV-uninfected controls (HIV-neg). The two groups of ECs presented inflammation and activation profiles similar to HIV-neg individuals, and there was no evidence of CD4+ T cell decline over time. VCs, by contrast, had higher levels of IL-18, IP-10, and CRP and a lower CD4/CD8 ratio than that of HIV-neg ($P < 0.05$). Plasma levels of IL-18 and IP-10 correlated positively with CD8+ T cell activation and negatively with both CD4/CD8 and CD4% in HIV-1 controllers. These results suggest that most ECs, defined using stringent criteria in relation to the cutoff level of viremia (≤50 copies/mL) and a minimum follow-up time of >5 years, show no evidence of persistent inflammation or immune activation. This study further suggests that plasmatic levels of IL-18/IP-10 combined with the frequency of CD8+CD38+HLA-DR+ T cells can be important biomarkers to identify models of a functional cure among HIV-1 ECs.

Keywords: HIV-1, inflammation, immune activation, elite controller, IP-10, IL-18

INTRODUCTION

HIV controllers (HICs) are HIV-1-infected individuals able to control viral replication in the absence of combined antiretroviral therapy (cART) (1). According to the level of control, the HICs are divided into two groups: elite controllers (ECs), individuals able to keep viremia below the limit of detection of viral load (VL) commercial kits (currently, <40–50 RNA copies/mL), and viremic