INSTITUTO OSWALDO CRUZ

Doutorado em Biologia Parasitária

AVALIAÇÃO DE MARCADORES DE ATIVAÇÃO IMUNE E INFLAMAÇÃO ASSOCIADOS AO RISCO DE DESENVOLVIMENTO DE DOENÇA CARDIOVASCULAR EM CONTROLADORES DO HIV

DIOGO GAMA CAETANO

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Ministério da Saúde

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Avaliação de marcadores de ativação imune e inflamação associados ao risco de desenvolvimento de doença cardiovascular em controladores do HIV

.

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

Orientadoras: Drª Monick Lindenmeyer Guimarães e Fernanda Heloise Côrtes

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O que prevemos raramente ocorre; o que menos esperamos geralmente acontece.

Benjamin Disraeli

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RESUMO
TESE DE DOUTORADO EM BIOLOGIA PARASITÁRIA

Diogo Gama Caetano

A infecção pelo HIV-1 é caracterizada pela desregulação do sistema imune, resultando em níveis aumentados de ativação imune e inflamação persistente. Alguns indivíduos denominados controladores do HIV-1 (HICs), no entanto, apresentam controle espontâneo da replicação viral e indícios de preservação da resposta imune. Entre HICs, dados discordantes existem acerca dos níveis de ativação imune e da frequência de subpopulações de linfócitos T, incluindo Treg e Th17. Além disso, alguns estudos indicaram inflamação persistente e maior incidência de aterosclerose e hospitalização em HICs comparados com a população geral. Sabendo que fenômenos associados com ativação imune e inflamação participam da gênese de doenças cardiovasculares (CVD), o presente estudo avaliou a extensão da imunopatogênese em HICs através de diversos marcadores de ativação imune e inflamação e caracterizados como alterados na infecção pelo HIV-1 e em indivíduos com CVD. Para tal, perfis de ativação e diferenciação de linfócitos T, níveis de ativação plaquetária, frequência de agregados monócitos plaquetas, o balanço de subpopulações de monócitos e a concentração de marcadores séricos foram avaliados em dois grupos de HICs com diferentes níveis de controle da viremia – Controladores de Elite (EC) e Controladores Virêmicos (VC) – e comparados com os dados de dois grupos controle - indivíduos infectados pelo HIV-1 em tratamento antirretroviral (cART) e indivíduos HIV-1 negativos (HIVneg). Ao avaliar parâmetros da ativação imune, ECs demonstraram níveis similares de linfócitos T ativados em comparação com HIVneg, enquanto cART e VCs apresentaram níveis elevados dessas células. Nas análises de subpopulações de células T $CD4^+$ em ECs, apenas células T de memória transicional apresentaram frequências alteradas em relação a HIVneg. No entanto, VCs apresentaram frequências maiores de linfócitos terminalmente efetores, naive e T de memória tronco, além de frequências menores de linfócitos de memória transicional e memória central comparado com HIVneg. Entre os linfócitos T $CD8^+$, ECs apresentaram frequências aumentadas de linfócitos T de memória tronco, enquanto VCs apresentaram altas frequências de células terminalmente efetoras. ECs apresentaram frequências altas de Tregs ativadas em comparação com os grupos controle, apesar dos HICs apresentarem menores frequências de Tregs totais em comparação com HIVneg. Altas frequências de linfócitos Th17 e razões Th17/Treg ainda foram observadas em ECs, indicando preservação de células Th17 neste grupo. Ao avaliar parâmetros inflamatórios, os dois grupos de HICs apresentaram maior expressão de CD62P em plaquetas comparados com HIVneg. Diferenças na frequência de agregados monócitos plaquetas não foram observadas. Menores frequências de monócitos clássicos em VCs e maiores frequências de monócitos não clássicos em VCs e ECs em comparação com HIVneg foram observadas mesmo após ajuste para variáveis confundidoras. Entre os marcadores séricos, níveis elevados em comparação com HIVneg foram observadas para D-dímero, ST2,VCAM-1 e ICAM-1 em VCs, VCAM-1 e ICAM-1 em cART e VCAM-1 em ECs, mesmo após ajuste para fatores confundidores. No conjunto, VCs apresentaram níveis elevados de diversos marcadores de ativação imune e inflamação, enquanto ECs apresentam evidências de equilíbrio do sistema imune e controle da ativação celular, apesar de inflamação persistente. Os níveis de inflamação em ECs, no entanto, são comparáveis ao de cART indicando que esses indivíduos podem se beneficiar de terapias alternativas à cART que visem diminuir a inflamação.

Palavras-chave: HIV-1, Controladores, Inflamação, Ativação imune, doença cardiovascular

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ABSTRACT
PhD Thesis in Parasitary Biology

Diogo Gama Caetano

The HIV-1 infection is characterized by immune system deregulation, resulting in increased levels of immune activation and persistent inflammation. However, some individuals denominated HIV-1 Controllers (HICs) present spontaneous control of viral replication and preservation of immune response. Among HICs, discordant data exist about the levels of immune activation and the frequency of T lymphocytes subsets, including Treg and Th17. Moreover, some studies indicated persistent inflammation and a higher incidence of atherosclerosis and hospitalization in HICs compared to the general population. Knowing that phenomena associated with immune activation and inflammation participate in the genesis of cardiovascular diseases (CV), this study evaluated the extension of immunopathogenesis in HICs through several markers associated with the immune activation and inflammation and characterized as increased in the HIV-1 infection and individuals with CVD. For this, activation and differentiation profiles of T lymphocytes, levels of platelet activation, frequency of monocyte-platelet aggregates, the balance of monocyte subsets, and the concentration of serum markers were evaluated in two groups of HICs with different levels of viremia control – Elite controllers (ECs) and Viremic Controllers (VCs) – and compared with the data obtained from two control groups - HIV-1 infected individuals under antiretroviral therapy (cART) and non-infected individuals (HIVneg). For immune activation analyses, ECs showed similar levels of activated T lymphocytes when compared to HIVneg, while cART and VCs showed elevated levels of these cells. In T CD4+ subset analyses on ECs, only transitional memory T cells had altered frequencies in comparison to HIVneg, while VCs displayed higher frequencies of terminally effector, naïve and stem memory T cells and lower frequencies of transitional memory and central memory T cells when compared to HIVneg. Among T CD8+ T cells, ECs showed increased frequencies of stem memory T cells, while VCs presented higher frequencies of terminally effector T cells. ECs also presented higher frequencies of activated Tregs in comparison to control groups, although HICs had lower frequencies of total Tregs compared to control groups. Higher frequencies of Th17 cells and increased Th17/Tregs ratios were also observed for ECs, indicating preservation of Th17 cells in this group. For inflammatory parameters, both HICs groups presented higher expression. Of CD62P in platelets when compared to HIVneg. Differences in the frequencies of monocyte-platelet aggregates were not observed among the groups. Lower frequencies of classical monocytes in VCs and higher frequencies of nonclassical monocytes in VCs and ECs when compared to HIVneg were observed even after statistical adjustment for confusing variables. Among serum markers concentrations, increased levels when compared to HIVneg were observed for D-dimer, ST2, VCAM-1, ICAM-1 in VCs, VCAM-1, and ICAM-1 in cART and VCAM-1 in ECs after statistical adjustments. As a whole, VCs presented alterations at several markers of immune activation and inflammation, while ECs presented evidence an equilibrated immune system and control of cellular activation, despite persistent inflammation. The levels of inflammation in ECs, however, are comparable to the observed in cART, indicating that those individuals can benefit themselves from alternative therapies that intend to lower inflammation.

Keywords: HIV-1, Controllers, Inflammation, Immune activation, cardiovascular disease

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%	Porcentagem
α	Alfa
β	Beta
γ	Gama
^{o}C	Graus Celsius
<LDL	Abaixo do limite de detecção
ACD	Anticoagulante a base de Ácido Cítrico, Citrato de Sódio e Dextrose
ANOVA	Do inglês Analysis of Variance
ANRS	Agência Nacional Francesa de Pesquisa em HIV/Aids e Hepatites Virais
APOBEC3G	Apolipoprotein B MRNA Editing Enzyme Catalytic Subunit 3G
AST	Aspartato transaminase
AUDIT	Alcohol Use Disorders Identification Test
cART	Terapia Antirretroviral combinada
CCR	C-C receptor de quimiocina
CD	Cluster de Diferenciação
CD62P	Cluster de Diferenciação 62P/P-selectina
cIMT	Espessura da camada Intima-média da carótida
CMV	Citomegalovírus
CO_2	Gás carbônico
CSF	Fluido cerebrospinal
CTLA	Proteína associada a Linfócito T citotóxico
DNA	Ácido desoxirribonucleico
EC	Controlador de Elite
EDTA	Ácido etilenodiamino tetra-acético
EUA	Estados Unidos da América
FACS	Fluorescence Activated Cell Sorter
FMO	Fluorescência menos um
GALT	Tecido linfoide associado ao intestino
GM-CSF	Fator Estimulador de Colônias de Granulócitos e Macrófagos
H2PO4	Ácido fosfórico
HDL	Lipoproteína de alta densidade
HIC	Controladores do HIV
HIV	Vírus da Imunodeficiência Humana
HLA-B	Antígeno Leucocitário Humano
HSD	Teste de significância honesta de Tukey
HSH	Homens que fazem sexo com homens
ICAM-1	Molécula de adesão intercelular 1
IFN	Interferon
IL	Interleucina
IMC	Índice de massa corporal
INI	Instituto nacional de Infectologia

IP-10	Proteína 10 induzida por interferon gama
IQR	Intervalo interquartil
KCl	Cloreto de potássio
KIR	Receptores semelhantes à imunoglobulina de células killer
LDL	Lipoproteína de baixa densidade
LEDGF	Fator de crescimento celular derivado do cristalino
LPS	Lipopolissacarídeo
LTNP	Não progressor de longo termo
MCP-1	Proteína quimioatraente de monócitos 1
MFI	Média de intensidade de fluorescência
MgCl2	Cloreto de magnésio
MIP	Proteína inflamatória de macrófagos
mL	Mililitro
mm^3	Milímetro cúbico
MPA	Agregados monócitos plaquetas
NA	Não disponível
NaCl	Cloreto de sódio
NaH2PO4	Fosfato monossódico
NaHCO3	Bicarbonato de sódio
NI	Não informado
NK	Natural Killer
PBMC	Células mononucleares do sangue periférico
PBS	Tampão salino de fosfato
PCR	Proteína C-reativa
PD-1	Proteína de morte celular programada 1
PFA	Paraformaldeído
PMA	Acetato Forbol Miristato
PSGL-1	Glicoproteína ligante 1 de P-selectina
PVHIV	Pessoas vivendo com HIV
RANTES	Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted
SAMHD-1	Proteína 1 contendo domínio SAM e HD
SFB	Soro fetal bovino
SINAM	Sistema de Informação de Agravos de Notificação
SIV	Vírus da Imunodeficiência Símia
TARV	Terapia antirretroviral
TCM	Linfócito T de memória central
TEFF	Linfócito T terminalmente efetor
TEM	Linfócito T de memória efetora
TF	Fator tecidual
TGF-β	Fator de crescimento transformador beta
Th17	Linfócito T auxiliar 17
TLR	Receptor semelhante a Toll
TN	Linfócito T naïve
TNF	Fator de necrose tumoral

Treg	Linfócito T regulatório
TRIM-5	Proteína 5 contendo motivo Tripartite
TSCM	Linfócito T tronco de memória
TTM	Linfócito T de memória transicional
UNAIDS	Programa Conjunto das Nações Unidas sobre HIV/Aids
VC	Controladores virêmicos
VCAM	Molécula de adesão vascular
VDRL	Teste laboratorial de pesquisa de doença venérea
VLDL	Lipoproteína de densidade muito baixa

ÍNDICE

1. INTRODUÇÃO

Desde sua identificação no início dos anos 80 (Barré-Sinoussi et al. 1983; Gallo et al. 1984), o vírus da imunodeficiência humana (HIV-1) já infectou aproximadamente 75,7 milhões de indivíduos em todo o mundo e foi associado à morte de cerca de 32,7 milhões desses (UNAIDS 2020), se tornando um dos patógenos de maior impacto social, econômico e de saúde pública dos últimos 40 anos . Segundo o último boletim epidemiológico da UNAIDS (Figura 1), 1,7 milhões de novas infecções foram estimadas em todo o mundo em 2019, somando um total de 38 milhões de pessoas vivendo com HIV (PVHIV) em 2018, e as doenças relacionadas com a aids foram responsáveis pela morte de 690 mil de indivíduos no mesmo ano. Dessa totalidade de casos, as regiões leste e sul da África concentram a maior proporção de casos (54% do total de PVHIV e 42% do total de novas infecções em 2019) e a maior taxa de mortalidade (43% do total global de mortes em 2019).

A gravidade histórica da epidemia demandou o desenvolvimento e aperfeiçoamento de diferentes estratégias de controle, principalmente em relação a ampliação da oferta de tratamento com antirretrovirais. Os dados mais recentes mostram que o acesso ao tratamento com a terapia antirretroviral combinada (cART ou TARV) mais que triplicou na última década, atingindo atualmente cerca de 26 milhões de PVHIV (UNAIDS 2020). Esta ampliação, por sua vez, é impulsionada pela implantação de políticas de oferecimento de início do tratamento antirretroviral concomitante com o diagnóstico positivo e independente da contagem de células T $CD4^+$ ou estágio da doença do indivíduo. Estas estratégias vem sendo priorizadas e estimuladas com base nos estudos que demonstram o aumento da expectativa de vida em indivíduos tratados (Teeraananchai et al. 2017) e que o início precoce da terapia é associado com melhores desfechos clínicos na fase crônica da doença (Danel et al. 2015; Ding et al. 2015; Lundgren et al. 2015; Okulicz et al. 2015).

Os antirretrovirais vêm demonstrando serem essenciais tanto para a prevenção quanto para o manejo clínico da infecção pelo HIV-1. Estudos nos últimos anos demonstraram que maiores coberturas de tratamento de indivíduos infectados podem refletir diretamente nas taxas de transmissão, visto que a supressão viral suprime a chance de transmissão (Cohen et al. 2011; Rodger et al. 2019). Além disso, a comprovação da eficácia de diversos regimes de antirretrovirais para uso como profilaxia pré-exposição (PreP) têm levado a implementação de tais regimes como forma de prevenção em diversos lugares do mundo, principalmente entre populações

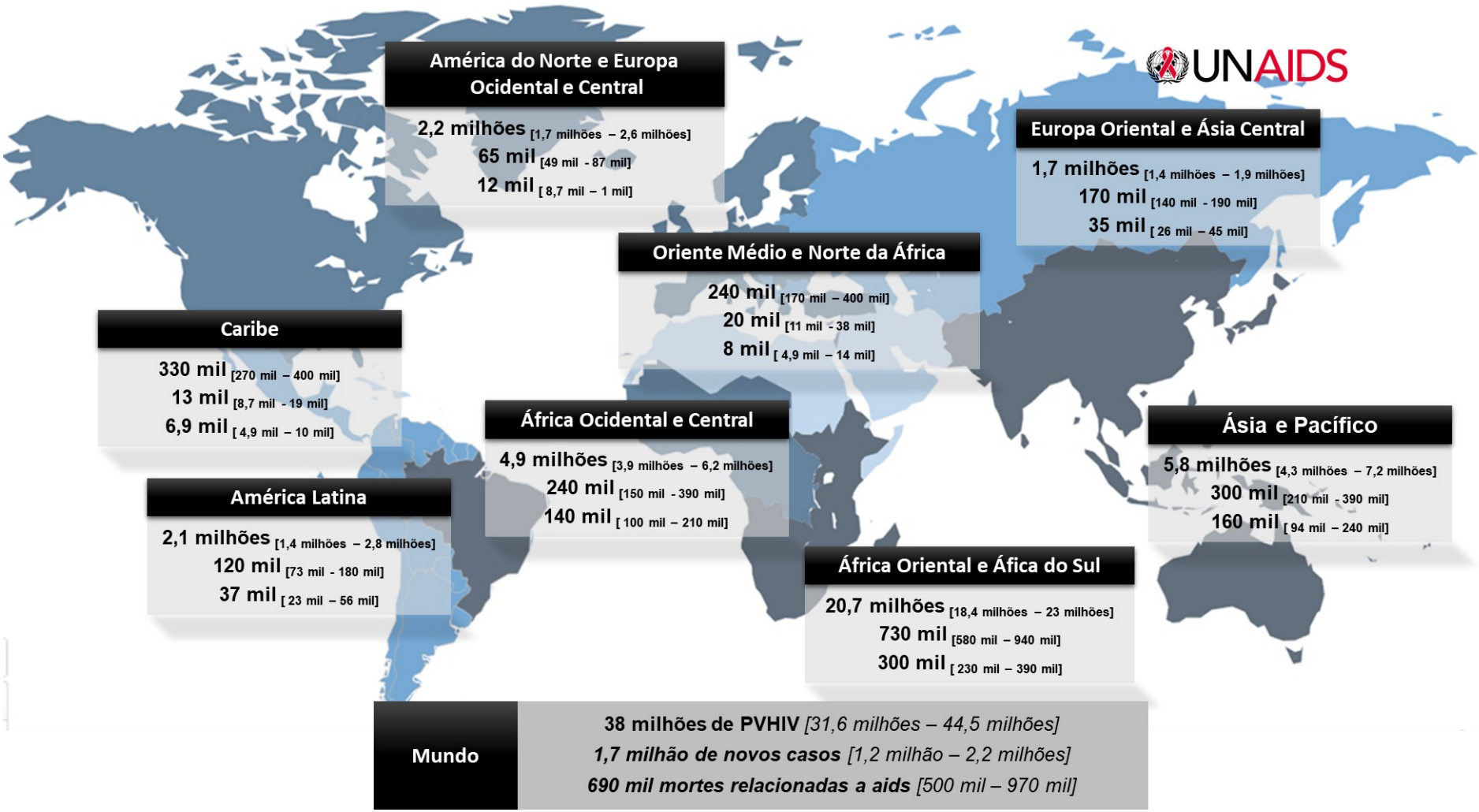

Figura 1. Estimativas de número de pessoas vivendo com HIV-1, novas infecções e mortes relacionadas à aids no mundo e de acordo com as diferentes regiões.
Os números detalhados referem-se aos dados da UNAIDS para o ano de 2019, de acordo com o boletim epidemiológico de 2020 (UNAIDS, 2020)

chaves, que apresentam maior risco de contrair o vírus, como homens que fazem sexo com homens, usuários de drogas injetáveis, trabalhadores do sexo e transgêneros (Grinsztejn et al. 2018; Grulich et al. 2018). Estes grupos concentram mais da metade das novas infecções globais, chegando a mais de 90% dessas no norte da África e Europa Oriental, demandando políticas especiais para prevenir a disseminação do HIV (UNAIDS 2019).

Em soma, a consolidação de estratégias com prévio sucesso segue aliada ao desenvolvimento de novos fármacos com maiores barreiras genéticas e menos efeitos colaterais (Cihlar and Fordyce 2016), bem como ação prolongada (Gulick and Flexner 2019), os quais tem contribuído como estratégias para melhorar a aderência ao tratamento.

A adoção destas estratégias em combinação com campanhas de prevenção focadas em populações chaves, por sua vez, reflete em melhores estatísticas epidemiológicas. Em comparação com 2010, observa-se uma queda de 23% no número de novas infecções em 2019 (2.1 milhões/ano para 1.7 milhões/ano) e de 39% na mortalidade (1.2 milhões/ano para 690 mil/ano) em todo o mundo (UNAIDS 2020).

De modo a estabelecer uma meta para orientar as políticas de saúde visando controlar a infecção pelo HIV, a UNAIDS estabeleceu em 2015 um programa de metas denominado “90-90-90” (Figura 2) (UNAIDS 2015). Este programa consiste no

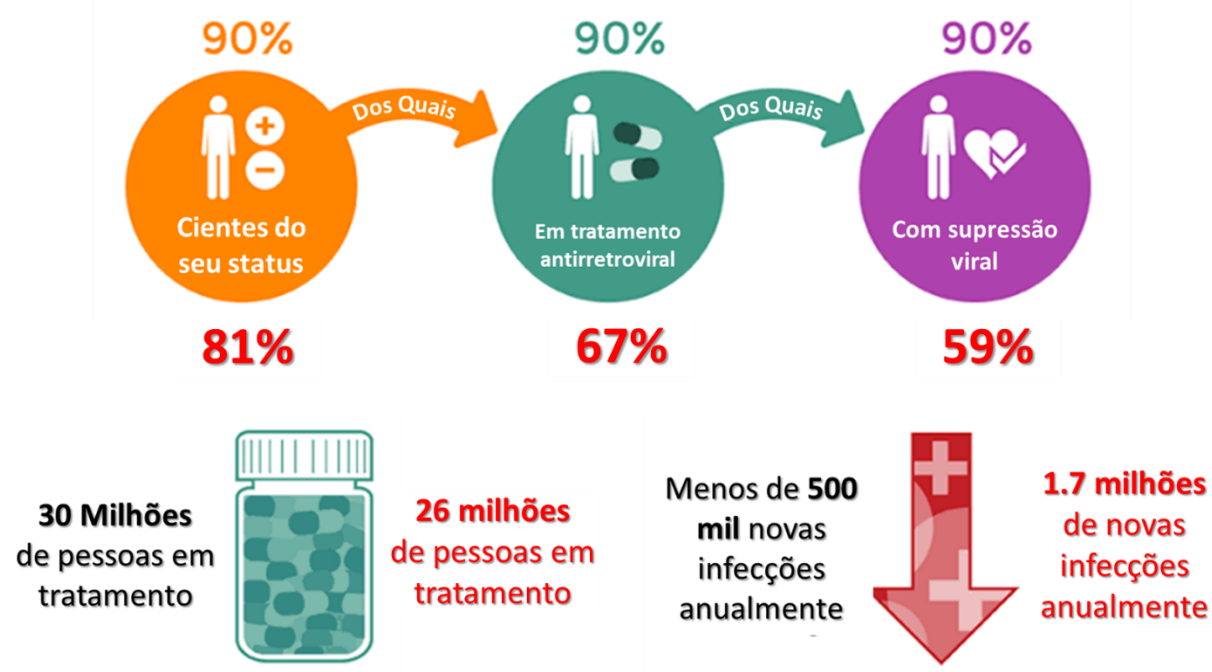

Figura 2. Metas estabelecidas pelo programa 90-90-90 da UNAIDS.
Em vermelho, estão indicados os patamares em 2020 para cada uma das metas. (2019? 2020?)

escalonamento e ampliação das políticas de testagem e tratamento para indivíduos infectados com HIV de modo que, em 2020, 90% de todas as pessoas vivendo com HIV estariam diagnosticadas, 90% das pessoas diagnosticadas estariam em cART e 90% das pessoas tratadas apresentam supressão viral. As estimativas, porém, são de que, atualmente, 81% dos indivíduos infectados tenham conhecimento do seu status, com 67% desses tem acesso a cART e 59% dos indivíduos tratados alcançaram supressão viral (UNAIDS 2020), indicando que novas medidas e a ampliação das atuais políticas são necessárias para que as estimativas correspondam aos números reais.

No Brasil, o número de casos de HIV notificados ao Sistema de Informação de Agravos de Notificação (SINAN) no período entre 2007-2020 foi de mais de 340 mil casos, sendo a região sudeste o local de maior concentração de indivíduos infectados, possuindo 44,4% desse total de casos. Considerando o mesmo período, observa-se um aumento no número de casos registrados principalmente no período 2012-2015 (de 13 mil casos para 38 mil casos no ano), refletindo o esforço de ampliação da testagem e a melhora nas políticas de diagnóstico. Somente em 2019, cerca de 42 mil novas infecções foram notificadas (Ministério da Saúde 2019). Apesar de uma média de 39 mil novos casos de aids por ano, a taxa de detecção de aids caiu 17,2% entre 2009 e 2019 em todo o país. Estratificando por regiões, observou-se uma queda no mesmo período de 33,6% e 30,3% nas taxas de detecção da região sudeste e sul, respectivamente. No entanto, as taxas de detecção aumentaram 11,3% na região nordeste e 24,4% na região norte (Ministério da Saúde 2019).

Em relação a mortalidade por doenças relacionadas a aids, mais de 349 mil mortes foram notificadas no Brasil entre 1980 e 2019. Desses óbitos, a região sudeste concentra também a maioria (57,7% do total). Em termos de coeficiente de mortalidade no Brasil, a taxa segue a tendência observada para a taxa de detecção de aids: redução geral de 29,3% no país inteiro entre 2009-2019, refletindo quedas observadas para a região sudeste e sul, mas aumento nas regiões norte e nordeste (Ministério da Saúde 2019).

Apesar de 40 anos já terem se passado desde a descrição da aids e da identificação de seu agente etiológico, até o momento ainda não existem vacinas capazes de controlar a pandemia. Vários fatores têm limitado o avanço na área de vacinas sendo o polimorfismo viral e a falta de correlatos de imunidade associados à

proteção contra a infecção ou evolução para a aids os obstáculos mais relevantes.

1.1. A Patogênese da infecção pelo HIV-1

A infecção pelo HIV-1 é caracterizada por uma série de eventos influenciados pelo tropismo específico desse vírus para importantes células do sistema imune. Após o evento de transmissão, a infecção se estabelece em linfócitos T CD4$^+$, macrófagos e células dendríticas e é disseminada para diferentes tecidos, incluindo órgãos linfoides, através dos processos de migração dessas células (Moir et al. 2011). A partir disso, uma série de fenômenos característicos são observados a curto e a longo prazo caracterizando três fases na infecção: fase aguda, fase crônica e fase de aids (Figura 3). A fase aguda engloba os primeiros meses da infecção e, apesar de ser caracterizada por cargas virais plasmáticas que vão de centenas de milhares a milhões de cópias por mililitro durante as primeiras semanas, esta fase se apresenta clinicamente com ausência de sintomas ou sintomas leves e inespecíficos como febre, fadiga, mialgias, cefaleias, náusea e diarreia (Kahn and Walker 1998; Lavreys et al. 2002; Selik and Linley 2018). Durante o período, no entanto, a alta taxa de replicação viral permite o estabelecimento da infecção em diferentes tipos celulares e a disseminação do vírus para diversos tecidos. Dentre esses tecidos, se destacam os

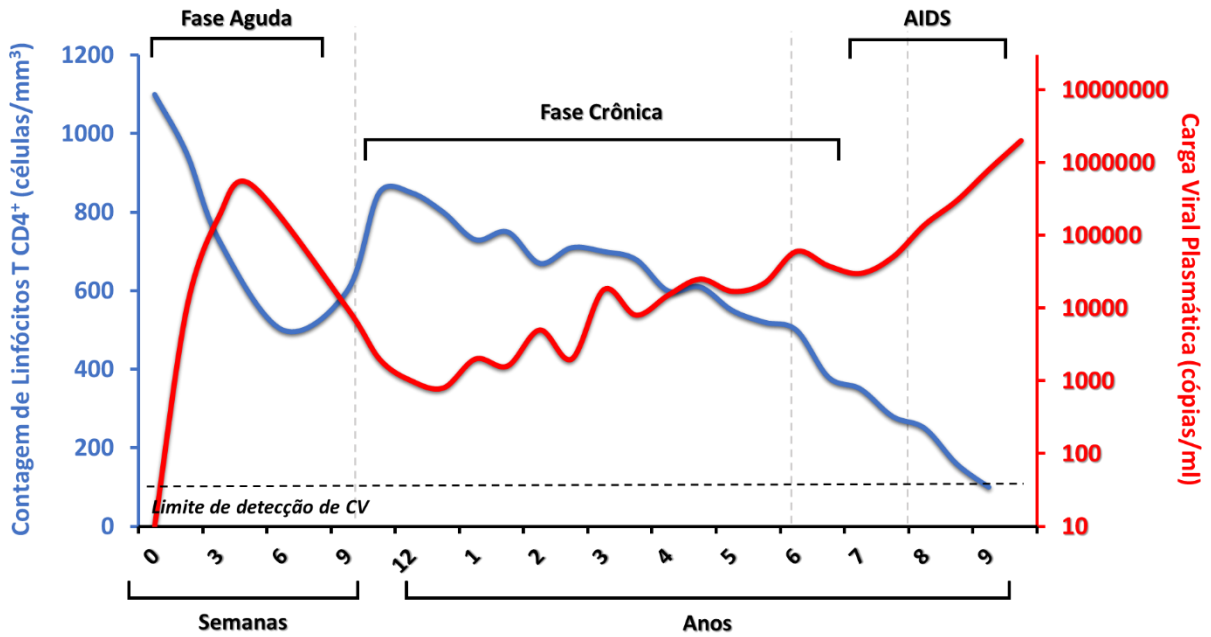

Figura 3. A história Natural da infecção pelo HIV-1 e suas 3 fases (aguda, crônica e aids).
O esquema exemplifica as dinâmicas de carga viral plasmática e de contagem de células T CD4$^+$ ao longo das fases da infecção. Baseado em Pantaleo e colaboradores, 1993.

tecidos linfoides associados ao intestino (GALTs), o qual constituirá um dos principais sítios de replicação viral durante a fase crônica.

Nos GALTs, estudos demonstraram uma depleção quase total de células T $CD4^+$, em nível muito maior ao observado no sangue periférico, ainda durante as primeiras semanas de infecção (Guadalupe et al. 2003; Brenchley et al. 2004b). Esta depleção parece ser particularmente importante para a patogênese da infecção, visto que a manutenção da integridade da mucosa ou melhor recuperação da subpopulação de células T $CD4^+$ desse tecido é associada com uma melhor resposta imune nas fases seguintes da infecção (Guadalupe et al. 2003; Macal et al. 2008; Ciccone et al. 2011).

Associado ao pico de viremia das primeiras semanas da fase aguda, também é observado o desenvolvimento da resposta imune inicial contra o vírus. Inicialmente, o surgimento de células T $CD8^+$ HIV-1-específicas é observado e correlacionado com a diminuição da viremia dias depois do início da resposta (Borrow et al. 1994; Wilson et al. 2000; Goonetilleke et al. 2009; Streeck et al. 2014). Em seguida à resposta celular, a resposta humoral e a produção de anticorpos contra o HIV-1 também é observada entre 3-12 semanas pós infecção (Busch and Satten 1997).

O fim da fase aguda é marcado pela soroconversão, incluindo a presença de anticorpos anti-p31 (referente a proteína viral Integrase), uma baixa recuperação dos níveis de células T $CD4^+$ no sangue periférico e o declínio da carga viral (Kassutto and Rosenberg 2004). Cerca de quatro a seis meses pós infecção, a carga viral alcança um nível relativamente estável denominado *set point* viral (Huang et al. 2012). Estudos demonstram associações entre a duração do período até o alcance do *set point*, bem como a sua magnitude, com a eficiência da resposta imune durante a fase aguda e a velocidade de progressão para a aids durante a fase crônica (Mellors et al. 1997; Lavreys et al. 2006). A partir do estabelecimento do *set point* viral, é iniciada a fase crônica (Figura 3). Este período é caracterizado por um desequilíbrio funcional crescente e progressivo do sistema imune que se reflete em uma gradual depleção de linfócitos T $CD4^+$ ao longo de anos de infecção. Paralelamente, ocorre aumento da viremia causando, após anos de infecção, um colapso do sistema imune que se traduz na imunodeficiência observada na fase de aids. Durante esse período, distintos perfis de progressão para a aids podem ser observados e estes serão detalhados na seção 1.3.

A fase de aids (Figura 3) é caracterizada por valores de células T CD4$^+$ abaixo de 200 células/mm^3 refletindo numa maior vulnerabilidade ao desenvolvimento de infecções oportunistas como tuberculose, toxoplasmose, pneumocistose, entre outras, e pode levar o indivíduo à morte na ausência de tratamento (Kaslow et al. 1996). A introdução da cART a qualquer momento do curso do clínico da infecção leva a supressão da carga viral plasmática a níveis abaixo do limite de detecção das metodologias de quantificação mais comumente empregadas e a recuperação gradual das contagens de linfócitos T CD4$^+$, servindo como estratégia para impedir ou reverter a progressão para a aids

1.2. desequilíbrio imune na infecção pelo HIV-1

De forma geral, a infecção pelo HIV-1 é caracterizada por um desequilíbrio do sistema imune cujo efeitos mais marcantes observados são uma ativação imune persistente e a depleção de células T CD4$^+$. Esses fenômenos se encontram associados a diversos eventos, tais como a alteração na representatividade e na dinâmica de maturação de diferentes subpopulações de células T, um favorecimento de respostas pró-inflamatórias, alterações estruturais em órgãos linfoides, a exacerbação de mecanismos de exaustão celular, entre outros (Pantaleo and Fauci 1996; McCune 2001; Brenchley et al. 2006a; Moir et al. 2011; Okoye and Picker 2013). No conjunto, todos esses fenômenos atuam de forma complexa, retroalimentando uns aos outros, e influenciando na imunopatogênese da doença.

1.2.1. Mecanismos de depleção de linfócitos T CD4$^+$

Um dos exemplos que evidenciam as interações complexas na infecção pelo HIV-1 é a proporção de participação direta do vírus na morte de linfócitos T CD4$^+$ induzindo efeitos citopáticos e apoptose nas células hospedeiras (Sylwester et al. 1997; Lenardo et al. 2002; Nie et al. 2002), embora esse não seja o principal mecanismo que leva a morte dessas células. Estudos já demonstraram que a proporção de células infectadas é muito menor do que a proporção de células mortas (Harper et al. 1986), com uma elevada proporção de células morrendo por mecanismos de morte celular induzida por ativação (Finkel et al. 1995; Garg and Joshi 2017). Além disso, células T CD8$^+$ apresentam taxas de morte e proliferação tão altas quanto as de células T CD4$^+$ na infecção pelo HIV-1 (Mohri et al. 1998; Hellerstein et al. 1999), apesar de não serem infectadas pelo vírus (Brenchley et al. 2004a).

Não sendo o vírus diretamente responsável pela massiva e progressiva depleção de células T CD4$^+$ observada na infecção pelo HIV-1, estudos identificaram que a infecção viral é capaz de alterar toda a dinâmica de produção e diferenciação de células T atingindo principalmente uma subpopulação celular. Apesar da sua ausência em grande parte das subpopulações de células T CD4$^+$, o CCR5 apresenta alto nível de expressão em linfócitos T CD4$^+$ de memória efetora (TEM), fazendo com que essas células sejam alvos preferenciais do HIV-1 (Brenchley et al. 2004a). Estes linfócitos T CD4$^+$CCR5$^+$ se distribuem de maneira heterogênea no corpo, sendo os principais constituintes da população de células T em mucosas, mas menos frequentes no sangue periférico e nos órgãos linfoides secundários (Poles et al. 2001; Grossman et al. 2006). Por esse motivo, ocorre uma depleção massiva de linfócitos T CD4$^+$ na mucosa intestinal na fase aguda (Poles et al. 2001; Mattapallil et al. 2005) (Guadalupe et al. 2003; Brenchley et al. 2004b).

A depleção de linfócitos TEM acaba por impactar indiretamente na população de linfócitos TCD4$^+$ naive (TN) e de memória central (TCM), os quais atuam como células precursoras das populações efetoras. Como forma de repor a população TEM, as TCM e TN recebem estímulos de proliferação e diferenciação, contribuindo para a ativação imune crônica observada em decorrência da infecção. Com a progressão da infecção, observa-se a exaustão de tais mecanismos linfoproliferativos e de reposição das subpopulações de células T mais diferenciadas (McCune 2001; Douek et al. 2003; Grossman et al. 2006; Okoye and Picker 2013).

Uma das razões para o prejuízo nos mecanismos de renovação das células T está associado ao desarranjo e perda da funcionalidade em estruturas linfoides. Além da supressão de hematopoiese, a fase crônica tardia da infecção pelo HIV-1 é associada a depleção de timócitos, a perda de demarcação corticomedular e ao desenvolvimento de folículos de células B nas regiões medulares do timo (McCune 2001; Dion et al. 2004). Os órgãos linfoides periféricos, por sua vez, sofrem de um desarranjo estrutural levando a descompartimentalização e degradação da rede de células dendríticas foliculares, da rede de células reticulares fibroblásticas e alteração do tráfico de células T (Rosenberg and Janossy 1999; McCune 2001; Schacker et al. 2002; Grossman et al. 2006; Dimopoulos et al. 2017). No conjunto, estas alterações afetam a capacidade de linfopoiese do organismo, prejudicando a geração de linfócitos TN e contribuindo para a imunodeficiência.

Uma vez que linfócitos TN são precursores naturais de todas as subpopulações de linfócitos, as alterações nos mecanismos de geração dessas células podem influenciar na dinâmica de linfócitos TCM. Apesar de linfócitos TCM constituírem um dos principais reservatórios na infecção pelo HIV-1 e a longevidade dessas células ser associada com a persistência viral em humanos (Chomont et al. 2009; Buzon et al. 2014; Song et al. 2020), estudos de infecção natural com o vírus da imunodeficiência símia (SIV) em macacos sooty mangabey demonstraram que a estabilidade e a preservação do conjunto de células TCM é um fator importante para o manejo da infecção, visto que estes macacos se infectam, mas não desenvolvem patogenia (Paiardini et al. 2011; McGary et al. 2014). Nesses animais, a perda de células TCM é diretamente ligada a inabilidade de repor o conjunto de células TEM e a progressão da doença (Okoye et al. 2007). A mesma associação é observada na infecção em humanos, sendo menores frequências de TCM e TN associadas a progressão para aids (Rabin et al. 1995; Roederer et al. 1995; Lederman et al. 2011; Moir et al. 2011; Fromentin et al. 2013). Com isso, estima-se que parte da imunodeficiência observada na infecção pelo HIV-1 seja um resultado do desequilíbrio na homeostase de TCM, que torna estas células menos funcionais e diminui suas proporções, prejudicando a reposição de perfis maduros como os de TEM (McCune 2001; Okoye and Picker 2013).

1.2.2. Ativação imune na infecção pelo HIV-1

Um dos fatores mais importantes que levam à perda da homeostase de todos os linfócitos T na infecção é a ativação imune crônica. A ativação imune engloba um conjunto de estados alterados caracterizados pelo aumento do processo de renovação celular, da proporção de linfócitos T e B com perfil fenotípico de célula ativada, da concentração de citocinas e quimiocinas pró-inflamatórias e desregulação do ciclo celular (Douek et al. 2003; Moir et al. 2011). Embora a ativação celular seja um mecanismo importante na reposição das células TEM depletadas pelo HIV-1, como discutido anteriormente, a exacerbação desse mecanismo influencia na persistência viral e na progressão da doença de diferentes formas. Uma dessas formas é a intensificação da replicação viral decorrente, da indução de transcrição viral em células infectadas ativadas e promovendo a produção de novas partículas virais a partir dos reservatórios virais(Biancotto et al. 2008; Hatano et al. 2013a; Klatt et al. 2013).

Os níveis de células ativadas, caracterizadas pela expressão simultânea de CD38 e HLA-DR, e marcadores séricos de ativação, como a neopterina e a β2-microglobulina (β2M) são alguns dos maiores preditores da progressão para a aids (Giorgi et al. 1993, 1999; Liu et al. 1997; Zangerle et al. 1998; Mildvan et al. 2005). Além disso, a ativação imune é associada a uma queda mais acelerada na contagem de células T $CD4^+$ durante a fase crônica (Deeks et al. 2004), uma recuperação mais lenta da população de linfócitos T $CD4^+$ após o início da terapia antirretroviral (Hunt et al. 2003) e a uma maior taxa de mortalidade e morbidade (Hunt et al. 2011a; Erlandson et al. 2013).

Outra forma importante pela qual a ativação imune influencia na progressão da doença está na indução de mecanismos de exaustão celular e senescência os quais levam linfócitos T a estados caracterizados por alteração na capacidade proliferativa e produção de citocinas e perda de capacidade efetora (Saeidi et al. 2018). A exaustão celular é causada por estimulação antigênica excessiva e constante, típica de infecções crônicas, e é capaz de induzir apoptose, levando a depleção de linfócitos (Hayashi et al. 2002; Brenchley et al. 2003; Saeidi et al. 2018). Na infecção pelo HIV-1, a exaustão de linfócitos é tão importante para a patogênese que a expressão de proteína de morte celular programada 1 (PD-1), um regulador negativo do sistema imune, é diretamente associada à ineficiência de linfócitos T $CD8^+$ HIV-1-específicos e com a progressão da doença (Day et al. 2006; Trautmann et al. 2006). A senescência, por sua vez, é um fenômeno associado ao envelhecimento que é caracterizado pela perda da capacidade proliferativa de linfócitos T, apesar de manutenção da capacidade de secreção de citocinas (Desai and Landay 2010). Na infecção pelo HIV-1, os mecanismos de senescência são acelerados e caracterizados pelo aumento na proporção de linfócitos T expressando CD57, cuja frequência correlaciona com a progressão da doença e que apresentam menor capacidade de proliferação em resposta a estímulos antigênicos e maior susceptibilidade a apoptose (Brenchley et al. 2003; Le Priol et al. 2006; Cao et al. 2009)..

Além das subpopulações citadas anteriormente, outras duas populações de linfócitos são alteradas durante a infecção pelo HIV-1 e possuem funções que podem influenciar nos níveis de ativacão imune no organismo. As células T reguladoras (Treg), uma subpopulação de linfócitos T caracterizada pela expressão de FoxP3 e CD25 que atua como moduladora da resposta imune, inibindo a proliferação de outras células imunes e atuando na indução de tolerância (Chevalier and Weiss 2013;

Valverde-Villegas et al. 2015; Hasenkrug et al. 2018). Devido a sua capacidade imunossupressora, a possível participação dos linfócitos Treg na modulação da ativação imune crônica na infecção pelo HIV-1 vem sendo amplamente estudada. Alguns estudos mostraram que altas frequências de células Treg poderiam ter um efeito positivo associado a diminuição da ativação imune (Jiao et al. 2009; Schulze Zur Wiesch et al. 2011; Angin et al. 2012). Por outro lado, em outros estudos a baixa atividade de células Treg foi associada com o aumento da atividade antiviral de células T $CD8^+$ (Aandahl et al. 2004; Eggena et al. 2005; Hryniewicz et al. 2006; Kinter et al. 2007; Zelinskyy et al. 2009).

Embora na infecção pelo HIV-1 o papel das Treg ainda não esteja bem definido, diversos estudos indicam que esta população pode contribuir para a progressão da doença, já que observa-se aumento da sua frequência ao longo da fase crônica da infecção na ausência de tratamento (Andersson et al. 2005; Tsunemi et al. 2005; Nilsson et al. 2006; Baker et al. 2007; Chase et al. 2008; Bi et al. 2009; Suchard et al. 2010; Hunt et al. 2011b; Nikolova et al. 2011; Schulze Zur Wiesch et al. 2011). Corroborando essa hipótese, a administração da cART é associada a menores frequências dessa população em comparação com indivíduos não tratados (Bi et al. 2009; Jiao et al. 2009; Suchard et al. 2010; Montes et al. 2011; Schulze Zur Wiesch et al. 2011).

A outra subpopulação importante são as células Th17. Estas células são abundantes em regiões de mucosa, sendo caracterizadas pela expressão de IL-17 e pela produção de diversas citocinas pró-inflamatórias (Raffatellu et al. 2008; Conti et al. 2009; Dandekar et al. 2010). De maneira geral, estas células apresentam importante função na defesa do organismo contra patógenos, na manutenção da integridade das mucosas ao estimular a expressão de peptídeos antimicrobianos (Liang et al. 2006), no recrutameto de neutrófilos (Griffin et al. 2012; Flannigan et al. 2017) e na indução da regeneração epitelial (Pickert et al. 2009). Considerando que a translocação de produtos microbianos nas regiões de mucosa é um dos mecanismos que contribuem para a ativação imune na infecção pelo HIV-1 (Brenchley et al. 2006b), linfócitos Th17 vem sendo associados ao controle da ativação sistêmica. Um desafio para isso, no entanto, é o fato de que estas células fazem parte do conjunto de células T $CD4^+$ que são depletadas ainda no início da infecção. Estudos com primatas não humanos infectados por SIV mostraram que células Th17 são preferencialmente depletadas em modelos patogênicos da infecção (Cecchinato et al. 2008; Raffatellu et

al. 2008; Favre et al. 2009), mas preservadas em modelos não patogênicos (Brenchley et al. 2008; Favre et al. 2009). Seguindo essas observações, menores frequências de Th17 foram observadas com a progressão da infecção pelo HIV-1 (Brenchley et al. 2008; Macal et al. 2008; Prendergast et al. 2010; Salgado et al. 2011), com algum nível de restauração em indivíduos tratados com cART (Macal et al. 2008).

Apesar de funções e efeitos opostos na patogênese do HIV-1, as alterações nessas duas subpopulações apresentam um aspecto antagônico, de modo que a baixa razão Th17/Treg é um parâmetro associado a progressão da doença (Favre et al. 2009; Brandt et al. 2011; Jenabian et al. 2013; Falivene et al. 2015) e a alta razão ao perfil de controle espontâneo da infecção (Brandt et al. 2011; Jenabian et al. 2013; Falivene et al. 2015). Parte dessa associação pode ser decorrente da origem dessas células, visto que ambas são derivadas de células TN e sua diferenciação estimulada por diferentes níveis de expressão de Interleucina 6 (IL-6) e do fator de crescimento transformador β (TGF-β). Mais especificamente, a diferenciação de Th17 e Treg dependem da sinalização por TGF-β, porém a expressão concomitante de IL-6 favorece o desenvolvimento de células Th17 em detrimento de Treg (Bettelli et al. 2006).

1.2.3. Inflamação Persistente na Infecção pelo HIV-1

Uma das consequências da ativação imune persistente e do desbalanço homeostático de células do sistema imune é a manutenção de um estado de inflamação crônica. Processos inflamatórios atuam como resposta a patógenos em diferentes frentes, englobando ações que visam o recrutamento de fagócitos e outras células do sistema imune para sítios de infecção, o aumento da expressão de citocinas e quimiocinas, a contenção do patógeno no tecido afetado e o reparo tecidual em caso de dano físico (Murphy and Weaver 2017). Tais processos, no entanto, quando prolongados irão influenciar no agravamento e progressão da infecção pelo HIV-1, assim como em outras doenças, como as cardiovasculares.

1.2.3.1. Influência das Citocinas

A alteração no padrão de secreção de citocinas e quimiocinas causados pelo HIV-1 é observada desde o início da infecção e é um dos principais combustíveis para a inflamação sistêmica. Um dos eventos mais característicos da fase aguda da infecção pelo HIV-1 é o aumento rápido de expressão de diversas citocinas inflamatórias ou

reguladoras, como os interferons α e γ (IFN-α e IFN-γ), o fator de necrose tumoral α (TNF-α), a proteína induzida por IFN-γ 10 (IP-10), a proteína quimioatraente de monócitos 1 (MCP-1), as interleucinas IL-6, IL-8, IL-18 e IL-10, entre outras (Stacey et al. 2009; Freeman et al. 2016; Muema et al. 2020).

Essa “tempestade de citocinas” característica se intensifica em paralelo ao aumento da carga viral (Stacey et al. 2009) e apresenta tanto efeitos positivos, como negativos. Algumas dessas citocinas, como os Interferons e o TNF-α, claramente atuam na tentativa de resolução da viremia, induzindo a ativação de células do sistema imune através de seu efeito antiviral. Outras, como MCP-1, IL-6, IP-10 e IL-18, apresentam importante função reguladora, agindo como fatores de recrutamento de células imunes e estimulando a sobrevivência das mesmas, aumentando a expressão de genes que atuam na homeostase celular e inibindo vias apoptóticas (Kedzierska and Crowe 2001; Freeman et al. 2016; Luis Muñoz-Carrillo et al. 2019).

Apesar de suas funções importantes para eliminação do patógeno, o aumento exacerbado e prolongado destas citocinas é associada a imunopatogênese do HIV-1. A intensidade da tempestade de citocinas foi associada a progressão para a doença no modelo de primatas não humanos (Keating et al. 2016) e a piores prognósticos em humanos (Roberts et al. 2010). Uma vez encerrada a fase aguda, os níveis das diferentes citocinas decaem, mas não são normalizados (Stacey et al. 2009; Freeman et al. 2016; Muema et al. 2020), uma vez que a ativação imune persistente ativa mecanismos de retroalimentação positiva para muitas dessas citocinas (Luis Muñoz-Carrillo et al. 2019). Além disso, citocinas como TNF-α, IL-1, IL-6 e IL-18 atuam potencializando a replicação viral (Kedzierska and Crowe 2001). No conjunto, todos estes fatores atuam sinergicamente e reciprocamente intensificando a patogênese da doença de forma crônica. Não obstante, níveis elevados de algumas citocinas, como IP-10 e IL-6, foram associados com menores contagens de células T $CD4^+$ e com a progressão para a doença (Lafeuillade et al. 1991; Mildvan et al. 2005; Liovat et al. 2012; Valverde-Villegas et al. 2018). Em soma, níveis aumentados de algumas citocinas persistem mesmo após início da cART (Osuji et al. 2018).

1.2.3.2. Influência dos perfis celulares

O desbalanço entre as citocinas favorecendo um perfil pró-inflamatório, influencia diretamente nos fenótipos celulares e na funcionalidade das células do sistema imune. De maneira geral, a migração de diversas células do sistema imune para os tecidos é

estimulada nas condições de inflamação causadas pelo HIV-1. Um dos fatores essenciais para essa migração é a ativação de células endoteliais, a qual é mediada por citocinas pró-inflamatórias, em especial IL-1β e TNF-α, e produtos do processo inflamatório, como a proteína C-reativa (PCR) (Szmitko et al. 2003). Esta ativação induz vasodilatação, permeabilidade vascular e a expressão de moléculas de adesão celular como a E-selectina, a molécula de adesão celular vascular 1 (VCAM-1) e a molécula de adesão intercelular 1 (ICAM-1), as quais medeiam os processos de transmigração das células do sistema imune através da lâmina interna dos vasos sanguíneos (Szmitko et al. 2003). Assim como as moléculas que estimulam esse processo, a expressão de marcadores de ativação endotelial também é aumentada na infecção pelo HIV-1, favorecendo a transmigração celular (Graham et al. 2013; Kamtchum-Tatuene et al. 2019)

Este fenômeno influencia de maneira marcante na dinâmica de monócitos e macrófagos. Em condições de inflamação, monócitos são recrutados da corrente sanguínea para diferentes tecidos do organismo em resposta a um gradiente de MCP-1 (Deshmane et al. 2009), o qual também tem sua expressão elevada na infecção pelo HIV-1 (Weiss et al. 1997; Coll et al. 2006; Westhorpe et al. 2014).

Em humanos, três subpopulações de monócitos são observadas com base no padrão de expressão do correceptor de LPS CD14 e do receptor Fcγ-III CD16, apresentando funcionalidade distinta. A maioria dos monócitos presentes no organismo apresentam o fenótipo clássico, com alta expressão de CD14 e nenhuma expressão de CD16 ($CD14^{++}CD16^{-}$). No outro lado do espectro, monócitos não clássicos são definidos pela alta expressão de CD16 e menor expressão de CD14 ($CD14^{+}CD16^{++}$). Monócitos intermediários, por sua vez, são caracterizados como um perfil de transição de monócitos clássicos para não clássicos, apresentando maiores níveis de expressão de CD14 e menor de CD16 ($CD14^{++}CD16^{+}$) (Ziegler-Heitbrock et al. 2010). Enquanto monócitos clássicos são caracterizados por um maior potencial fagocítico e pela diferenciação mais frequente em células dendríticas, monócitos não clássicos são caracterizados por um perfil voltado para resposta contra infecções virais e mais inflamatório, com maior capacidade de expressão de TNF-α e IL-1β (Belge et al. 2002; Cros et al. 2010; Mukherjee et al. 2015; Boyette et al. 2017).

Na infecção pelo HIV-1, um aumento na frequência de populações de monócitos expressando CD16 é observado e relacionado com a magnitude da carga viral (Abel et al. 1992; Thieblemont et al. 1995; Pulliam et al. 1997; Amirayan-Chevillard et al.

2000; Han et al. 2009; Funderburg et al. 2012; Liang et al. 2015; Chen et al. 2017; Luo et al. 2018), com níveis anormais persistindo algumas vezes mesmo após o início da cART (Amirayan-Chevillard et al. 2000; Han et al. 2009; Chen et al. 2017). Maiores frequências de marcadores de ativação dessa população, como CD14 e CD163 solúveis (sCD14 e sCD163), também são observados e correlacionam com outros marcadores inflamatórios, indicando a contribuição desta população para o estado de inflamação crônica (Kuller et al. 2008; Burdo et al. 2011; Pereyra et al. 2012; Kelesidis et al. 2012; Fitch et al. 2013; Masiá et al. 2013; Castley et al. 2014; Westhorpe et al. 2014; Li et al. 2015; Liang et al. 2015; Chen et al. 2017; de Paula et al. 2018).

Além de influenciarem na patogênese da infecção através de seu perfil mais inflamatório, monócitos também são suscetíveis a infecção (Crowe and Sonza 2000). Apesar da estimativa de que apenas 0,1% dos monócitos estejam infectados, as subpopulações que expressam CD16 são mais suscetíveis e preferencialmente infectadas devido a seus maiores níveis de CCR5 (Ellery et al. 2007; Jaworowski et al. 2007).

O cenário de inflamação crônica do HIV-1 também pode influenciar a diferenciação de macrófagos. Em relação a sua origem, estudos dos últimos anos em camundongos demonstraram que, em condições normais, a renovação de macrófagos residentes em tecido é fruto de auto-proliferação na grande maioria dos tecidos, exceto na derme, coração e intestino (Ginhoux et al. 2016), sendo esse último um dos principais sítios de replicação viral como discutido anteriormente. Em situações de inflamação patogênica um aumento no influxo de monócitos e sua posterior diferenciação em macrófagos é observada (Ginhoux and Jung 2014). Estes dois fatos apontam para uma importância nesse processo de diferenciação de monócitos em macrófagos nas condições de inflamação causadas pelo HIV-1.

Na patogênese da infecção, macrófagos atuam em duas linhas diferentes. A primeira delas ocorre devido a sua susceptibilidade ao vírus. Em soma aos linfócitos TEM, macrófagos são as principais células infectadas pelo HIV-1 nos tecidos e atuam como importantes reservatórios virais sendo capazes de sustentar a replicação viral na ausência de linfócitos (Honeycutt et al. 2016). Estas células também são mais resistentes aos efeitos citopáticos do HIV-1 (Swingler et al. 2007; Reynoso et al. 2012; Yuan et al. 2017) e a morte mediada por linfócitos T $CD8^+$ quando infectadas (Vojnov et al. 2012; Clayton et al. 2018), contribuindo para a persistência viral.

A ativação de macrófagos na infecção pelo HIV-1 também é outro mecanismo que contribui diretamente para a manutenção da inflamação no tecido. Funcionalmente, estas células apresentam uma diversidade de fenótipos que se distribuem no em um espectro entre dois polos extremos de ativação. De um lado, em condições de homeostase ou a influência de citocinas clássicas de resposta Th2, como IL-4 e IL-13, levam ao desenvolvimento de macrófagos M2, os quais possuem um perfil anti-inflamatório e atuam em processos de reparo tecidual, intensificação de fagocitose e supressão da inflamação. Do outro lado, citocinas associadas a resposta Th1, como IFN-γ, levam ao desenvolvimento de macrófagos M1, os quais são associados a um perfil pró-inflamatório e a um aumento na expressão de citocinas como IL-1β, IL-12, IL-23 e TNF-α (Cassol et al. 2010). Este último perfil é induzido pela replicação viral (Porcheray et al. 2006; Brown et al. 2008; Cassol et al. 2009; Chihara et al. 2012), contribuindo para o aumento da inflamação. Apesar de macrófagos M1 serem fenótipos associados a resistência contra a replicação viral e a infecção de novo (Cassol et al. 2009; Schlaepfer et al. 2014; Graziano et al. 2018), estas células também permitem a propagação viral através do recrutamento de outras células suscetíveis a infecção e do estimulo da replicação viral em linfócitos infectados devido ao contexto inflamatório associado (Saïdi et al. 2007).

Por fim, a inflamação mediada pelo HIV-1 também pode se constituir em um dos mecanismos principais de depleção de células T $CD4^+$. Além das citocinas pro-inflamatórias contribuírem para a ativação imune através de mecanismos de ativação *"bystander"* de linfócitos (Finkel et al. 1995; Garg and Joshi 2017) e promoverem o recrutamento de novas células que podem ser infectadas pelo vírus (Biancotto et al. 2008), o HIV-1 pode levar a ativação do inflamassoma (Feria et al. 2018) e se torna gatilho para a indução de piroptose em células T $CD4^+$. Esta modalidade de morte celular é caracterizada pela ativação de caspase-1 e pelo aumento da expressão celular de pró-IL-1β, a qual é ativada e liberada após a morte celular e contribui para a inflamação (Doitsh et al. 2014).

1.2.3.3. Inflamação e comorbidades cardiovasculares

Além de favorecer os efeitos negativos da própria infecção pelo HIV-1 e influenciar na progressão para a aids, a inflamação persistente atua também na patogênese de uma série de comorbidades. O aumento da expectativa de vida entre PVHIV decorrente da eficiência da cART permitiu observar que uma série de morbidades não

relacionadas a aids, como síndrome metabólica, dislipidemia, hipertensão, diabetes e doença cardiovascular, são mais frequentes nesses indivíduos em comparação com a população não infectada pelo HIV-1 (Naidu et al. 2017; Nansseu et al. 2018; Dominick et al. 2020). Parte dessa maior frequência de comorbidades não está relacionada diretamente a patogênese da infecção, visto que fatores de risco tradicionais para doenças cardiovasculares relacionados ao estilo de vida, como o fumo e obesidade, são mais frequentes em PVHIV em comparação com a população geral (Triant et al. 2007; Freiberg et al. 2013; Diaz et al. 2016; Frazier et al. 2018; Moreira et al. 2018). Além disso, diversos estudos observaram a relação entre alguns antirretrovirais, principalmente inibidores de protease, e um risco aumentado para o desenvolvimento de doenças cardiovasculares (Kaplan et al. 2007; Diaz et al. 2016; Hatleberg et al. 2018). Mesmo assim, a infecção pelo HIV-1 se apresenta como componente de risco independente de tais fatores, visto que pacientes infectados pelo HIV-1 apresentam risco duas vezes maior de infarto do miocárdio (Triant et al. 2007; Lang et al. 2010; Paisible et al. 2015) e aproximadamente quatro vezes maior de morte por doença cardíaca repentina quando comparados com a população geral (Tseng et al. 2012).

A relação entre ativação imune e inflamação e o desenvolvimento de complicações cardiovasculares vem sendo amplamente estudada nos últimos anos, indicando que esses podem ser os componentes principais influenciando no risco aumentado para essas morbidades em PVHIV. Linfócitos T ativados, por exemplo, foram identificados como importantes mediadores de infarto isquêmico em camundongos (Bansal et al. 2017). De forma semelhante, marcadores celulares e séricos de ativação imune já foram associados a processos relacionados a doenças cardiovasculares tanto na população geral (Weiss et al. 1994; Fernandez et al. 2019), como em PVHIV (Kaplan et al. 2011; Fitch et al. 2013; Longenecker et al. 2013; Siedner et al. 2016). Associações semelhantes foram observadas entre hipertensão e aterosclerose e a ativação de linfócitos T específicos para citomegalovírus (CMV), o qual é o causador da coinfecção mais prevalente entre PVHIV (Hsue et al. 2006; Masiá et al. 2013; Hui et al. 2016; Ballegaard et al. 2020).

A inflamação, por sua vez, também é um componente importante para o desenvolvimento de doenças cardiovasculares. As concentrações de IL-6, proteína C-reativa e D-dímero são aumentadas em decorrência da infecção pelo HIV-1 e amplamente associadas ao desenvolvimento e a gravidade de doenças

cardiovasculares (Kuller et al. 2008; Boulware et al. 2011; Sandler et al. 2011; Duprez et al. 2012; Hunt et al. 2014; Tenorio et al. 2014; Grund et al. 2016; Baker et al. 2017b; Carvalho et al. 2018; Peterson et al. 2018). TNF-α também foi identificado como um participante em processos de geração de aterosclerose em camundongos (Gao et al. 2016) e altos níveis dessa citocina foram observados como um fator preditor de maiores pontuações na escala de risco Framingham, a qual utiliza dados de idade, gênero, hábitos de fumo, níveis de colesterol e valores de pressão sistólica para estimar o risco de um indivíduo desenvolver doenças cardiovasculares dentro de 10 anos (Mooney et al. 2015).

A relação entre D-dímero, inflamação e doenças cardiovasculares ressalta a influência da infecção viral nos processos de homeostase vascular e endotelial. O endotélio de PVHIV apresenta sinais de dano tecidual associado com os processos de regeneração e ativação (Zietz et al. 1996) e sinais de disfunção endotelial (Nolan et al. 2003; Solages et al. 2006). Este efeito parece estar associado diretamente ao vírus, ao passo que proteínas como a Gp120, Tat e Nef já foram associadas com disfunção e apoptose de células endoteliais (Anand et al. 2018). Um estudo com camundongos transgênicos que expressavam *proteínas virais* observou danos funcionais no endotélio dos animais e o aumento de diversos marcadores de aterosclerose, como um aumento da rigidez arterial e diminuição da espessura da camada intima-média da carótida (cIMT) (Hansen et al. 2013).

A aterosclerose é um processo patogênico causado pela deposição de lipídios nas camadas internas da parede arterial, levando a formação de placas que obstruem o fluxo sanguíneo. Este processo apresenta uma grande influência da resposta inflamatória do organismo, visto que é fruto de uma resposta do sistema imune inato à formação e acúmulo de lipoproteínas de baixa densidade oxidadas. O acúmulo dessas proteínas na parede arterial induz uma inflamação que estimula o recrutamento de monócitos da corrente sanguínea para o tecido e a sua posterior diferenciação em macrófagos. Os macrófagos recrutados para a placa, por sua vez, fagocitam as lipoproteínas de baixa densidade oxidadas acumuladas e se convertem em células espumosas. Estas últimas, apresentam perda da mobilidade e ficam presas no tecido, morrendo eventualmente e levando a um acúmulo de cristais de colesterol e células apoptóticas/necróticas no tecido (Gisterå and Hansson 2017).

Em PVHIV, menores valores de cIMT são observados (Bonnet et al. 2004; Hsue et al. 2004, 2009; McComsey et al. 2007; Oliviero et al. 2009; van Vonderen et al. 2009;

Bergmann et al. 2018; Low et al. 2019; Msoka et al. 2019), mesmo em indivíduos tratados (Hsue et al. 2004, 2009; van Vonderen et al. 2009) ou crianças (Bonnet et al. 2004; McComsey et al. 2007; Idris et al. 2016), indicando que o desenvolvimento da aterosclerose nesses indivíduos independe do tratamento antirretroviral ou de fatores de risco associados. Devido a participação da inflamação no processo de formação da placa aterosclerótica, diversos pontos de ligação entre a infecção pelo HIV-1 e a aterosclerose vem sendo identificados.

Um dos pontos em comum na patogênese do HIV-1 e da aterosclerose é a expressão de moléculas indicadores de ativação das células endoteliais. Moléculas de adesão, como VCAM-1 e ICAM-1, são essenciais para os processos de recrutamento de monócitos e são superexpressas durante a formação da placa aterosclerótica (Nakashima et al. 1998), assim como na infecção pelo HIV-1 (Graham et al. 2013; O'Halloran et al. 2015; Baker et al. 2017a; Mosepele et al. 2018; Kamtchum-Tatuene et al. 2019; Sereti et al. 2019; Angelovich et al. 2020). Além dessas, a interação entre o receptor CD40 (expresso em células endoteliais, macrófagos, monócitos, células dendríticas e linfócitos B) e CD40L (expresso principalmente em linfócitos T ativados e plaquetas) é considerada uma importante via de ativação do endotélio, levando a expressão de moléculas de adesão e de citocinas pró-inflamatórias (Szmitko et al. 2003; Hassan et al. 2012). Esta capacidade de mediação da interação entre monócitos, plaquetas e linfócitos ativados provocado pela ligação CD40/CD40L mostra que esta associação pode influenciar na aterogênese, com as duas moléculas sendo mais expressas na placa aterosclerótica e em indivíduos com complicações cardiovasculares (Bruemmer et al. 2001; Lievens et al. 2010; Bosmans et al. 2020). Em um estudo com indivíduos infectados pelo HIV-1, os níveis de sCD40L ainda correlacionaram com a concentração de ST2 (Mehraj et al. 2016; Younas et al. 2017), um importante biomarcador de estiramento do miocárdio que funciona como um preditivo de mortalidade independente de outros fatores de risco (Ky et al. 2011; Kohli et al. 2012; Braunwald 2013).

IL-6 e IL-1β também são citocinas importantes na aterosclerose e na infecção pelo HIV. Além de atuarem como combustíveis do processo inflamatório relacionado ao HIV-1, a expressão dessas citocinas também é aumentada durante a aterogênese. No processo, os cristais de colesterol acumulados em células espumosas atuam como ativadores de moléculas do inflamassoma, ligado diretamente a produção de IL-1β (Duewell et al. 2010). A IL-1β produzida nesse processo, por sua vez, estimula a

produção de IL-6 por células do músculo liso (Loppnow and Libby 1990). Nesse sentido, os processos inflamatórios gerados por HIV-1 e aterosclerose independentemente podem atuar sinergicamente, inflamando mais o organismo.

Outro ponto em comum é a participação de monócitos nas duas patogenias. Apesar de monócitos clássicos serem a principal população que migra para a placa aterosclerótica (Swirski et al. 2007), monócitos não clássicos apresentam características de patrulhamento do tecido endotelial e manutenção da homeostase vascular (Narasimhan et al. 2019). Estes monócitos não clássicos são ainda observados em maiores quantidades no lúmen de artérias durante os processos iniciais de formação de placa em camundongos (McArdle et al. 2015; Marcovecchio et al. 2017), indicando uma participação importante na aterogênese recente. Em humanos, populações $CD16^{+}$ foram correlacionadas com aterosclerose em diversos estudos (Wildgruber et al. 2009; Poitou et al. 2011; Rogacev et al. 2012; Höpfner et al. 2019; SahBandar et al. 2019), inclusive em indivíduos infectados pelo HIV-1 (Fitch et al. 2013; Chow et al. 2016; Luo et al. 2018). Além disso, os marcadores de ativação de monócitos sCD14 e sCD163 também estão correlacionados com o desenvolvimento e progressão da aterosclerose na população geral e em PVHIV (Aristoteli et al. 2006; Sandler et al. 2011; Pereyra et al. 2012; Fitch et al. 2013; McKibben et al. 2015; Hanna et al. 2017; Subramanya et al. 2019)

Outro marcador de ativação de monócitos relevante para a infecção pelo HIV-1 e a aterosclerose é o fator tecidual (TF). O TF é uma proteína transmembrana expressa principalmente por células perivasculares e epiteliais e que atua como receptor para os fatores de coagulação FVII e FVIIa, formando um dos complexos ativadores básicos da cascata de coagulação no organismo (Grover and Mackman 2018). Em condições de estímulo, monócitos expressam quantidades consideráveis de TF (Egorina et al. 2005; Butenas et al. 2008; Schechter et al. 2017), indicando que em cenários inflamatórios essas células podem ser produtoras de TF e contribuir para os efeitos patogênicos decorrentes dessa inflamação. Valores aumentados de TF são observadas no curso da infecção pelo HIV-1 e também se correlacionam com o desenvolvimento mais rápido da aterosclerose (Funderburg et al. 2012; Barska et al. 2017; Schechter et al. 2017; Luo et al. 2018a; Lin et al. 2019; Hsu et al. 2020). A alta expressão de TF também influencia na ocorrência de trombose associada a ruptura de placas, contribuindo para a patogênese de doenças cardiovasculares (Grover and Mackman 2018).

As correlações entre aumento de TF, aterosclerose e infecção pelo HIV-1 ainda indicam outro processo associado a infecção que pode contribuir para o quadro de susceptibilidade a doença cardiovascular. A infecção pelo HIV-1 é associada a um estado de hipercoagulopatia relacionado a inflamação persistente que pode ser observado através do aumento de expressão de TF e D-dímero, como discutido anteriormente. Além desses dois marcadores, hiper-reatividade e maiores níveis de ativação plaquetária também são observados entre indivíduos infectados pelo HIV-1 (Karmochkine et al. 1998; Satchell et al. 2010; Mayne et al. 2012; O'Brien et al. 2013; Tunjungputri et al. 2014; Nkambule et al. 2015b, 2015c, 2015a; O'Halloran et al. 2015; Pastori et al. 2015; van der Heijden et al. 2018; Mesquita et al. 2018), atingindo níveis mais baixos, porém anormais, mesmo após o tratamento com cART (Tunjungputri et al. 2014; O'Halloran et al. 2015; Mesquita et al. 2018; van der Heijden et al. 2018).

A ativação concomitante de plaquetas e monócitos ainda pode atuar na formação de agregados monócitos-plaquetas (MPA). As plaquetas podem se ligar aos leucócitos através da interação da P-selectina (CD62P) e o ligante 1 da glicoproteína P-selectina (PSGL-1) (Evangelista et al. 1999). Em monócitos, essa interação com plaquetas intensifica o perfil inflamatório dessas células (Thomas and Storey 2015), aumenta a sua adesão ao endotélio (Martins et al. 2006) e estimula a produção de MCP-1 e TNF-α (Weyrich et al. 1995). Devido a contribuição para o processo inflamatório e para a infiltração de monócitos no tecido, frequências aumentadas de MPA são associadas ao desenvolvimento de doenças cardiovasculares, incluindo infarto, derrame e aterosclerose avançada (Furman et al. 2001; Michelson et al. 2001; Htun et al. 2006; Smout et al. 2009; Czepluch et al. 2014). Na infecção pelo HIV-1, frequências aumentados de MPAs também foram observados (Singh et al. 2012; Tunjungputri et al. 2014; Liang et al. 2015; Nkambule et al. 2015b; van der Heijden et al. 2018), indicando mais uma ligação entre coagulação, inflamação e a susceptibilidade a doenças cardiovasculares em PVHIV.

1.3. Os perfis de progressão para aids e controle da infecção pelo HIV-1

A duração da fase crônica da infecção pelo HIV-1 na ausência de tratamento antirretroviral varia entre os indivíduos, caracterizando distintos perfis de progressão para a aids (Figura 4). A maioria dos indivíduos infectados (70% a 80%) progridem para a aids em cerca de oito a 10 anos, sendo denominados progressores típicos. No entanto, uma porcentagem significativa dos indivíduos (10% a 15%) evolui para a aids em um espaço de tempo mais curto, que varia de dois a cinco anos, sendo então chamados de progressores rápidos (Pantaleo and Fauci 1996). Embora nenhum fator determinante e único seja comum a esses indivíduos com pior prognóstico, a progressão rápida já foi associada a infecção com variantes virais com maior capacidade replicativa, maior percentual de vírus X4-trópicos no início da infecção (Dalmau et al. 2014), superinfecção (Gottlieb et al. 2004; Luan et al. 2017), maiores níveis de DNA viral durante a fase aguda (Jiao et al. 2014) (Jarrin et al. 2015), além de fatores genéticos do hospedeiro (Casado et al. 2010; Teixeira et al. 2014).

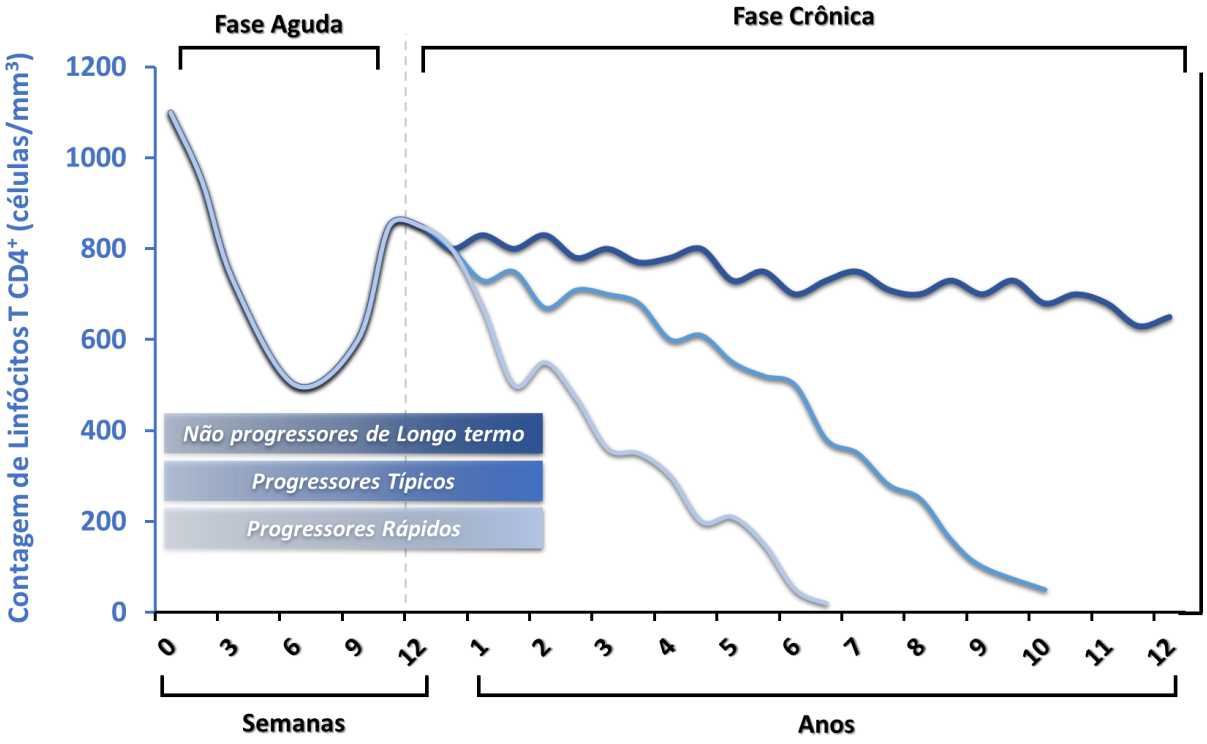

Figura 4. Perfis de progressão para a aids.
O gráfico exemplifica as dinâmicas de contagens de células T-CD4⁺ durante a fase aguda e crônica da infecção pelo HIV-1 que podem ser associadas aos perfis de progressores típicos, rápidos e não progressores de longo termo (LTNPS).

No outro extremo dos perfis de progressão, uma pequena fração de indivíduos infectados (5-10%) permanece clinicamente assintomática e com altos valores de T CD4$^+$ (>500 células/μL) durante um tempo mínimo de acompanhamento de 7 anos de infecção na ausência de tratamento. Estes indivíduos são denominados progressores lentos ou não progressores de longo prazo (LTNP, do inglês long-term non progressors). Alguns indivíduos são capazes de controlar a replicação viral na ausência da terapia antirretroviral, sendo denominados controladores do HIV (HIC, do inglês HIV Controllers) (Figura 5). Devido a raridade deste perfil controlador, diferentes critérios de classificação são observados na literatura. O principal critério de classificação destes é a manutenção de cargas virais plasmáticas baixas (<2000 cópias/mL) ou indetectáveis por no mínimo um ano (Gurdasani et al. 2014). Com base no nível de controle da viremia, estes indivíduos ainda são subdivididos em dois grupos. A maioria apresenta carga viral baixa (>Limite de detecção até 2000 cópias/mL) ao longo de anos e são denominados controladores virêmicos (VC). Uma fração menor desses indivíduos (<1%) apresenta majoritariamente cargas virais plasmáticas indetectáveis pelas metodologias de quantificação mais usadas no monitoramento clínico regular (<40 cópias/mL) e são denominados de controladores de elite (EC). Para alguns desses EC, episódios intermitentes de picos de viremia, denominados de "*blips*" ainda podem ser observados.

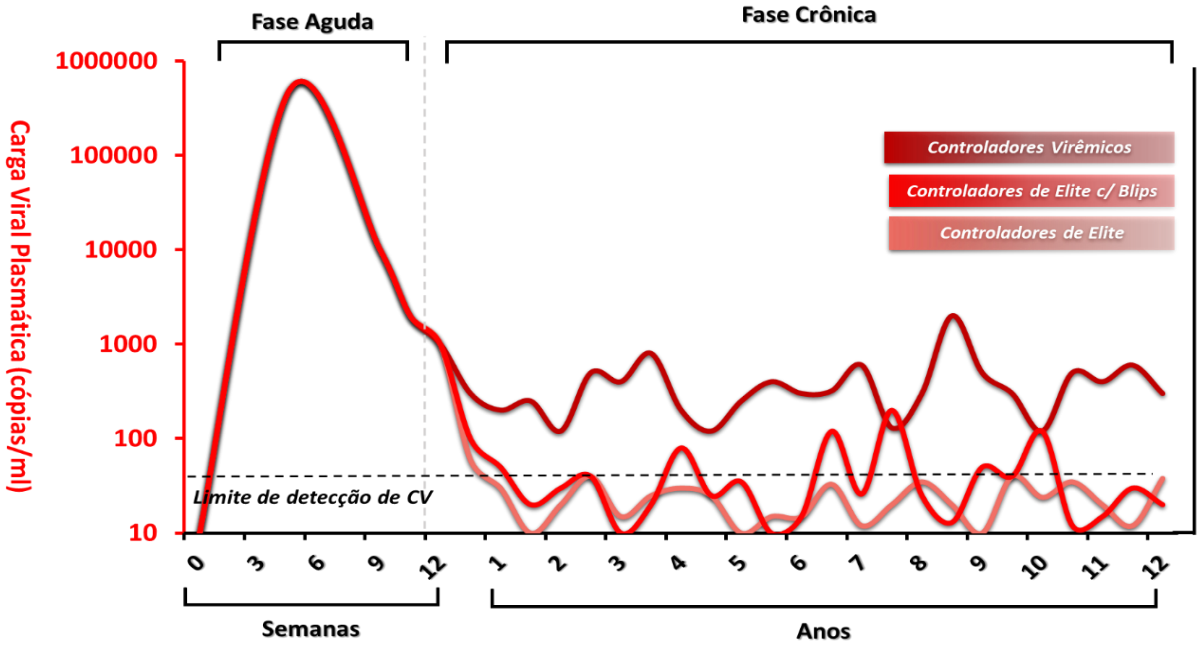

Figura 5. Perfis de controle espontâneo da viremia na infecção pelo HIV-1.
O gráfico exemplifica as dinâmicas de carga viral plasmática durante a fase crônica da infecção pelo HIV-1 em indivíduos Controladores do HIV-1, com diferentes níveis de controle.

Ao longo das últimas décadas algumas coortes principais de HIC foram constituídas, sendo referências para o estudo desses indivíduos, embora utilizem definições diferentes de classificação. Na França, a coorte CO21 CODEX é gerenciada pela Agência Nacional Francesa de Pesquisa sobre o HIV/Aids e as Hepatites Virais (ANRS) e inclui HIC como indivíduos infectados pelo HIV e assintomáticos por pelo menos cinco anos e que apresentem ao menos cinco mensurações de carga viral plasmática consecutivas <400 cópias/mL, na ausência de terapia antirretroviral, independente da contagem de células T $CD4^+$ (Lecuroux et al. 2013). Nos Estados Unidos, o Departamento de defesa americano classificou HIC como indivíduos com níveis de carga viral menores que o limite de detecção (no caso de ECs) ou menores que 2000 cópias/mL (no caso de VCs), por pelo menos um ano (Okulicz et al. 2009). Seguindo a mesma definição, coortes são estudadas por grupos na universidade da California, em São Francisco, (Hunt et al. 2008) e no Hospital Geral de Massachussets (Pereyra et al. 2008). Na Espanha, o Centro Nacional de Microbiologia de Madri identificou LTNP com perfil de EC e VC com os mesmos critérios do Departamento de defesa americano, mas somente mediante comprovação de infecção assintomática por pelo menos 10 anos (Casado et al. 2010). Além disso, um consórcio internacional denominado “HIV Controller Consortium” apresentou estudos entre 2007 e 2015 que reuniram indivíduos controladores de elite de diversas coortes, incluindo as anteriormente citadas (Walker 2007).

Embora seja comum que indivíduos com perfil LTNP apresentem concomitantemente o status de controlador, se faz necessário a separação dos dois perfis visto que existem casos de indivíduos LTNP que não mantêm controle da carga viral plasmática (Casado et al. 2010; Klatt et al. 2014), assim como alguns indivíduos controladores, com carga viral suprimida e que apresentam progressão para a aids num tempo inferior ao de sete anos e queda de células T $CD4^+$ (Okulicz et al. 2009; Noel et al. 2015).

1.3.1. Fatores Associados com a não progressão e o Controle da Viremia

A grande heterogeneidade de definições do perfil de controle da progressão e da replicação viral refletem também a heterogeneidade dos fatores associados com tais perfis. Ao longo das últimas décadas, fatores virológicos, genéticos e imunológicos que contribuem para a proteção durante a infecção foram identificados.

1.3.1.1. Fatores genéticos

Com base em estudos de perfil genético de indivíduos infectados pelo HIV-1, vários fatores já foram associados ao controle da progressão e/ou da replicação viral. Dentre eles, se destacam: a presença de alelos CCR5-Δ32, o qual gera uma proteína truncada que impede a infecção por variantes virais R5-trópicas em indivíduos homozigotos e é associada a progressão mais lenta em indivíduos heterozigotos (Dean et al. 1996; Liu et al. 1996); presença de alelos HLA-B protetores tais como HLA B*57, B*27 e B*52 (Kaslow et al. 1996; Hendel et al. 1999; Altfeld et al. 2003; Teixeira et al. 2014); presença de alelos KIR3DS1 e 3DL1 (Tomescu et al., 2011); e presença de polimorfismos em genes de proteínas celulares que atuam como fatores de restrição do vírus, como APOBEC, Teterina, TRIM-5-alpha, SAMHD-1 e LEDGF/p75 (Messiaen et al. 2012; Santa-Marta et al. 2013; Passaes et al. 2014).

Além desses, já foram descritos perfis de controle associados a polimorfismos de base única (SNP) presentes em genes de receptores tipo Toll (TLR) (Dalmasso et al. 2008; Nissen et al. 2018; Shaikh et al. 2019) e em alelos HLA (Dalmasso et al. 2008; Pereyra et al. 2010; Guergnon et al. 2012; Nissen et al. 2018). Tais polimorfismos afetam processos celulares diversos, como tráfego nuclear e resposta inflamatória (Nissen et al. 2018; Reis et al. 2019). Apesar disso, o caráter facultativo de fatores genéticos associados ao controle, assim como a presença de genes associados ao risco para progressão em alguns HIC/LTNP, demonstra que o fenótipo protetor não é definido apenas por fatores genéticos.

1.3.1.2. Fatores virológicos

O perfil de progressão da doença também pode ser decorrente de características inerentes do vírus. Uma das primeiras descrições de controle espontâneo da infecção foi decorrente da transmissão via transfusões de sangue de variantes virais contendo deleções no gene viral *nef* (Learmont et al. 1992; Deacon et al. 1995; Huang et al. 1995). Desde então, casos isolados de atenuação causada por mutações em *nef* (Kirchhoff et al. 1999; Toro et al. 2004) ou outras variações genômicas virais também foram descritas, incluindo alterações atribuídas a genes acessórios (Michael et al. 1995; Toro et al. 2004; Mologni et al. 2006; Rajan et al. 2006) e estruturais (Huang et al. 1998; Casado et al. 2018). Mais recentemente, uma relação entre uma menor funcionalidade da proteína viral Vif, resultando em falha no antagonismo de

APOBEC3G, um importante fator de restrição viral, também foi associada aos perfis de controle (Cruz et al. 2013; Kikuchi et al. 2015).

Apesar de tais mutações levarem a menor capacidade replicativa, uma grande parte dos indivíduos com perfil de controle espontâneo apresentam vírus competentes para replicação (Blankson et al. 2007; Lamine et al. 2007; Salgado et al. 2014). Além disso, diversos estudos identificaram pares de transmissão entre indivíduos progressores e não progressores (Bailey et al. 2008; Buckheit et al. 2012; Yue et al. 2015), demonstrando que as a presença de alguma destas características virológicas, assim como as genéticas, não são totais determinantes da não progressão ou controle.

1.3.1.3. Fatores Imunológicos

Apesar dos fatores virais e genéticos descritos anteriormente, o perfil imunológico do hospedeiro parece ser a peça essencial que influencia no controle da infecção. Basicamente, HIC e LTNP apresentam características que indicam uma menor descompensação do sistema imune durante a fase crônica da infecção e que os ajudam a driblar os efeitos patogênicos causados pela hiperativação e inflamação.

Em primeiro lugar, o sistema imune de HIC e LTNP parece ser mais eficiente em combater o vírus. Estes indivíduos têm sido caracterizados por apresentarem células T $CD8^+$ HIV-1 específicas com maior capacidade de supressão da replicação viral e maior frequência de polifuncionalidade que a de indivíduos progressores (Betts et al. 2006; Almeida et al. 2007; Saez-Cirion et al. 2009; Ferrando-Martinez et al. 2012). Além disso, estas células em HICs apresentam maior capacidade citotóxica (Migueles et al. 2002; Sáez-Cirión et al. 2007; Taborda et al. 2015), maior resistência a apoptose (Franceschi et al. 1997; Yan et al. 2013) e manutenção da capacidade proliferativa mesmo após um longo tempo de infecção (Migueles et al. 2002; Day et al. 2007). Ademais, também são mais eficientes em reconhecer e matar linfócitos T $CD4^+$ autólogos infectados (Sáez-Cirión et al. 2007; Buckheit et al. 2013), mesmo em condição de infecção latente (Monel et al. 2019), demonstrando sua contribuição para o controle tanto da infecção produtiva, como da infecção latente de reservatórios.

Além dessas características, células T $CD8^+$ de controladores também se diferenciam pelo alvo de suas respostas. A avaliação da resposta citotóxica contra diferentes proteínas virais demonstrou que respostas Gag-específicas são melhor correlacionadas com menores cargas virais e maiores contagens de células T $CD4^+$

(Edwards et al. 2002; Kiepiela et al. 2007; Chen et al. 2009), sendo as principais contribuintes para a resposta citotóxica em indivíduos controladores (Saez-Cirion et al. 2009; Ferre et al. 2010; Berger et al. 2011). A resposta observada contra Gag também se correlaciona diretamente a contribuição de alelos HLA-B considerados protetores, como B*57 e B*27, os quais possuem grande eficiência na apresentação de epítopos imunodominantes de Gag em indivíduos controladores (Chen et al. 2012; Ladell et al. 2013). Embora esta restrição motive o surgimento de variantes virais e de escape da resposta imune, HICs já foram descritos também por apresentarem grande plasticidade de resposta imune, montando novas respostas eficientes contra epítopos mutados rapidamente (O'Connell et al. 2011; Pohlmeyer et al. 2013).

Entre células T $CD4^+$, a manutenção da funcionalidade e particularidades em HIC e LTNP também são observadas. Linfócitos T $CD4^+$ de indivíduos com perfil de controle também apresentam polifuncionalidade, expressando principalmente IFN-γ e IL-2 em resposta a estímulos e maior eficiência de resposta T $CD4^+$ HIV-1 específica (Potter et al. 2007; Owen et al. 2010; Ferrando-Martinez et al. 2012; Van Braeckel et al. 2013). Essas células ainda apresentam manutenção da capacidade proliferativa (Rosenberg et al. 1997; Dyer et al. 2008), da funcionalidade citotóxica em células $CD57^+$ (Phetsouphanh et al. 2019) e menores níveis de expressão de marcadores de exaustão, como PD-1 e a proteína associada a linfócitos T citotóxicos 4 (CTLA-4) (Cockerham et al. 2014; Bansal et al. 2015; Noyan et al. 2018; Saxena et al. 2018), em comparação com células de indivíduos progressores em uso de cART ou não.

Fatores protetores também são observados em relação a proporção das diferentes subpopulações de células T. HIC/LTNP apresentam maiores frequências de linfócitos TCM (Potter et al. 2007; Elrefaei et al. 2014; Klatt et al. 2014; Bansal et al. 2015; Saxena et al. 2017) e Th17 (Ciccone et al. 2011; Salgado et al. 2011; Jenabian et al. 2013; Falivene et al. 2015) em comparação com progressores, indicando maior preservação da homeostase imune. Para linfócitos TCM, menores níveis de DNA proviral nessa subpopulação também são associados com perfis de controle espontâneo (Descours et al. 2012; De Masson et al. 2014).De forma semelhante, o a preservação do repertório de células Treg também parece ser um fator de proteção entre esses indivíduos, visto que LTNP/HIC mantém baixas frequências dessas células durante a fase crônica da infecção(Jiao et al. 2009; Brandt et al. 2011; Jenabian et al. 2013; Falivene et al. 2015). Além das frequências individuais, o balanço entre células Th17 e Treg é mantido, sendo avaliado pela razão entre células

Th17/Treg em indivíduos com perfil de controle espontâneo (Brandt et al. 2011; Jenabian et al. 2013; Falivene et al. 2015).

No contexto de ativação imune, menores frequências de linfócitos ativados são observados em HIC/LTNP em comparação com indivíduos progressores (Hunt et al. 2008, 2011b; Owen et al. 2010; Brandt et al. 2011; Shaw et al. 2011; Ciccone et al. 2011; Card et al. 2012; Bansal et al. 2015; Côrtes et al. 2015; Falivene et al. 2015; Noel et al. 2015; Gonzalez et al. 2016; Saxena et al. 2017). Esta associação, no entanto, depende dos níveis de controle virológico apresentados pelos indivíduos estudados e da correta classificação deles. Normalmente, controladores virêmicos são caracterizados por apresentar níveis mais elevados de ativação imune comparáveis aos de indivíduos progressores (Groves et al. 2012; Gaardbo et al. 2013; Côrtes et al. 2015), enquanto controladores de elite apresentam níveis menores desses marcadores (Hunt et al. 2008, 2011b; Brandt et al. 2011; Ciccone et al. 2011; Kamya et al. 2011; Shaw et al. 2011; Bansal et al. 2015; Côrtes et al. 2015; Falivene et al. 2015).

Além disso, ainda há controvérsias sobre o quanto controladores de elite conseguem manter a ativação imune sob controle na fase crônica da infecção, visto que enquanto alguns estudos identificaram em EC níveis de ativação imune similares aos de indivíduos não infectados pelo HIV (Brandt et al. 2011; Ciccone et al. 2011; Shaw et al. 2011; Bansal et al. 2015; Côrtes et al. 2015), outros observaram níveis elevados comparáveis ou mais altos aos de indivíduos em tratamento (Hunt et al. 2008, 2011b; Kamya et al. 2011; Falivene et al. 2015). Estas divergências podem estar relacionadas aos critérios de classificação dos indivíduos nos diferentes estudos, visto que EC com menores contagens de células T $CD4^+$ (Bansal et al. 2015) ou episódios esporádicos de carga viral (Côrtes et al. 2015) apresentam maiores frequências de células ativadas.

1.3.2. Durabilidade do controle da infecção

A observação de progressão para a aids em alguns HIC levanta dúvida sobre o quão persistente, duradouro e confiável pode ser o controle espontâneo da infecção. HIC/LTNP podem apresentar doença progressiva e experimentar perda do controle virológico, com aumento na replicação viral, e imunológico, com perda de células T $CD4^+$. Entre os fatores contribuindo para a progressão da doença nesses indivíduos,

a carga viral parece ser essencial visto que comumente observa-se o aumento da carga viral em indivíduos com carga viral detectável (Lefrère et al. 1997; Toro et al. 2004; Leon et al. 2016) e da frequência de Blips de carga viral antes da perda de controle (Madec et al. 2013; Noel et al. 2015; Yang et al. 2017). Outros fatores associados a perda do controle espontâneo da infecção, mas que se confundem como causa ou consequência do aumento da carga viral, ainda incluem o surgimento de variantes de escape da resposta imune como resultado da evolução natural do vírus (Goulder et al. 1997; Feeney et al. 2004; Bailey et al. 2007), ocorrência de superinfecção ou dupla infecção (Braibant et al. 2010; Clerc et al. 2010; Pernas et al. 2013) e aumento da ativação imune ou exaustão celular (Hunt et al. 2008; Pernas et al. 2017).

Além da possibilidade de progressão para a aids, a persistência de alguns marcadores inflamatórios e de ativação elevados em alguns HIC também contribui para a dúvida sobre a qualidade e durabilidade do controle espontâneo da infecção nesses indivíduos. Li e colaboradores, em 2015, demonstraram níveis elevados de 15 marcadores inflamatórios, incluindo IP-10, IL-1β TNF-α, sCD14, GM-CSF, IFN-γ, sCD40L, RANTES e MIP-1α, em EC quando comparados com indivíduos em uso de cART (Li et al. 2015). Da mesma forma, marcadores como IP-10, sCD14 e sCD163 são observados em níveis não normalizados em outros estudos com HIC (Brenchley et al. 2006b; Pereyra et al. 2012; Noel et al. 2014; León et al. 2015; Côrtes et al. 2018; Prabhu et al. 2019; Brusca et al. 2020).

A observação de níveis anormais de marcadores de ativação de monócitos, como sCD14 e sCD163, em EC é preocupante devido a correlação entre marcadores inflamatórios e o desenvolvimento de doenças cardiovasculares. Além desses marcadores, níveis aumentados de monócitos $CD16^{+}$ (Spivak et al. 2011; Krishnan et al. 2014; Prabhu et al. 2019), maior expressão de TF (Krishnan et al. 2014) e D-dímero (Kim et al. 2014), alterações no metabolismo lipídico (Vidal et al. 2012; Tort et al. 2018) e maiores valores de cIMT (Hsue et al. 2006; Pereyra et al. 2012; Brusca et al. 2020) já foram descritos em HIC/LTNP. Estudos avaliando a frequência de desfechos clínicos não associados a aids em HIC/LTNP tiveram resultados discordantes, com alguns observando maiores riscos de desenvolvimento desses desfechos para controladores de elite (Crowell et al. 2015) e controladores virêmicos em comparação com indivíduos em cART (Dominguez-Molina et al. 2016), enquanto outros não

observaram diferenças entre os dois grupos (Lucero et al. 2013; Crowell et al. 2016; Noël et al. 2019a).

Apesar de tais indicadores de risco, resultados positivos foram observados em estudos demonstrando que EC apresentam perfil transcriptômico caracterizado por menor expressão de genes associados à vias inflamatórias (Hocini et al. 2019) e que níveis aumentados de marcadores inflamatórios são característicos de indivíduos com menor eficiência de controle da viremia (Noel et al. 2014; Platten et al. 2016; Côrtes et al. 2018).

As correlações entre carga viral e a persistência de marcadores de ativação imune e inflamação elevados em alguns HICs, associado aos estudos demonstrando melhores prognósticos em indivíduos tratados precocemente, levaram a suposição de que esse grupo poderia se beneficiar da cART (Noël et al. 2019b). Estudos avaliando os efeitos da cART em HIC observaram efeitos imunológicos e virológicos associados ao tratamento. Em alguns estudos aumentos na contagem de linfócitos T $CD4^+$ foram observadas (Okulicz et al. 2009; Boufassa et al. 2014), enquanto outros não observaram influência da terapia nesse aspecto (Chun et al. 2013; Hatano et al. 2013b; Kim et al. 2014; Li et al. 2019). Reduções na carga viral plasmática foram observadas na grande maioria dos indivíduos, assim como para a carga viral retal (Hatano et al. 2013b), carga proviral de reservatórios (Chun et al. 2013) e carga viral plasmática avaliada através de metodologias de quantificação ultrassensíveis (Li et al. 2019). Além desses parâmetros, diminuições na frequência de células T ativadas em resposta a terapia também foram observadas (Hatano et al. 2013b; Li et al. 2019). Apesar disso, alguns estudos não observaram nenhum impacto na redução de marcadores inflamatórios, como D-dímero e IL-6 (Hatano et al. 2013b; Kim et al. 2014).

2. JUSTIFICATIVA

No conjunto, os estudos demonstram uma associação entre a infecção pelo HIV-1 e o desenvolvimento de doenças cardiovasculares, principalmente devido a processos associados com altos níveis de ativação imune e inflamação. Essa relação tem sido evidenciada mesmo em estudos incluindo indivíduos com carga viral suprimida por cART e em indivíduos capazes de controlar naturalmente a infecção. No entanto, a raridade destes indivíduos na população infectada pelo HIV, bem como a heterogeneidade nos critérios de classificação de indivíduos capazes de controlar naturalmente a infecção , torna necessário o desenvolvimento de mais estudos para avaliar o impacto da infecção pelo HIV-1 no perfil de ativação imune e de inflamação e sua correlação com o risco de desenvolvimento de comorbidades não associadas a aids nesta população. Além disso, tais indivíduos são considerados um modelo de cura funcional da infecção e a caracterização de diferentes aspectos do sistema imune nos mesmos são uma oportunidade para identificar elementos importantes para a imunopatogênese da infecção, bem como os possíveis determinantes para o controle da infecção a longo prazo. Ainda, os dados provenientes do presente estudo são importantes para subsidiar as discussões atuais acerca da necessidade e da indicação da cART para esses indivíduos com controle espontâneo da replicação viral, movido pelas políticas recentes de oferecimento precoce da cART, ou adoção de tratamentos alternativos que tenham como alvo direto a redução da inflamação e ativação imune.

3. OBJETIVOS

3.1. Objetivo Geral

Caracterizar aspectos relacionados a ativação imune e inflamação em indivíduos com diferentes perfis de controle natural da replicação pelo HIV-1 e sua correlação com marcadores associados ao desenvolvimento de doenças cardiovasculares

3.2. Objetivos específicos

- Comparar as frequências de linfócitos T ativados em indivíduos controladores de elite (EC) e controladores virêmicos (VC) com indivíduos em cART suprimidos e com indivíduos não infectados pelo HIV-1;
- Avaliar as proporções de linfócitos T com perfil naive, de memória, efetor, Th17 e Treg em EC e VC comparados com indivíduos em cART e indivíduos não infectados pelo HIV-1;
- Comparar os níveis de inflamação em indivíduos EC e VC com indivíduos em cART suprimidos e com indivíduos não infectados pelo HIV-1;
- Avaliar os níveis de ativação plaquetária e formação de agregados monócitos-plaquetas nos EC e VC comparados com indivíduos em cART e indivíduos não infectados pelo HIV-1;
- Avaliar a proporção de populações de monócitos, bem como seus níveis de ativação, em EC e VC comparados com indivíduos em cART e indivíduos não infectados pelo HIV-1;
- Verificar se existe uma correlação entre padrões de ativação e inflamação observados nos indivíduos EC e VC e os dados clínicos associados com risco ou ocorrência de doenças cardiovasculares;

4. MATERIAIS E MÉTODOS

4.1. Desenho experimental e populações de estudo

Para o presente trabalho foram utilizadas amostras biológicas de indivíduos que integram uma coorte de controladores do HIV-1 (HICs) identificados e acompanhados no Instituto Nacional de Infectologia Evandro Chagas/Fiocruz (INI-Fiocruz). Este grupo de indivíduos teve infecção pelo HIV-1 diagnosticada há pelo menos cinco anos e apresenta manutenção de cargas virais plasmáticas menores que 2000 cópias/mL, na ausência de cART, durante a maior parte do acompanhamento. Com base na eficiência de controle da carga viral plasmática, esses indivíduos foram ainda classificados em dois grupos: (1) Controladores de Elite (ECs) apresentavam carga viral plasmática mantida abaixo do limite de detecção (<LDL) durante o acompanhamento clínico e laboratorial (<50-80 cópias/mL dependendo do kit comercial de quantificação disponível na época); (2) Controladores Virêmicos (VCs) apresentavam cargas virais plasmáticas >LDL e abaixo de 2000 cópias/mL (> 50-80 a > 2000) em pelo menos 90% das mensurações realizadas durante o acompanhamento. Medidas ocasionais de carga viral acima do limite de ambos os grupos foram aceitas durante o acompanhamento, desde que não ultrapassassem 30% das mensurações.

A partir dessa coorte, dois subgrupos foram selecionados com base na disponibilidade de amostras ou disponibilidade para nova coleta para diferentes etapas do presente projeto. Para cada subgrupo de HICs, dois grupos controle, um composto por indivíduos infectados pelo HIV-1 em tratamento com cART por pelo menos dois anos e com carga viral suprimida (cART) e outro por indivíduos não infectados pelo HIV-1 (HIVneg), foram recrutados independentemente. Os dois subgrupos se caracterizam da seguinte forma:

- O subgrupo (A) incluiu 14 ECs e 13 VCs que apresentavam amostras de PBMCs criopreservadas disponíveis em biorrepositório e que foram utilizadas para avaliação ativação imune e de frequência de subpopulações. Para comparação dos resultados obtidos com HICs, um grupo de 18 indivíduos cART e um grupo de 18 indivíduos HIVneg foram utilizados como grupos controle.

- O subgrupo (B) incluiu oito ECs e cinco VCs que aceitaram realizar nova coleta de sangue direcionada a análise dos níveis de ativação plaquetária, subpopulações de monócitos e marcadores inflamatórios plasmáticos. Para comparação dos resultados obtidos para esse subgrupo, novos grupos de controles, cART (n=18) e HIVneg (n=18) foram utilizados. No momento de coleta de material biológico para o estudo, todos os participantes foram submetidos a medida de altura, peso, circunferência abdominal, pressão sanguínea e frequência respiratória e cardíaca. Os participantes também foram entrevistados para avaliação de risco para doenças cardiovasculares e diabetes (incluindo histórico familiar e doenças prévias), regularidade de atividade física, hábitos de fumo e uso de drogas, além da avaliação de padrões de risco ou prejuízo pessoal relacionado ao consumo de álcool através do questionário AUDIT (Alcohol Use Disorders Identification Test). Exames clínicos laboratoriais incluindo hemograma completo, hepatograma, lipidograma e medidas dos níveis de glicose e creatinina também foram realizados para todos os indivíduos.

O desenho experimental resumindo os grupos de estudo e as análises realizadas para cada subgrupo estão representados na Figura 6. Todas as análises referentes ao subgrupo A foram aprovadas para serem conduzidas pelo Comissão Nacional de Ética em Pesquisa (Registro CONEP 14430, Parecer 001/2011) e pelo Comitê de Ética em pesquisa do Instituto Nacional de Infectologia Evandro Chagas (CAAE 1717.0.000.009-07), enquanto as análises referentes ao subgrupo B foram aprovadas pelos Comitês de Ética em pesquisa do Instituto Oswaldo Cruz (CAAE 56306116.6.0000.5248) e do Instituto Nacional de Infectologia Evandro Chagas (CAAE 56306116.6.3001.5262). Todos os indivíduos recrutados para ambos os subgrupos forneceram consentimento por escrito para participação dos estudos.

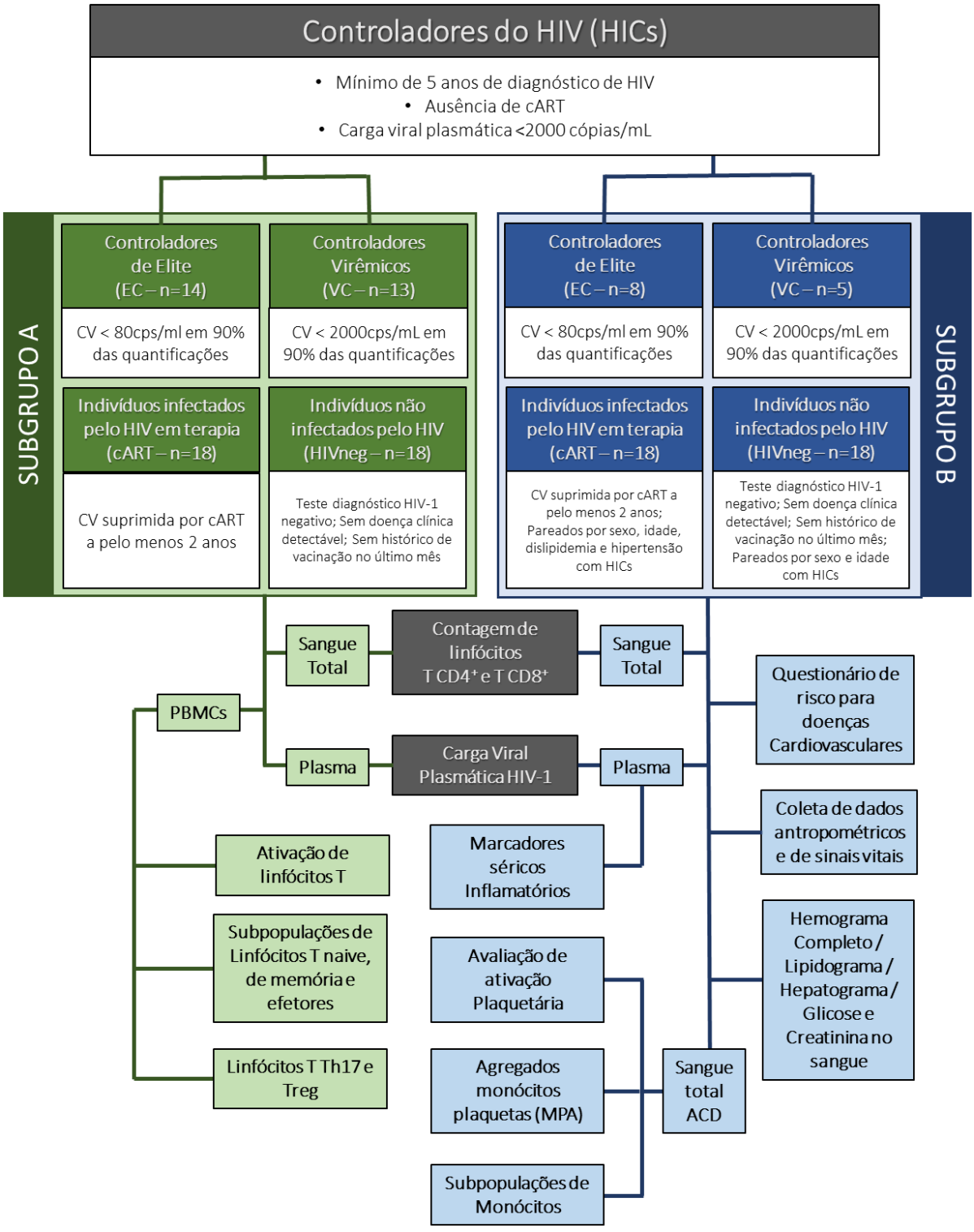

Figura 6. Desenho experimental do presente estudo.
Grupos analisados e os critérios de inclusão de participantes em cada grupo e subgrupo estão representados nos fluxogramas, bem como as análises realizadas a partir das amostras obtidas.

4.2. Preparo e coleta de amostras

Para ambos os subgrupos, os indivíduos foram submetidos a coleta de sangue em tubos a vácuo contendo EDTA e heparina. O sangue coletado foi utilizado para realização de exames clínico-laboratoriais, quantificação de carga viral plasmática do HIV-1, contagem de linfócitos T $CD4^+$, obtenção de alíquotas de plasma e sangue total e para isolamento de células mononucleares do sangue periférico (PBMCs).

As PBMCs foram isolados a partir do sangue total utilizando centrifugação em gradiente de densidade com histopaque-1077, como descrito anteriormente (Côrtes et al. 2013), e armazenados em nitrogênio líquido até o uso.

Indivíduos do subgrupo B foram ainda orientados para realização de um período de jejum de pelo menos oito horas antes da coleta do sangue em tubos contendo heparina e EDTA. Visando impedir a ativação de plaquetas durante o processamento, estes indivíduos também tiveram amostras de sangue coletadas por punção venosa lentamente em uma seringa contendo 15% volume a volume (v/v) de ACD (38mM Citrato, 85mM citrato de sódio, 135mM Dextrose anidra; pH 5,1) pré-aquecido a 37ºC. Amostras de sangue em ACD foram processadas em menos de uma hora após coleta.

4.3. Contagem de Linfócitos T $CD4^+$ e T $CD8^+$ e Quantificação de carga viral plasmática

Contagens absolutas de linfócitos T $CD4^+$ e $CD8^+$ foram obtidas a partir do sangue total utilizando o kit MultiTest TruCount-kit e o software MultiSet no citômetro de fluxo FACSCalibur (BD Biosciences, EUA). A carga viral plasmática do HIV-1 das amostras correspondentes a coleta realizada no presente estudo foi mensurada usando o kit Abbott RealTime HIV-1 assay (Abbott Laboratories, EUA).

4.4. Ativação e frequência de subpopulações de células T

Para as análises referentes a ativação de células T e frequências de subpopulações de células T, alíquotas de PBMCs criopreservadas de indivíduos do subgrupo A contendo cerca $1x10^7$ células com viabilidade >85% foram descongeladas, lavadas com RPMI 1640 (Sigma-Aldrich, EUA) suplementado com 10% de soro fetal bovino (SFB, Gibco - Thermo Fisher Scientific, EUA) e incubados durante a noite a 37ºC em estufa contendo 5% de CO_2 e umidade controlada.

Para avaliação de subpopulações de linfócitos naive, de memória, efetores e ativados entre células $CD4^+$ e $CD8^+$, 1-2 x 10^6 PBMCs foram marcados por 10 minutos com 0,125ul de FVS450 (BD Biosciences, EUA) para exclusão de células mortas. Após, as células foram lavadas com PBS-SFB 2% e marcadas com anticorpos anti-CD3 APC-H7, anti-CD4 PE-CF594, anti-CD8 APC, anti-CD45RA PE-Cy7, anti-CD27 BV510, anti-CCR7 Alexa Fluor 700, anti-CD95 PerCP-Cy5.5, anti- HLA-DR PE e anti-CD38 BB515 (todos da BD Biosciences, EUA) por 30 minutos. Após a marcação, as células foram lavadas com PBS-SFB 2%, fixadas com PBS-PFA 1% e posteriormente adquiridas no citômetro de fluxo BD FACSAria™ IIu (BD Biosciences, EUA) e analisadas usando o programa FlowJo v10. Um resumo dos anticorpos utilizados está disponível na Tabela Suplementar 1 (Anexo 9.1).

Para as análises de células T (Figura 7), os linfócitos vivos foram selecionados com base nos parâmetros de tamanho, granulosidade e expressão de CD3/FVS. O status de ativação de células T em ambos os compartimentos $CD4^+$ e $CD8^+$ foi avaliado baseado na coexpressão de CD38 e HLA-DR. As subpopulações naive, de memória e efetoras em ambos os compartimentos foram identificadas com base na expressão diferencial de CD45RA, CCR7, CD27 e CD95, sendo classificadas como: naïve (TN: $CD45RA^+CCR7^+CD27^+$ $CD95^-$), tronco de memória (TSCM: $CD45RA^+CCR7^+CD27^+CD95^+$), memória central (TCM:$CD45RA^-CCR7^+CD27^+$), memoria transicional (TTM:$CD45RA^-CCR7^-CD27^+$), memória efetora (TEM:$CD45RA^-CCR7^-CD27^-$), e efetora ou terminalmente diferenciada (TEFF:$CD45RA^+CCR7^-CD27^-$) (Figura 7). Controles Fluorescência menos um (FMO) foram utilizados para identificar as populações $CD45RA^+$, $CCR7^+$, $CD27^+$, $CD38^+$ e $HLA\text{-}DR^+$.

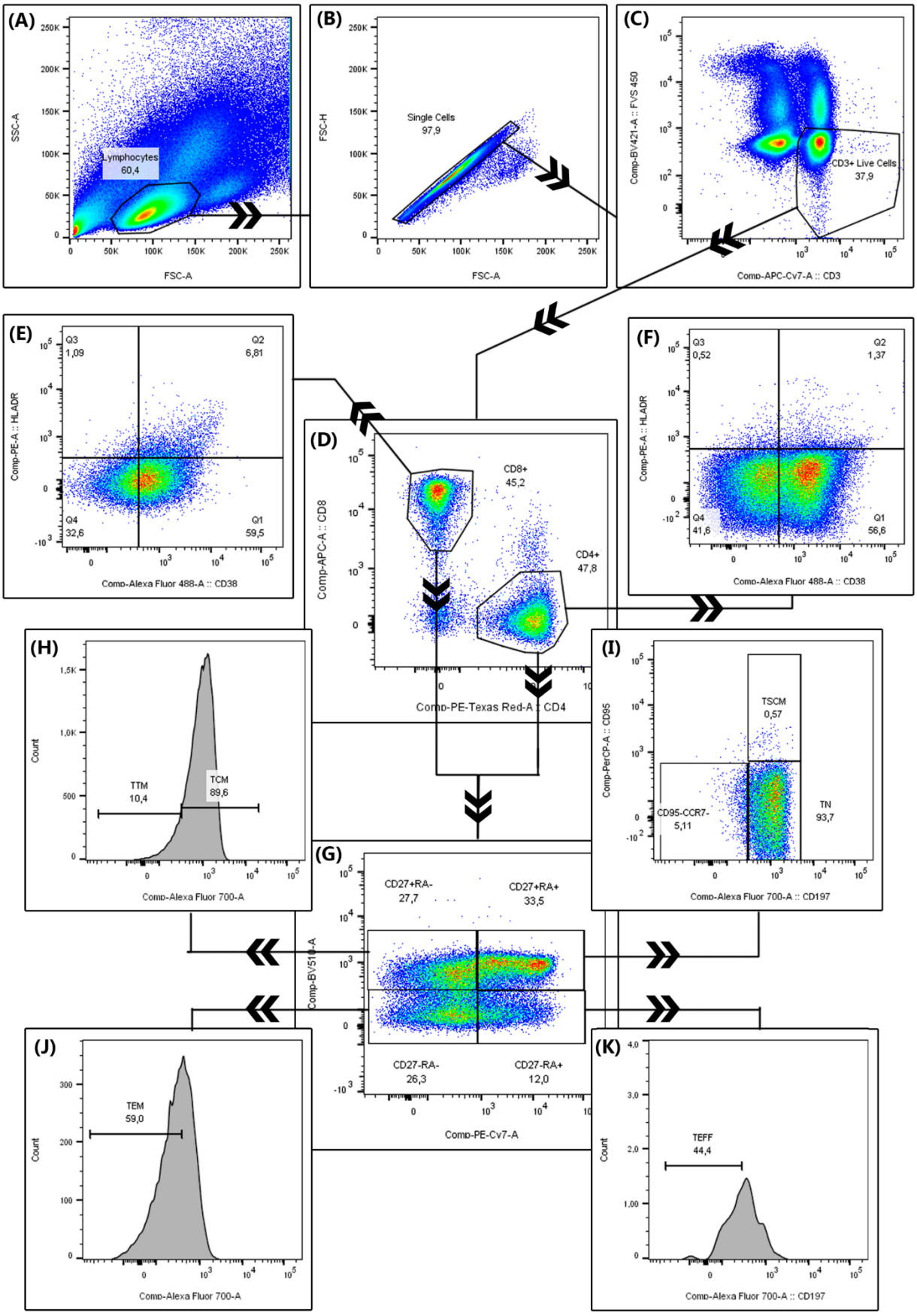

Figura 7. Estratégias de gate para análise de ativação e subpopulações naive, de memória e efetoras por citometria de fluxo.

Linfócitos foram identificados pelos padrões característicos de forward e side scatter seguido da seleção de células vivas FVS- e CD3+. Linfócitos ativados foram caracterizados como células CD38+HLA-DR+ nos compartimentos CD4+ e CD8+. As subpopulações TN, TSCM, TCM, TTM, TEM, e TEFF foram identificadas através da expressão diferencial de CD27, CD45RA, CD95 e CCR7. As setas representam o fluxo seguido para as análises.

Para determinação das frequências de células Treg e Th17, 5 x 10^6 PBMCs foram estimuladas por 5 horas com 50 ng/ml phorbol myristate acetate (PMA) e 1 µg/ml de ionomicina (Sigma-Aldrich, EUA) na presença de Golgi Stop (Human Th17/Treg Phenotyping Kit; BD Biosciences, EUA), conforme recomendado pelo fabricante. As células foram então marcadas com FVS450 e anticorpos anti-CD25 BB515 e anti-CD8 BV510 (Todos da BD Biosciences, EUA), como citado anteriormente. Posteriormente, as células foram lavadas com PBS-SFB 2% e fixadas usando o Human FoxP3 Buffer A (Human Th17/Treg Phenotyping Kit; BD Biosciences, EUA). Em seguida, as células foram lavadas e incubadas com PBS-SFB 2% a 4ºC durante a noite. Por fim, as células foram permeabilizadas usando o Human FoxP3 Buffer C (Human Th17/Treg Phenotyping Kit; BD Biosciences, EUA) e marcadas com anticorpos anti-CD3 APC-H7, anti-CD45RA PeCy7 e com o Human FoxP3 cocktail (Human Th17/Treg Phenotyping Kit; BD Biosciences, EUA). As amostras foram adquiridas no citômetro de fluxo BD FACSAria™ IIu (BD Biosciences, EUA) e analisadas usando o programa FlowJo v10. Um resumo dos anticorpos utilizados está disponível na Tabela suplementar 1 (Anexo 9.1). Células Th17 foram definidas como células T $CD4^+IL17^+$, enquanto células Treg foram definidas como linfócitos T $CD4^+CD25^{high}Foxp3^+$ (Figura 8). Subpopulações de células Treg foram ainda classificadas como Treg ativada ($CD45RA^-Foxp3^{high}$), Treg não supressora ($CD45RA^-Foxp3^{low}$) e Treg em repouso ($CD45RA^+Foxp3^{low}$). Controles FMO foram utilizadas para identificar as populações $CD45RA^+$, $CD25^+$, $IL17^+$ e $Foxp3^+$.

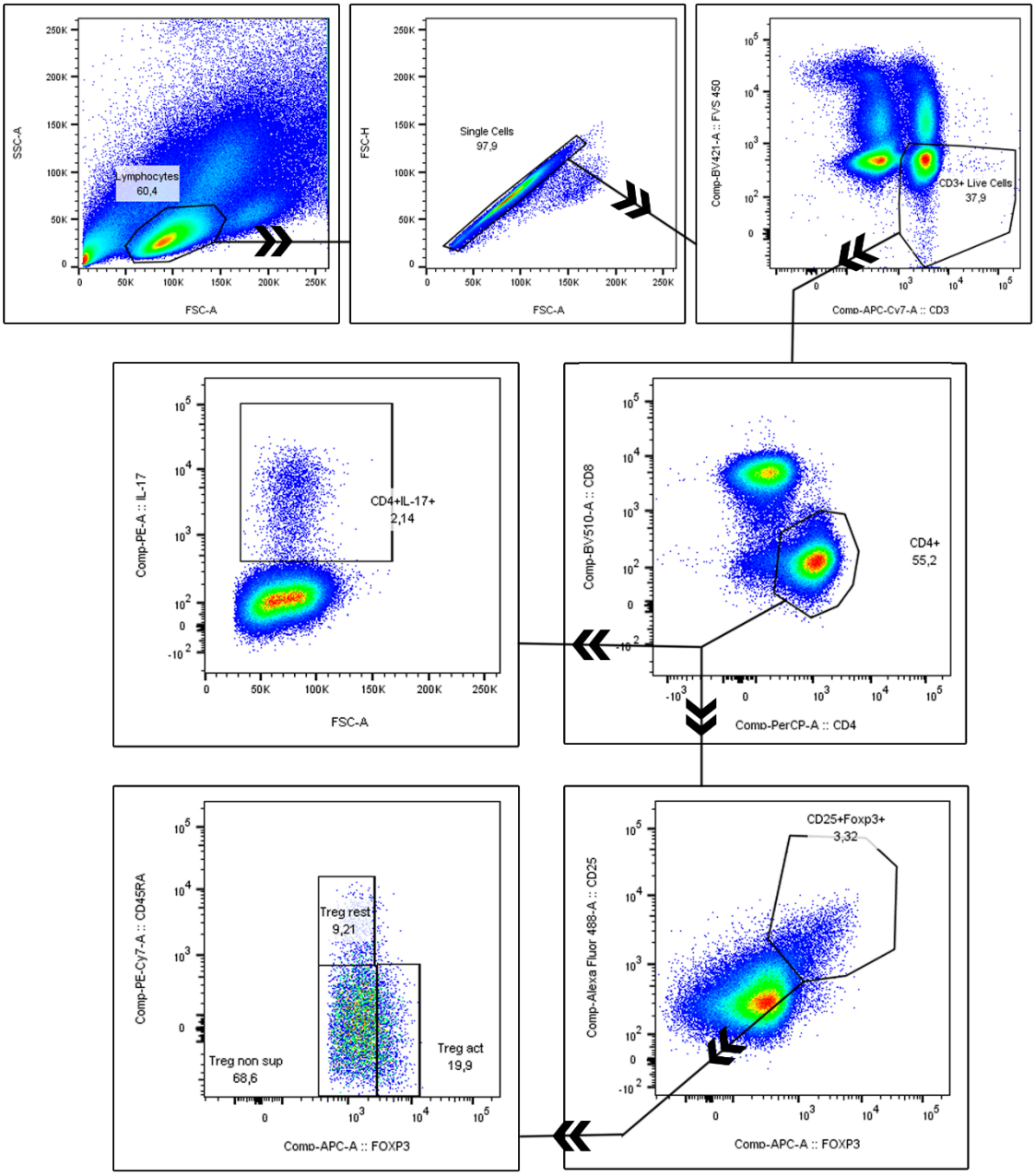

Figura 8. Estratégia de gate para identificação de linfócitos Treg e suas subpopulações e de linfócitos Th17 por citometria de Fluxo.
Linfócitos foram identificados pelos padrões característicos de forward e side scatter seguido da seleção de células viáveis FVS- e CD3+. Células Th17 foram identificadas como células CD4+IL17+. Células Treg totais foram identificadas com base na coexpressão de CD25 e FoxP3 na população CD4+. Células Treg ativadas, latentes e não supressoras foram ainda identificadas com base na expressão diferencial de FOXP3 e CD45RA na população de Treg totais. As setas representam o fluxo seguido para as análises.

4.5. Ativação plaquetária, agregados monócitos-plaquetas e subpopulações de monócitos

Para análises referentes a frequência de plaquetas ativadas, agregados monócitos plaquetas e as diferentes subpopulações de monócitos, foram utilizadas amostras de

sangue coletadas em ACD pertencentes aos indivíduos do subgrupo B. As amostras foram processadas logo após a coleta.

Para análises de ativação plaquetária, 20µl de sangue total coletado em ACD foi diluído 10x em tampão Hepes-Tyrode (137 mM NaCl, 2.8 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 12 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 5.5 mM glucose, 0,35% SFB, pH 7,4) e incubado a 37ºC por 30 minutos a temperatura ambiente. Em paralelo, uma replicata foi estimulada com160nM de PMA e incubada pelo mesmo período. Após a incubação, 15uL das amostras foram marcadas com anticorpos anti-CD41 APC e anti-CD62P PE por 20 minutos a temperatura ambiente. Amostras marcadas foram fixadas com 400 uL de PBS-PFA 4% e armazenadas a 4ºC até o momento de aquisição em citômetro de fluxo FACS Calibur (BD Biosciences, EUA). Um resumo dos anticorpos utilizados está disponível na Tabela suplementar 1 (Anexo 9.1).

As plaquetas foram identificadas com base no tamanho e granulosidade. A frequência de plaquetas ativadas foi observada mediante análise de coexpressão de CD41 e CD62P (*Figura 9*). Os limites entre a população positiva e negativa para CD62P foi definido com base na comparação entre amostras estimuladas e não estimuladas.

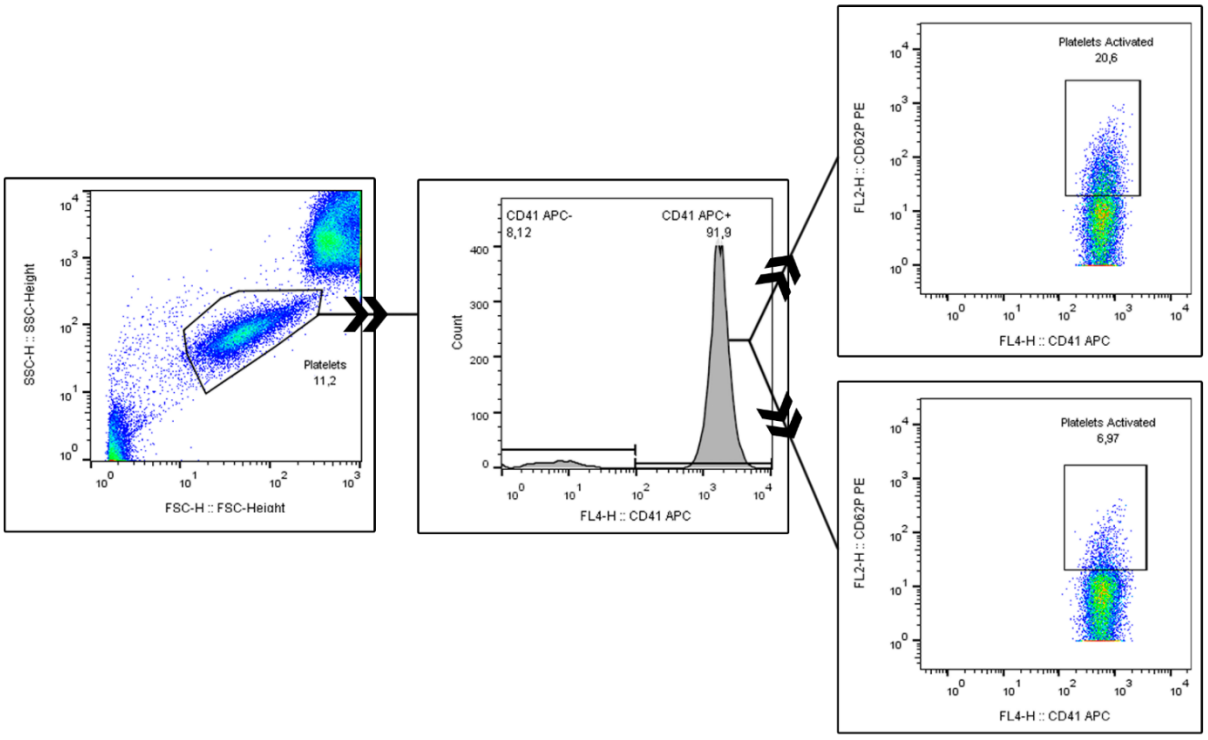

Figura 9. Estratégia de gate para identificação de plaquetas ativadas (CD62P⁺) por citometria de fluxo.
Plaquetas totais foram identificadas pelos padrões característicos de forward e side scatter seguido da seleção de células CD41⁺. Amostras estimuladas com PMA foram comparadas com amostras não estimuladas para delimitar as populações CD62P⁺ e CD62P-. As setas representam o fluxo seguido para as análises.

Para análise de agregados monócitos-plaquetas, 1mL de sangue total coletado em ACD foi lisado por 10 minutos com a solução de lise de hemácias (BD FACS Lysing Solution, BD Biosciences) e centrifugada a 200g por 15 minutos sem aplicação de freio. O precipitado obtido foi cautelosamente suspendido em 250 µl de tampão Hepes-Tyrode e marcado com anticorpos anti-CD3 FITC, anti-CD41 APC e anti-CD14 PercpCy5.5 por 20 minutos a temperatura ambiente. Amostras marcadas foram fixadas com tampão de lise e armazenadas a 4ºC até o momento de aquisição em citômetro de fluxo FACS Calibur (BD Biosciences, EUA). Um resumo dos anticorpos utilizados está disponível na Tabela suplementar 1 (Anexo 9.1).

Para avaliação de subpopulações de monócitos, 200 µl de sangue total foi lisado por 10 minutos com solução de lise. As células foram lavadas com PBS-SFB 5% e marcadas por 30 minutos com anticorpos anti-CD3 FITC, anti-CD14 Percp-Cy 5.5, anti-CD16 APC e anti-CD142 PE. As células marcadas foram lavadas com PBS-SFB 2%, fixadas com PBS-PFA 4% e armazenadas a 4ºC até o momento de aquisição em citômetro de fluxo FACS Calibur (BD Biosciences, EUA). Um resumo dos anticorpos utilizados está disponível na Tabela suplementar 1 (Anexo 9.1).

Para as análises de subpopulações de monócitos e agregados monócitos plaquetas (Figura 10), as quais foram realizadas no software Flowjo, a população de monócitos foi identificada com base no tamanho, granulosidade e filtrada a partir da ausência de expressão de CD3. Agregados monócitos plaquetas foram identificados com base na coexpressão de CD14 e CD41. As subpopulações de monócitos por sua vez foram identificadas a partir da expressão diferencial de CD14 e CD16, sendo classificadas como: monócitos não clássicos ($CD3^{-}CD14^{+}CD16^{+}$), intermediários ($CD3^{-}CD14^{++}CD16^{+}$) e clássicos ($CD3^{-}CD14^{++}CD16$-). Os limites entre cada subpopulação foram definidos com base em amostras FMO para CD14 e CD16.

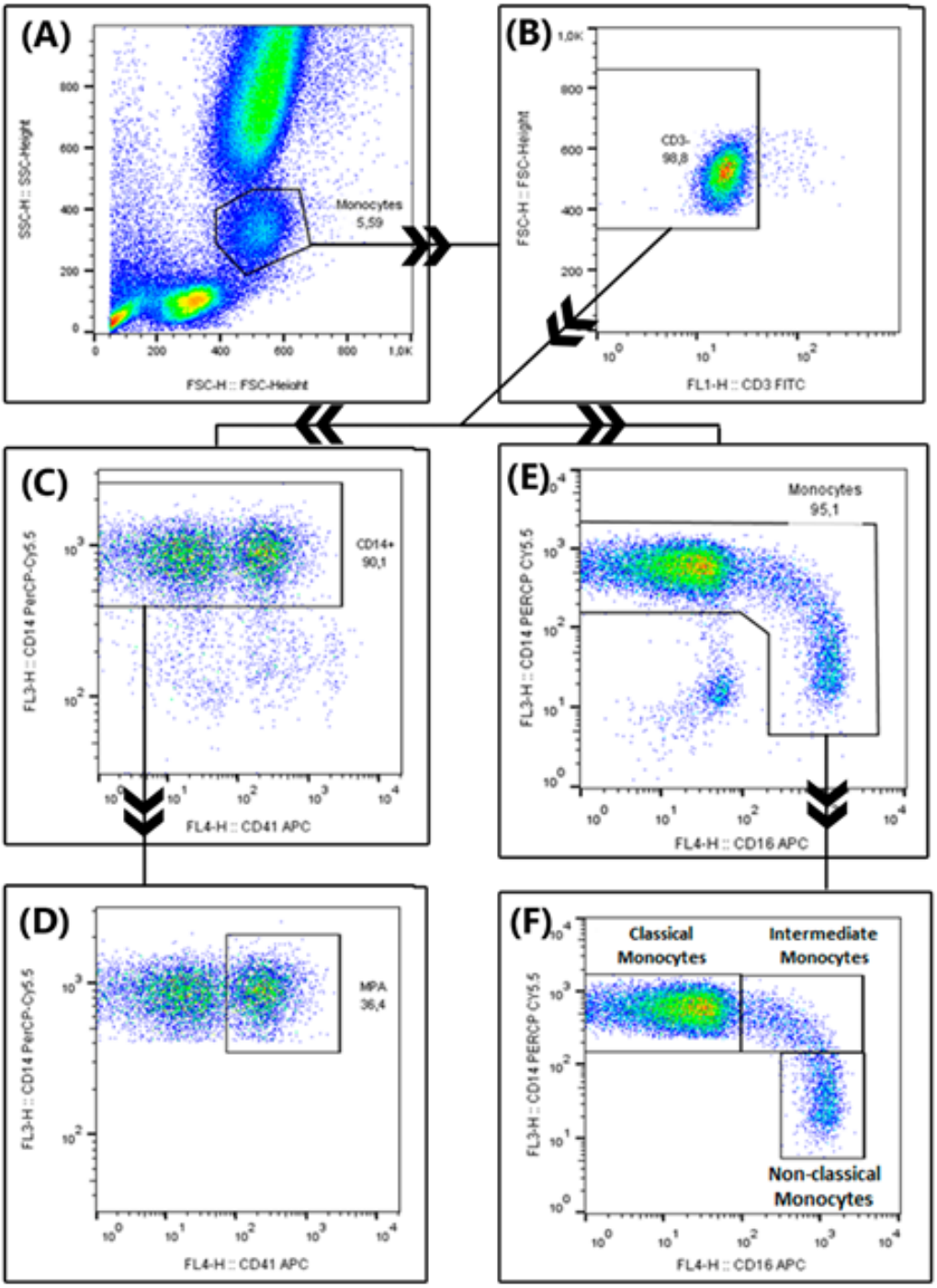

Figura 10. Estratégia de gate para identificação de agregados monócitos-plaquetas e de subpopulações de monócitos por citometria de fluxo.
Monócitos totais foram identificados pelos padrões característicos de forward e side scatter (A), seguido da seleção de células CD3- (B). MPAs foram caracterizados como células $CD14^{+}CD41^{+}$dentro da população de monócitos $CD14^{+}$ (C e D). Um gate de exclusão para células CD14-CD16- foi realizado para delimitar apenas monócitos e as frequências de monócitos clássicos, intermediários e não clássicos foi definido com base na expressão de CD14 e CD16 (E e F). As setas representam o fluxo seguido para as análises.

4.6. Perfis de Inflamação

Os níveis séricos dos marcadores IL-6, IL-10, CD40L, MCP-1, TNF-α, D-Dimer, ST2, VCAM-1 e ICAM-1 dos indivíduos do subgrupo B foram avaliados através de

Luminex usando um imunoensaio customizado ProcartaPlex multiplex (Invitrogen, EUA) de acordo com as instruções dos fabricantes. Amostras marcadas foram analisadas em um instrumento MAGPIX (Luminex Corp, EUA).

4.7. Estatística

O Risco Framingham de 10 anos para o desenvolvimento de doenças cardiovasculares foi avaliado através das calculadoras baseadas no estudo de D'Agostino e colaboradores, em 2008, e disponibilizadas pelo website do "Framingham Heart Study" (Framingham Heart Study, 2020). Os modelos utilizados levaram em conta informações de idade, sexo, pressão sistólica, tratamento para hipertensão, diabetes e fumo. Alternativamente, um modelo de cálculo foi complementado com os valores de IMC, enquanto o outro com valores de colesterol total e LDL.

Na avaliação das características sociodemográficas, clínicas e laboratoriais entre os diferentes grupos de HICs, cART e indivíduos não infectados pelo HIV-1, para variáveis numéricas contínuas, foram utilizados os testes Kruskal-Wallis ANOVA por Ordenação para avaliar a hipótese de que as diferentes amostras na comparação foram retiradas da mesma distribuição ou distribuições com a mesma mediana. Da mesma forma, para as variáveis nominais categóricas, foram utilizados os testes exatos de Fisher para avaliar as frequências entre os diferentes grupos para avaliar a hipótese de independência entre os grupos de indivíduos e estas variáveis. Além disso, as análises do coeficiente de correlação de classificação de Spearman foram estimadas para variáveis numéricas contínuas. Comparações em pares de médias dos parâmetros analisados entre grupos de interesse foram realizadas usando testes de Mann Whitney ou contrastes obtidos de modelos bi- e multivariados lineares ajustados por regressões ordinárias de mínimos quadrados. Para as análises ajustadas, os valores P foram corrigidos pelo método de Tukey Honest Significant Difference (HSD). As variáveis de confusão foram selecionadas por modelos lineares bivariados ajustados por regressões ordinárias de mínimos quadrados e incluídas em modelos multivariados se algum P-valor ajustado <0,2 para eliminar o viés amostral. Construção de gráficos e análises de Mann Whitney foram realizadas no software Graphpad Prism v7, enquanto o restante das análises estatísticas foi realizado no software R v. 3.6.1.

5. RESULTADOS

5.1. Características clínicas e demográficas dos grupos estudados

As características clínicas e demográficas dos indivíduos do subgrupo A encontram-se descritas na Tabela 1. Nenhuma diferença significativa de idade foi encontrada entre os grupos, no entanto a frequência de homens foi significativamente maior entre ECs, em comparação com VCs e cART (21% vs 69% vs 61%). A mediana de carga viral plasmática em VCs foi de 450 cópias/mL, enquanto níveis indetectáveis foram observados para todos os indivíduos dos grupos EC e cART. Contagens mais altas de linfócitos T $CD4^+$ foram observadas em ECs em comparação com cART (p = 0.0079). Perfis detalhados de linfócitos T $CD4^+$ e carga viral plasmática para os ECs e VCs durante o acompanhamento de longo termo foram previamente descritos (de Azevedo et al. 2017; Caetano et al. 2018). As medianas de tempo de diagnóstico de HIV-1 para ECs e VCs foi de 8,5 e 10,4 anos, respectivamente.

Tabela 1. Características clínicas e demográficas dos HICs incluídos no subgrupo A e seus respectivos grupos controle.

	HIVneg (n = 18)	**cART (n = 18)**	**EC (n = 14)**	**VC (n = 13)**
Idade (mediana [Q1-Q3])	37.1 [29.80-49.55]	44.5 [38.28-50.05]	42.8 [37.60-58.8]	42.7 [37.60-47.05]
Sexo (%Masculino)	50	61	**21**	69
Carga viral plasmática (copias/ml, mediana [Q1-Q3])	N/A	<40 [<40]	<40 [<40-87]	450 [224.0-881.5]
Contagem de linfócitos T $CD4^+$ (células/mm^3), mediana [Q1-Q3]	831 [741.80-1227]	853[a] [745-1006]	**1165**[a] **[888-1486]**	830 [605-1365]
Anos de diagnóstico de HIV-1 (mediana [Q1-Q3])	N/A	10.9 [8.7-15.1]	8.5 [4.0-15.4]	10.4 [5.4-14.55]

HIVneg: Indivíduos não infectados pelo HIV-1; EC: Controladores de Elite; VC: Controladores virêmicos; cART: Indivíduos infectados pelo HIV-1 em tratamento com cART. N/A: Não se aplica. Q1-Q3- Quartis 1 e 3 O P-valor foi obtido usando o teste Mann-Whitney. [a] p = 0.0079, comparando os grupos EC e cART.

Para os indivíduos do subgrupo B, as características clínicas e demográficas encontram-se descritas na Tabela 2. Diferenças significativas de idade, sexo e raça não foram encontradas para este segundo subgrupo. As medianas de tempo de diagnóstico para os grupos EC e VC foram de nove e 14 anos, respectivamente. Entre os indivíduos do grupo controle cART, a mediana de tempo de diagnóstico foi de 17 anos, com uma mediana de 11 anos de tratamento.

As contagens de linfócitos T $CD4^+$ foram similares entre os grupos, porém VCs e indivíduos tratados possuíam maiores contagens de linfócitos T $CD8^+$. VCs possuíam mediana de razão CD4/CD8 abaixo de 1, enquanto os grupos EC, cART e HIVneg apresentavam razões acima de 1, com este último grupo apresentando as maiores razões ($p<0,03$). A mediana de carga viral plasmática para VCs foi de 700 cópias/mL. Todos os indivíduos do grupo EC e cART apresentaram cargas virais <40 cópias/mL.

Para os indivíduos cART, os esquemas terapêuticos utilizados por cada indivíduo no momento de seu recrutamento estão disponíveis na Tabela suplementar 2, disponível no anexo 9.2. Os esquemas terapêuticos mais prevalentes foram combinações de dois Inibidores da transcriptase reversa nucleosídeos (ITRN) com 1 inibidor da transcriptase reversa não nucleosídeo (ITRNN) (44%) e de 2 Inibidores de transcriptase reversa nucleosídeos com 2 inibidores de protease (IP) (22%). Em resumo, 89% dos indivíduos estavam em uso de combinações duplas de inibidores da transcriptase reversa nucleosídeos (ITRN), com 81% desses em uso de Tenofovir/Lamivudina (TDF/3TC), enquanto 33% utilizavam combinações de dois Inibidores da protease, com 66% desses em uso de Darunavir/Ritonavir (DRV/RTV).

Nos grupos cART e EC, alguns indivíduos apresentavam histórico de doença cardíaca prévia. No grupo EC, uma mulher com idade avançada sofreu de insuficiência valvar aórtica, sendo submetida a cirurgia de troca da válvula há 9 anos. No grupo cART, pacientes com eventos cardiovasculares incluíam dois homens com histórico de infarto agudo do miocárdio, dois homens com histórico de cardiopatia isquêmica, uma mulher com histórico de insuficiência valvar aórtica e uma mulher com miocardiopatia dilatada.

Em relação a fatores de risco para doenças cardiovasculares, não houve diferença estatística para a frequência de hipertensos, diabéticos ou fumantes entre os quatro grupos, embora a frequência de diabéticos e fumantes fosse maior entre indivíduos do grupo cART e VC.

Tabela 2. Características clínicas e demográficas dos HICs incluídos no subgrupo B e seus respectivos grupos controle.

	HIVneg (n=18)	cART (n=18)	EC (n=8)	VC (n=5)	P-valor
Idade [mediana de anos (Q1-Q3)]	48 (40 - 53)	52 (47 - 57)	44 (40 - 48)	46 (42 - 54)	0,225
Sexo [%Masculino]	44,4%	61,1%	37,5%	60,0%	0,617
Raça					
- Branca [n (%)]	12 (66,7%)	9 (50%)	1 (12,5%)	1 (20%)	0,190
- Negra [n (%)]	2 (11,1%)	3 (16,7%)	3 (37,5%)	2 (40%)	
- Parda [n (%)]	4 (22,2%)	6 (33,3%)	4 (50%)	2 (40%)	
Parâmetros Clínicos da infecção pelo HIV-1					
- Tempo de diagnóstico [mediana de anos (Q1-Q3)]	N/A	17 (11 - 22)	9 (7 - 10)	14 (13 - 21)	**0,014**
- Tempo de cART [mediana de anos (Q1-Q3)]	N/A	11 (10 - 19)	N/A	N/A	NA
- Carga Viral [cópias/ml, mediana (Q1-Q3)]	N/A	<40	<40	700 (357 - 1480)	**p<0,001**
- CD4 [células/mm^3, mediana (Q1-Q3)]	1069 (841 - 1234)	1033 (860 - 1317)	982 (972 - 1158)	1043 (670 - 1262)	0,918
- CD8 [células/mm^3, mediana (Q1-Q3)]	571 (396 - 750)	967 (845 - 1074)	616 (550 - 734)	1087 (848 - 1104)	**0,003**
- Razão CD4/CD8 [mediana (Q1-Q3)]	1,88 (1,62 - 2,38)	1,11 (0,96 - 1,12)	1,46 (1,16 - 1,85)	0,84 (0,67 - 1,23)	**0,027**
Doença cardiovascular					
- Hipertensão [n (%)]	3 (16,7%)	10 (55,6%)	2 (25%)	2 (40%)	0,092
- Doença Cardiovascular prévia [n (%)]	0 (0%)	6 (33,3%)	1 (12,5%)	0 (0%)	**0,027**
- Histórico Familiar [n (%)]	13 (72,2%)	14 (77,8%)	6 (75%)	5 (100%)	0,621
- Risco Framingham (IMC) [% (Q1-Q3)]	4,55% (3% - 10%)	33% (15% - 42%)	7% (4% - 9%)	14,8% (6% - 16%)	**0,037**
- Risco Framingham (Colesterol) [% (Q1-Q3)]	3,8% (2% - 7%)	16,7% (11% - 21%)	4,4% (4% - 7%)	8,7% (5% - 12%)	0,076
Outros eventos clínicos					
- Diabetes Tipo II [n (%)]	1 (5,6%)	6 (33,3%)	2 (25%)	2 (40%)	0,165
- Dislipidemia [n (%)]	1 (5,6%)	10 (55,6%)	1 (12,5%)	2 (40%)	**0,006**
- Sífilis [VDRL positivo, n (%)]	0 (0%)	0 (0%)	0 (0%)	1 (20%)	0,329
Hábitos de Vida					
- Fumante [n (%)]	0 (0%)	2 (11,1%)	2 (25%)	1 (20%)	0,213

- Prática de Exercício [n (%)]		10	(55,6%)	7	(38,9%)	1	(12,5%)	1	(20%)	0,157
- Uso prévio de Cocaína [n (%)]		1	(5,6%)	3	(16,7%)	0	(0%)	1	(20%)	0,678
- Risco associado ao consumo de álcool	Baixo	17	(94,4%)	17	(94,4%)	6	(75%)	3	(60%)	
	Moderado	1	(5,6%)	1	(5,6%)	2	(25%)	1	(20%)	0,051
	Muito Alto	0	(0%)	0	(0%)	0	(0%)	1	(20%)	
Antropometria										
- IMC [kg/m2, (mediana (Q1-Q3)]		29	(27 - 32)	29	(25 - 31)	30	(28 - 33)	26	(25 - 28)	0,679
- Pressão sistólica [mmHg, (mediana (Q1-Q3)]		121	(113 - 125)	129	(113 - 140)	119	(113 - 131)	132	(114 - 145)	0,651
- Pressão diastólica [mmHg, (mediana (Q1-Q3)]		75	(68 - 80)	77	(67 - 81)	72	(65 - 75)	94	(80 - 95)	0,183
- Circunferência Abdominal [cm, (mediana (Q1-Q3)]		99	(90 - 107)	98	(89 - 110)	100	(96 - 101)	89	(89 - 104)	0,998
Bioquímica										
- Glicose [mg/dl, mediana (Q1-Q3)]		91	(87 - 96)	95	(90 - 102)	96	(93 - 105)	105	(101 - 125)	0,077
- Creatinina [mg/dl, mediana (Q1-Q3)]		0,83	(0,77 - 0,98)	0,90	(0,79 - 1,18)	0,77	(0,68 - 0,9)	1,07	(0,89 - 1,1)	0,267
Hemograma										
- Hemácias [x10^6 células/mm^3, mediana (Q1-Q3)]		4,6	(4,4 - 4,9)	4,6	(4,3 - 4,9)	4,6	(3,9 - 4,9)	4,8	(4,8 - 5,1)	0,370
- Monócitos [células/mm^3, mediana (Q1-Q3)]		476	(347 - 580)	482	(439 - 551)	383	(322 - 438)	343	(314 - 428)	0,059
- Plaquetas [x10^3 células/mm^3, mediana (Q1-Q3)]		276	(240 - 324)	231	(202 - 264)	304	(261 - 326)	221	(196 - 243)	**0,025**
Lipidograma										
- Colesterol Total [mg/dl, mediana (Q1-Q3)]		194	(175 - 212)	151	(137 - 203)	179	(170 - 200)	199	(166 - 242)	0,200
- HDL [mg/dL, mediana (Q1-Q3)]		52	(40 - 61)	53	(39 - 64)	52	(45 - 60)	44	(41 - 55)	0,988
- LDL [mg/dL, mediana (Q1-Q3)]		122	(109 - 134)	93	(60 - 118)	117	(99 - 129)	123	(104 - 141)	**0,044**
- Triglicerídeos [mg/dl, mediana (Q1-Q3)]		76	(61 - 92)	95	(81 - 160)	83	(74 - 104)	165	(95 - 176)	0,061
- VLDL [mg/dL, mediana (Q1-Q3)]		15	(12 - 18)	21	(17 - 34)	17	(15 - 21)	33	(19 - 35)	**0,022**

HIVneg - Indivíduos não infectados pelo HIV-1; EC - Controladores de Elite; VC - Controladores virêmicos; cART - Indivíduos infectados pelo HIV em tratamento com cART; N/A - Não se aplica; Q1-Q3 - Quartis 1 e 3; NI - Não informado; os p-valores foram obtidos utilizando o teste de Kruskal Wallis e p-valores <0,05 foram considerados significantes e destacados em negrito.

As contagens de linfócitos T $CD4^+$ foram similares entre os grupos, porém VCs e indivíduos tratados possuíam maiores contagens de linfócitos T $CD8^+$. VCs possuíam mediana de razão CD4/CD8 abaixo de 1, enquanto os grupos EC, cART e HIVneg apresentavam razões acima de 1, com este último grupo apresentando as maiores razões ($p<0,03$). A mediana de carga viral plasmática para VCs foi de 700 cópias/mL. Todos os indivíduos do grupo EC e cART apresentaram cargas virais <40 cópias/mL.

Para os indivíduos cART, os esquemas terapêuticos utilizados por cada indivíduo no momento de seu recrutamento estão disponíveis na Tabela suplementar 2, disponível no anexo 9.2. Os esquemas terapêuticos mais prevalentes foram combinações de dois Inibidores da transcriptase reversa nucleosídeos (ITRN) com 1 inibidor da transcriptase reversa não nucleosídeo (ITRNN) (44%) e de 2 Inibidores de transcriptase reversa nucleosídeos com 2 inibidores de protease (IP) (22%). Em resumo, 89% dos indivíduos estavam em uso de combinações duplas de inibidores da transcriptase reversa nucleosídeos (ITRN), com 81% desses em uso de Tenofovir/Lamivudina (TDF/3TC), enquanto 33% utilizavam combinações de dois Inibidores da protease, com 66% desses em uso de Darunavir/Ritonavir (DRV/RTV).

Nos grupos cART e EC, alguns indivíduos apresentavam histórico de doença cardíaca prévia. No grupo EC, uma mulher com idade avançada sofreu de insuficiência valvar aórtica, sendo submetida a cirurgia de troca da válvula há 9 anos. No grupo cART, pacientes com eventos cardiovasculares incluíam dois homens com histórico de infarto agudo do miocárdio, dois homens com histórico de cardiopatia isquêmica, uma mulher com histórico de insuficiência valvar aórtica e uma mulher com miocardiopatia dilatada.

Em relação a fatores de risco para doenças cardiovasculares, não houve diferença estatística para a frequência de hipertensos, diabéticos ou fumantes entre os quatro grupos. A ocorrência de dislipidemia, por sua vez, foi significativamente maior para os grupos cART e VC em comparação com os demais ($p<0,006$), com mais da metade do grupo cART apresentando dislipidemia.

Diferenças significativas também não foram observadas na comparação entre os grupos para os parâmetros antropométricos e para os valores de glicose e creatinina sanguíneos. No entanto, indivíduos VCs apresentaram níveis de glicose acima ou próximo do limite desejável (>100mg/dl).

Resultados de Lipidograma mostraram diferenças entre os grupos apenas para os níveis de colesterol LDL e VLDL. Para os grupos cART, HIVneg e EC, as medianas para todos os tipos de lipídios estavam dentro dos valores de referência. Para VCs, os valores de mediana de colesterol total estavam no limite e os de Triglicerídeos e VLDL estavam acima do limite de referência (Tabela 2).

5.2. Ativação de células T

Utilizando amostras de indivíduos do subgrupo A para avaliar o nível de ativação de linfócitos T CD4$^+$ (CD4$^+$CD38$^+$HLA-DR$^+$), foi observado que ECs apresentaram frequências de células ativadas similares aos observados para o grupo HIVneg. Por outro lado, VCs e cART apresentaram maiores frequências de células T CD4$^+$ ativadas em comparação EC ($p < 0.0001$ para ambos os grupos) e HIVneg ($p < 0.0001$ e $p = 0.0003$, respectivamente) (Figura 11-A). No compartimento T CD8$^+$ (CD8$^+$CD38$^+$HLA-DR$^+$), VCs apresentaram maiores níveis de ativação em comparação com todos os outros grupos ($p < 0.0001$ para ECs e HIVneg; $p = 0.0002$ para cART) (Figura 11-B). Indivíduos em tratamento apresentaram maiores frequências de linfócitos T CD8$^+$ ativadas apenas quando comparados com indivíduos HIVneg ($p = 0.0003$). Como observado para o compartimento T CD4$^+$, frequências similares de linfócitos T CD8$^+$ ativadas foram encontradas para os grupos EC e HIVneg.

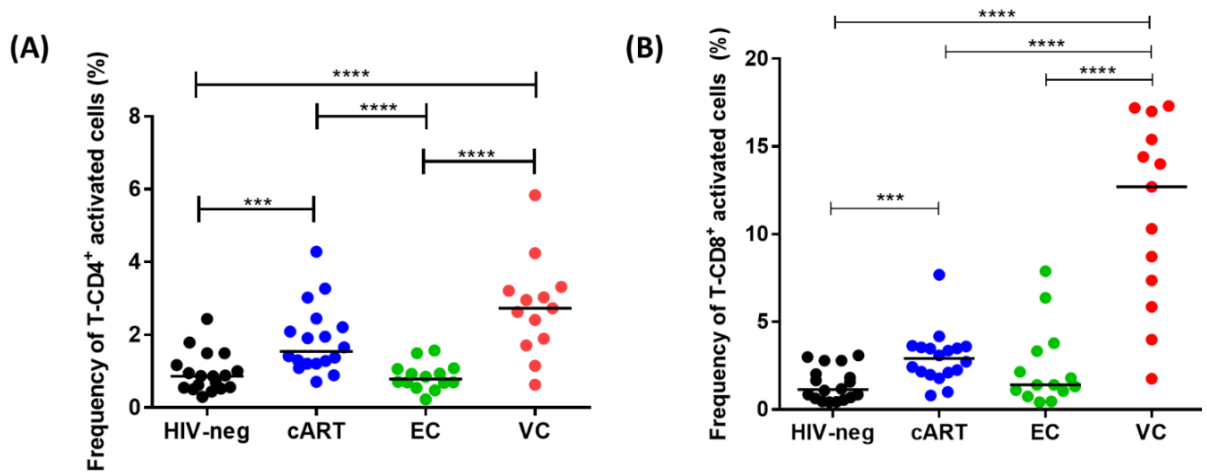

Figura 11. Níveis de ativação de células T em HICS e grupos controle.
*(A) Frequência de linfócitos T CD4$^+$ ativados (CD38$^+$HLA-DR$^+$). (B) Frequência de linfócitos T CD8$^+$ ativados (CD38$^+$HLA-DR$^+$). As linhas horizontais representam a mediana de cada grupo; P-valores foram calculados usando o teste de Mann-Whitney e estão representados como: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.*

Estes dados apontam para um controle da ativação de linfócitos T em ECs, contrastando com níveis mais elevados de ativação em VCs e em indivíduos em longo tempo de tratamento antirretroviral.

5.3. Subpopulações de células T

Análises fenotípicas também foram realizadas para comparar as frequências das distintas subpopulações de linfócitos T (TN, TSCM, TCM, TTM, TEM e TEFF) nas células T $CD4^+$ e T $CD8^+$ entre os grupos estudados. Para o compartimento $TCD4^+$ (**Figura 12**-A), ECs apresentaram frequências similares às observadas em HIVneg para todas as subpopulações, com a exceção de linfócitos TTM, para os quais níveis significativamente menores foram observados ($p = 0.0304$). Por outro lado, VCs apresentaram maiores frequências de linfócitos TEFF ($p = 0.0062$), TN ($p = 0.0111$) e TSCM ($p = 0.0315$), porém menores frequências de TTM ($p = 0.0032$) e TCM ($p = 0.0020$) quando comparados com o grupo HIVneg. No compartimento de linfócitos T $CD8^+$ (**Figura 12**-B), ECs apresentaram maiores frequências de linfócitos TSCM ($p = 0.0139$) enquanto VCs apresentam uma maior frequência de TEFF ($p = 0.0007$), ambos quando comparados com HIVneg, indicando que VCs apresentam frequência maior de linfócitos mais diferenciados enquanto ECs apresentam níveis mais elevados de algumas populações de memória, apontando para uma preservação maior dessas populações.

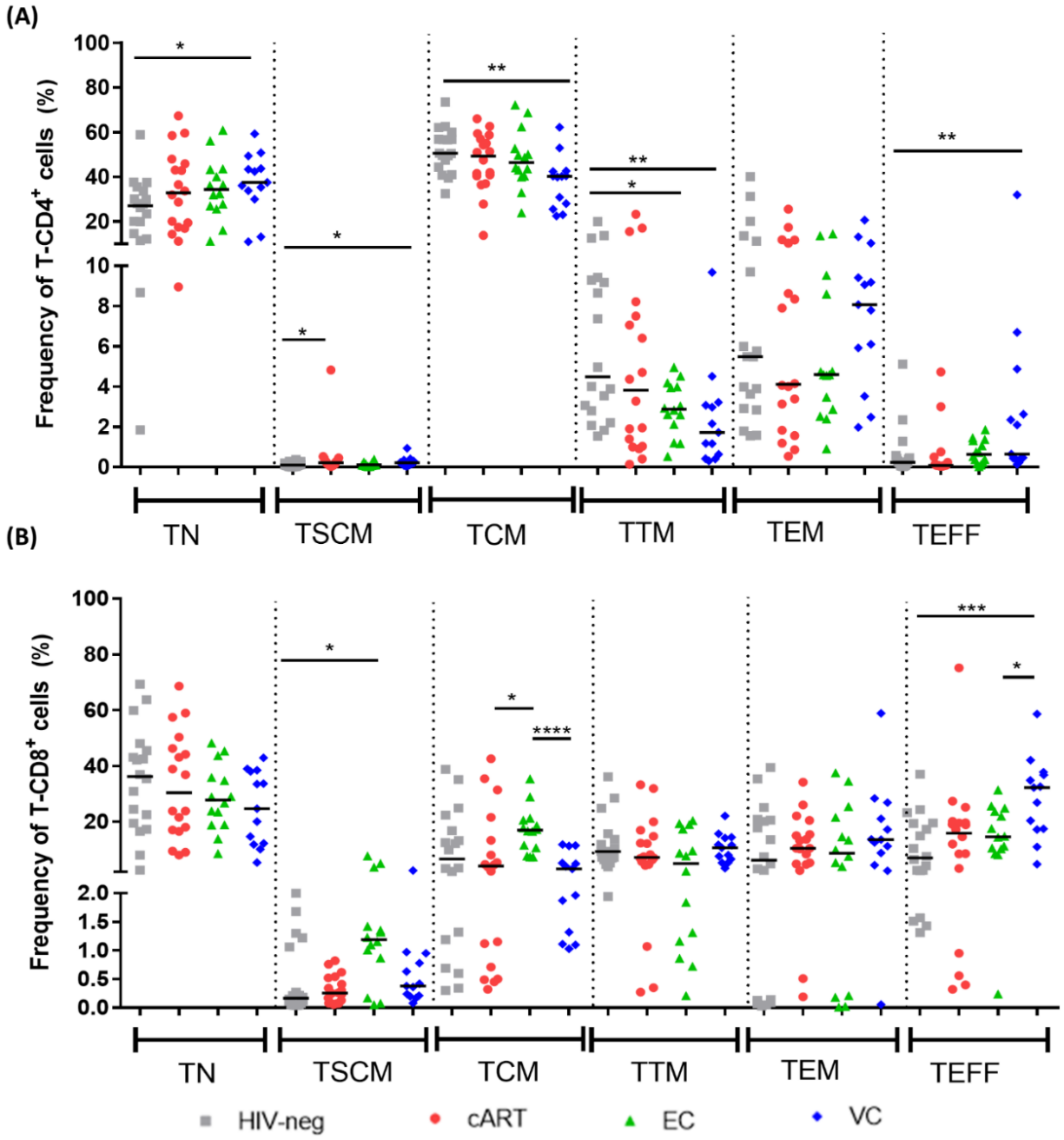

Figura 12. Perfis de subpopulações de linfócitos T em HICS e grupos controle.
*(A) Frequências de linfócitos T CD4⁺ naïve (TN; CD45RA⁺CCR7⁺CD27⁺CD95-), tronco de memória (TSCM; CD45RA⁺CCR7⁺CD27⁺CD95⁺), memória central (TCM; CD45RA-CCR7⁺CD27⁺), memória transicional (TTM; CD45RA-CCR7-CD27⁺), memória efetora (TEM; CD45RA-CCR7-CD27-) e terminalmente efetora (TEFF; CD45RA⁺CCR7-CD27-). (B) Frequências de linfócitos T CD8⁺ TN, TSCM, TCM, TTM, TEM e TEFF. Quadrados cinzas representam HIVneg, círculos vermelhos representam cART, triângulos verdes representam ECs e diamantes azuis representam VCs; as linhas horizontais representam a mediana de cada grupo; P-valores foram calculados usando o teste de Mann-Whitney e estão representados como: * p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.*

5.4. Células Treg e Th17

Foram avaliadas a frequência de células Treg totais, bem como de suas subpopulações ativadas, em repouso e não supressoras (Figura 13). Tanto ECs como VCs apresentaram menores níveis de Treg totais em comparação com indivíduos HIVneg ($p = 0.0018$ e $p = 0.0001$, respectivamente) e cART ($p = 0.032$ e $p = 0.006$, respectivamente) (Figura 13-A). Analisando as subpopulações, ECs apresentaram maiores frequências de Treg ativadas em comparação com os grupos cART ($p = 0.037$) e HIVneg ($p = 0.003$) (Figura 13-B). Inversamente, menores frequências de Treg em repouso também foram observadas para ECs em comparação com os grupos cART ($p = 0.008$) e HIVneg ($p = 0.036$) (Figura 13-C). Frequências similares de linfócitos Treg não supressores foram observadas para todos os grupos (Figura 13-D).

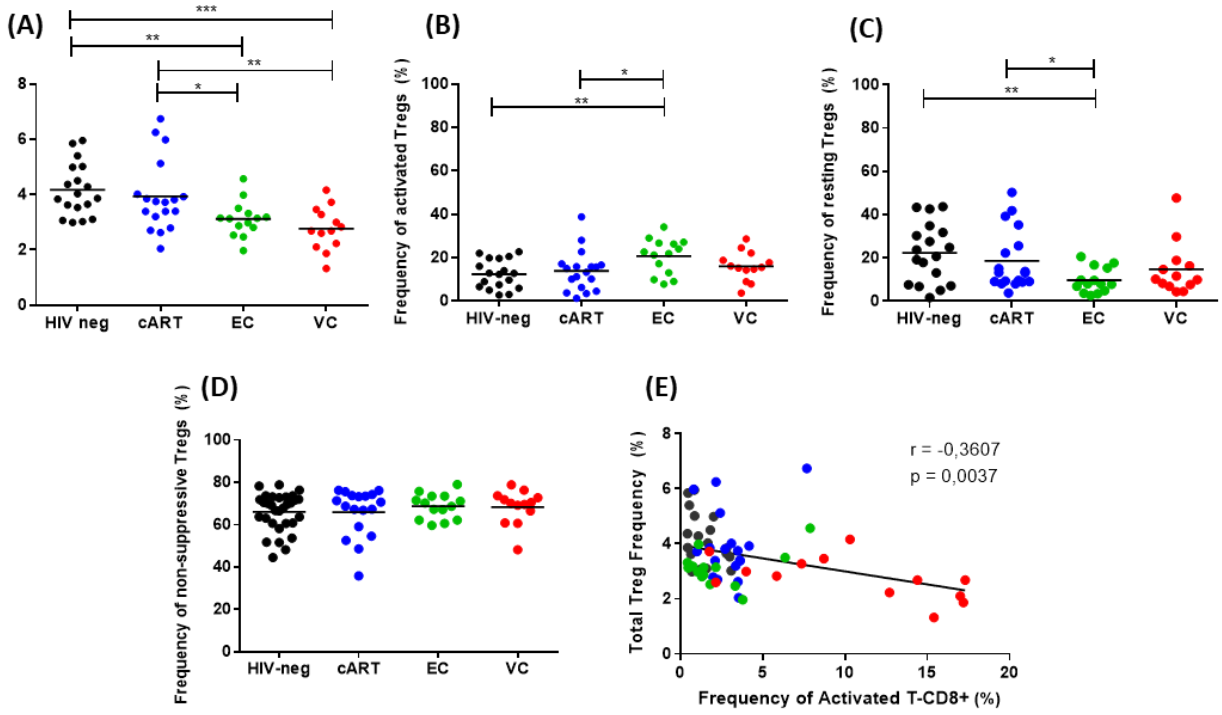

Figura 13. Perfis de subpopulação de Tregs em HICs e grupos controle.
*(A) Frequências de Treg totais ($CD4^+CD25^{high}Foxp3^+$). (B) Frequências de Tregs ativadas ($CD45RA^-Foxp3^{high}$). (C) Frequências de Treg latentes ($CD45RA^+Foxp3^{low}$); (D) Frequências de Treg Não supressoras ($CD45RA^-Foxp3^{low}$). (E) Correlação entre as frequências de Tregs totais e de linfócitos T $CD8^+$ ativados. Valores r e p estão demonstrados em cada correlação. P-valores foram calculados usando o teste de Mann-Whitney e estão representados como: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$*

Foi observada correlação inversa entre a frequência de Tregs totais e a ativação de linfócitos T $CD8^+$ ($r = -0.3607$; $p < 0.004$), no entanto nenhuma correlação entre as subpopulações de Treg e a ativação de linfócitos T foi encontrada.

Também foram avaliadas as frequências de linfócitos Th17 e a razão Th17/Treg entre os grupos estudados (Figura 14). ECs apresentaram maiores frequências de células Th17 e maiores razões Th17/Treg quando comparados com HIVneg (p = 0.048 e p = 0.002) e cART (p = 0.009 e p = 0.007) (Figura 14-A e B). Embora diferenças significativas em Th17 não tenham sido observadas para VCs quando comparados com os outros grupos, VCs apresentaram maiores razões Th17/Treg em comparação com os grupos cART (p = 0.004) e HIVneg (p = 0.001). Uma correlação inversa ainda foi observada entre a frequências de células Th17 e a frequência de Tregs totais (r = -0.2515; p = 0.04, Figura 14-C). Estes resultados se somam aos anteriores que indicam uma maior estabilidade e integridade do sistema imune de ECs, com controle da expansão das células Treg e preservação da população Th17.

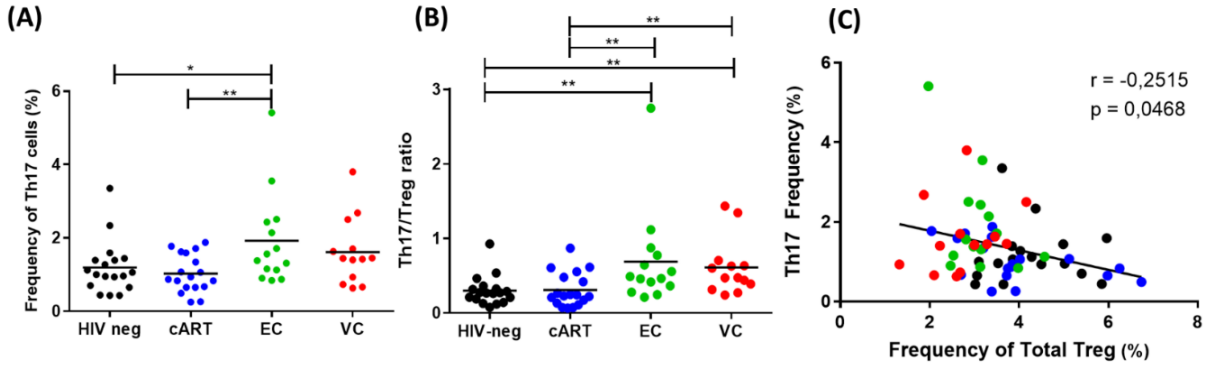

Figura 14. Frequências de linfócitos Th17 e razão Th17/Treg em HICs e grupos controle. *(A) Frequência de linfócitos Th17 ($CD4^+IL17^+$); (B) Razão Th17/Treg; Th17/Treg e (C) a correlação entre as frequências de Tregs totais e de linfócitos Th17 estão apresentadas nos gráficos. A razão Th17/Treg foi calculada através das frequências de ambas as populações em relação ao compartimento $CD4^+$. Valores r e p estão demonstrados em cada correlação. P-valores foram calculados usando o teste de Mann-Whitney e estão representados como descrito: * p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.*

5.1. Frequência de Plaquetas ativadas e formação de agregados monócitos plaquetas

De modo a avaliar o nível de ativação plaquetária e a frequência de agregados monócitos-plaquetas em indivíduos controladores, amostras de sangue dos indivíduos do subgrupo B foram coletadas em ACD e avaliadas para a expressão de CD62P em plaquetas $CD41^+$ e de CD41 em monócitos $CD14^+$, respectivamente (Figura 15). Todas as análises entre os grupos foram ajustadas por idade, gênero, e fatores

tradicionais de risco para doenças cardíacas: hipertensão, dislipidemia, diabetes, fumo, IMC, colesterol total e colesterol LDL.

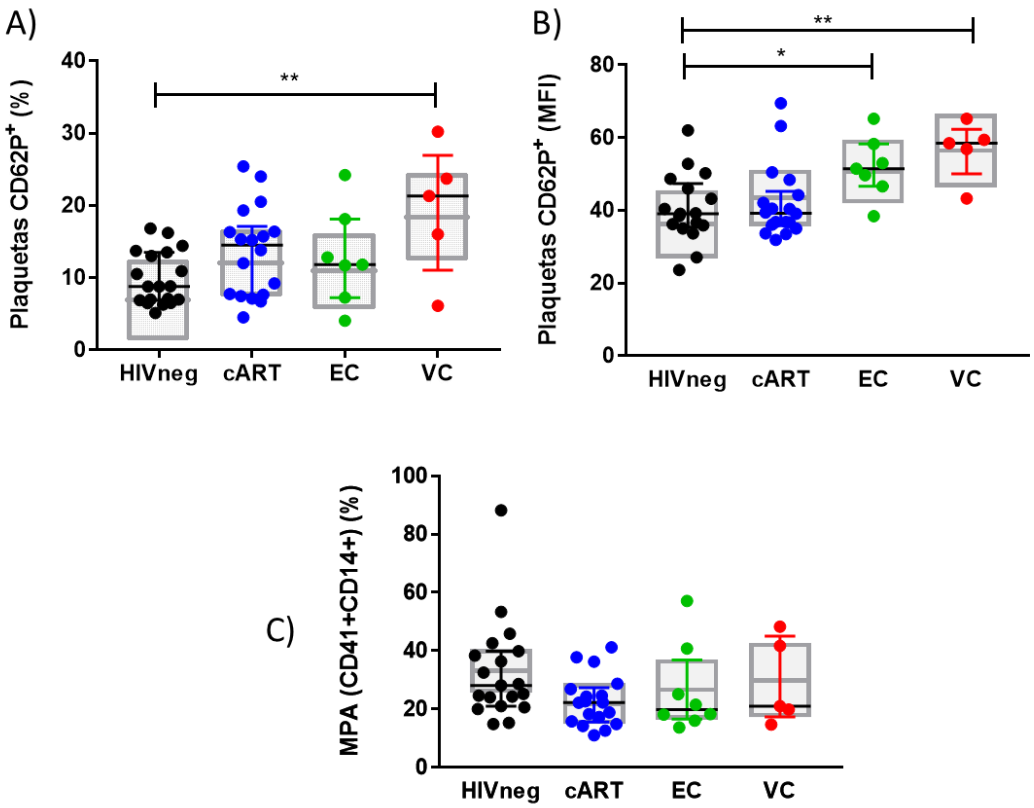

Figura 15. Frequência de plaquetas ativadas e agregados monócitos-plaquetas em HICs e grupos controle.
*(A) Frequências de plaquetas CD41⁺CD62P⁺; (B) MFI de CD62P na população de plaquetas; (C) Frequência de agregados Monócitos plaquetas (CD41⁺CD14⁺); Barras horizontais coloridas representam o IQR e medianas amostrais, enquanto boxplots cinza representam as mediana marginais estimadas ajustadas para viés amostral e seus intervalos de 95% de confiança. Comparações das medianas marginais entre os grupos foram realizadas através de testes T de contrastes obtidas a partir de modelos lineares multivariados ajustados por regressões de mínimos quadrados. P-valores foram corrigidos pelo método de Tukey Honest Significant Difference (HSD), sendo representados como: * p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.*

Analisando a frequência de plaquetas ativadas CD41⁺CD62P⁺ (Figura 15-A), diferenças significativas foram observadas apenas entre os grupos HIVneg e CV, com esse último apresentando maiores níveis de ativação mesmo após ajuste dos dados (p<0,01). Avaliando a ativação em função da média de intensidade de fluorescência (MFI) de CD62P dentro da população de plaquetas CD41⁺ (Figura 15-B), valores maiores foram observados para VCs (p<0,007) e também para ECs (p<0,03) em comparação com o grupo HIVneg, indicando que HICs apresentam níveis elevados de ativação plaquetária apesar das observações prévias indicando equilíbrio imunológico a partir de parâmetros relacionados a ativação imune

Para a análise de formação de agregados monócitos-plaquetas, foi calculada a frequência de células $CD41^{+}CD14^{++}$ dentro da população de monócitos de amostras dos indivíduos do subgrupo B (Figura 15-C). Diferenças significativas não foram encontradas entre os grupos, apesar dos indícios de aumento na ativação plaquetária para os HICs analisados.

5.2. Frequência de subpopulações de monócitos e sua ativação

De modo a avaliar a frequência de monócitos clássicos, intermediários e não clássicos em indivíduos controladores, o sangue total coletado em ACD para indivíduos do subgrupo B foi utilizado para marcação e identificação dessas células através de citometria de fluxo (Figura 16). Nas análises não ajustadas, os três grupos de indivíduos infectados pelo HIV-1 apresentaram frequências significativamente menores de monócitos clássicos em comparação com indivíduos não infectados pelo HIV-1 ($p<0,03$ para cART; $p<0,002$ para EC; $p<0,002$ para VC). Para o grupo VC, as frequências foram ainda significativamente menores também em comparação com o grupo cART ($p<0,009$), dados não apresentados. Ao ajustar as análises pelas variáveis citadas anteriormente, diferenças estatísticas só foram encontradas para o grupo VC em comparação com os outros três grupos ($p<0,00001$ vs HIVneg; $p<0,00002$ vs cART; $p<0,004$ vs EC) (Figura 16-A). As frequências de monócitos clássicos ainda correlacionaram negativamente com os dois valores de risco Framingham calculados, quantificação de glicose e a frequência de plaquetas ativadas (Figura 17).

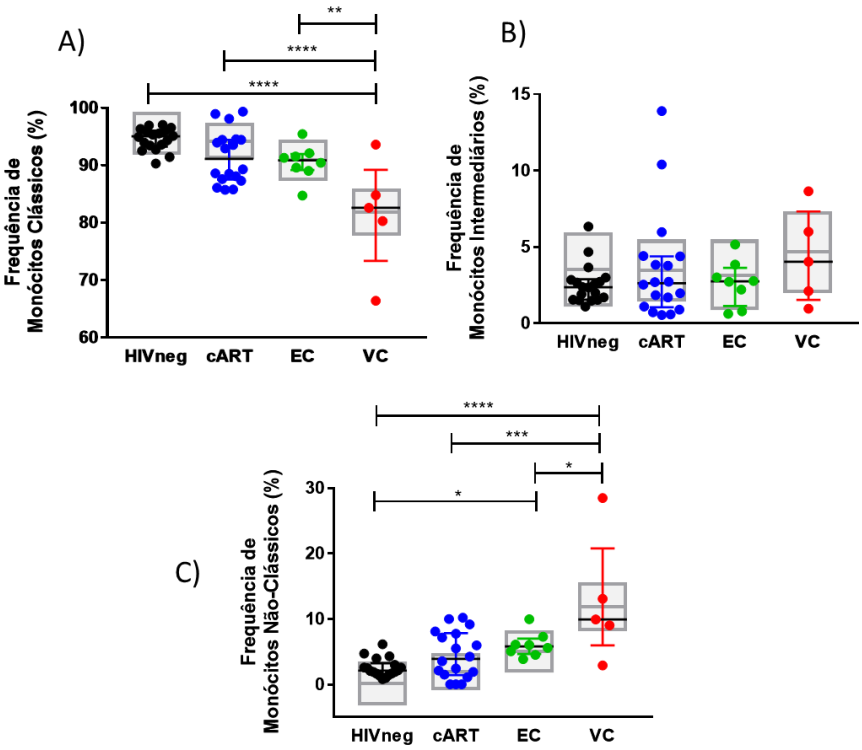

Figura 16. Frequência de subpopulações de monócitos em HICs e grupos controle.
*(A) Frequência de monócitos clássicos ($CD14^{++}CD16^{-}$); (B) frequência de monócitos Intermediários ($CD14^{++}CD16^{+}$); (C) Frequência de monócitos não clássicos ($CD14^{+}CD16$). Barras horizontais coloridas representam o IQR e medianas amostrais, enquanto boxplots cinza representam as mediana marginais estimadas ajustadas para viés amostral e seus intervalos de 95% de confiança. Comparações das mediaans marginais entre os grupos foram realizadas através de testes T de contrastes obtidas a partir de modelos lineares multivariados ajustados por regressões de mínimos quadrados. P-valores foram corrigidos pelo método de Tukey Honest Significant Difference (HSD), sendo representados como: * p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.*

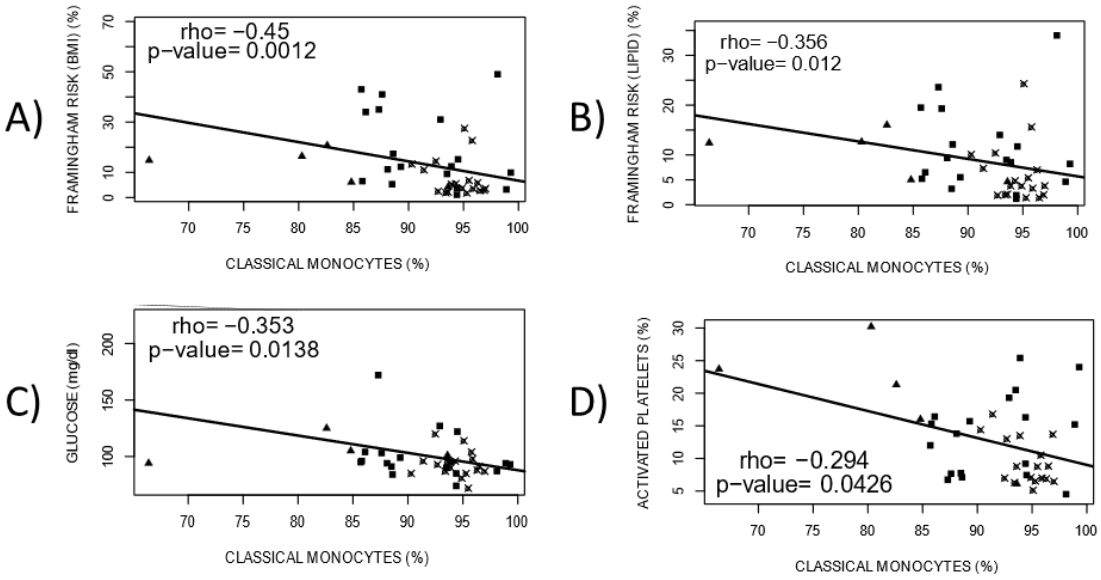

Figura 17- Correlações entre Monócitos clássicos e os diferentes parâmetros avaliados para o subgrupo B.
As frequências de monócitos clássicos obtidos por citometria de fluxo correlacionaram com os valores de: (A) Risco Framingham (IMC); (B) Risco Framingham (Lipídios); (C) Glicose; (D) Frequência de plaquetas ativadas. Correlações foram obtidas por meio de regressão de Spearman e os valores rho e p estão demonstrados em cada correlação.

Paralelamente, maiores frequências de monócitos não clássicos foram observadas para VCs em comparação com HIVneg e cART (p<0,002 e p<0,03, respectivamente) e ECs comparados com HIVneg (p<0,003). As análises corrigidas por fatores de risco mantiveram as mesmas diferenças, com valores de p maiores para VCs (p<0,00001 vs HIVneg; p<0,0002 vs cART; p<0,02 vs EC) e para ECs (p< 0,04 vs HIVneg) (Figura 16-C). As frequências de monócitos não clássicos correlacionaram positivamente com os dois valores de risco Framingham calculados, de pressão sistólica e concentração de AST (Figura 18). Diferenças significativas não foram observadas para a população de monócitos intermediários (Figura 16-B).

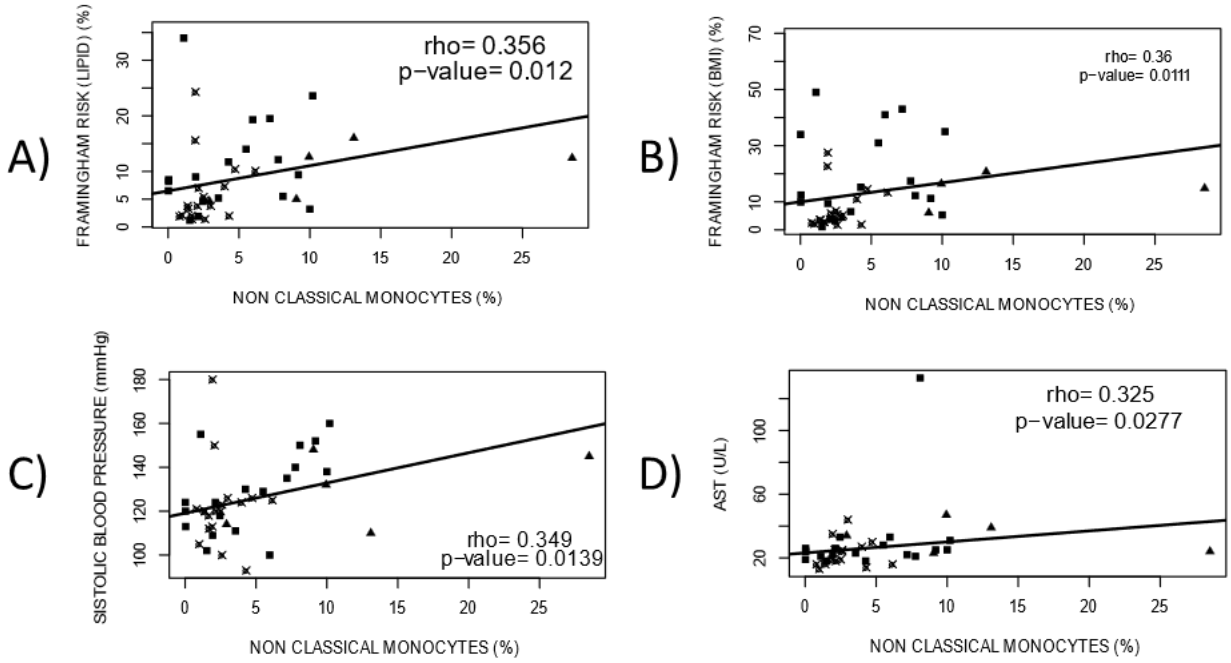

Figura 18- Correlações entre Monócitos não clássicos e os diferentes parâmetros avaliados para o subgrupo B.
As frequências de monócitos clássicos obtidos por citometria de fluxo correlacionaram com os valores de: (A) Risco Framingham (Lipídios); (B) Risco Framingham (IMC); (C) Pressão sistólica; (D) AST; Correlações foram obtidas por meio de regressão de Spearman realizada através do pacote R e os valores rho e p estão demonstrados em cada correlação.

Além da frequência de cada subpopulação de monócitos, as amostras do subgrupo B também foram marcadas para avaliar a frequência de monócitos expressando CD142, também conhecido como TF. As análises, no entanto, mostraram níveis extremamente baixos de expressão do TF nas células marcadas a fresco, independente do grupo analisado (Figura 19). Experimentos posteriores serão realizados para confirmar os padrões de expressão em células criopreservadas e estimuladas com LPS.

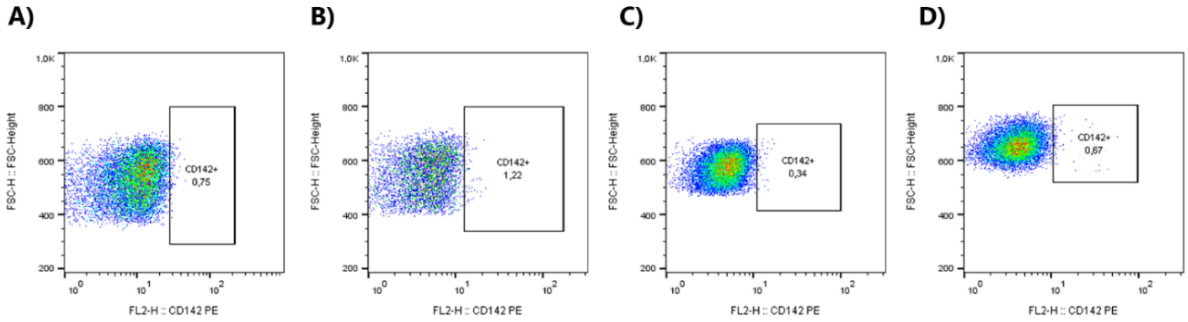

Figura 19 Gráficos representativos da expressão de TF em diferentes condições de marcação. *(A-D) Gráficos representativos da expressão de TF em amostras de sangue recém coletadas em indivíduos dos grupos EC (A), VC (B), cART (C) e HIVneg (D); Todos os gráficos representam a porcentagem de células positivas para TF dentro da população de monócitos CD3$^-$.*

5.3. Marcadores séricos de inflamação

De modo a avaliar os níveis séricos de alguns marcadores comumente associados com a resposta inflamatória ou anti-inflamatória e/ou com processos patogênicos que estão envolvidos no desenvolvimento de doenças cardiovasculares, foi feita a quantificação de VCAM-1, ICAM-1, CD40L, D-dímero, IL-6, IL-10, ST2/IL33-R, MCP-1 e TNF-α usando um ensaio multiplex (Figura 20). Para o marcador VCAM-1, foram observados níveis maiores, mesmo após correção para fatores de risco, nos grupos cART ($p<0,00001$), EC ($p<0,0001$) e VC ($p<0,005$) quando comparados com o grupo HIVneg. Para ICAM-1, as análises iniciais mostraram diferenças para os três grupos de indivíduos infectados pelo HIV-1 em comparação com HIVneg ($p<0,001$ para cART; $p<0,007$ para EC; $p=0,0005$ para VC). No entanto, após correção para fatores de risco, diferenças significativas só se mantiveram para os grupos cART e VC ($p<0,05$ e $p<0,02$, respectivamente).

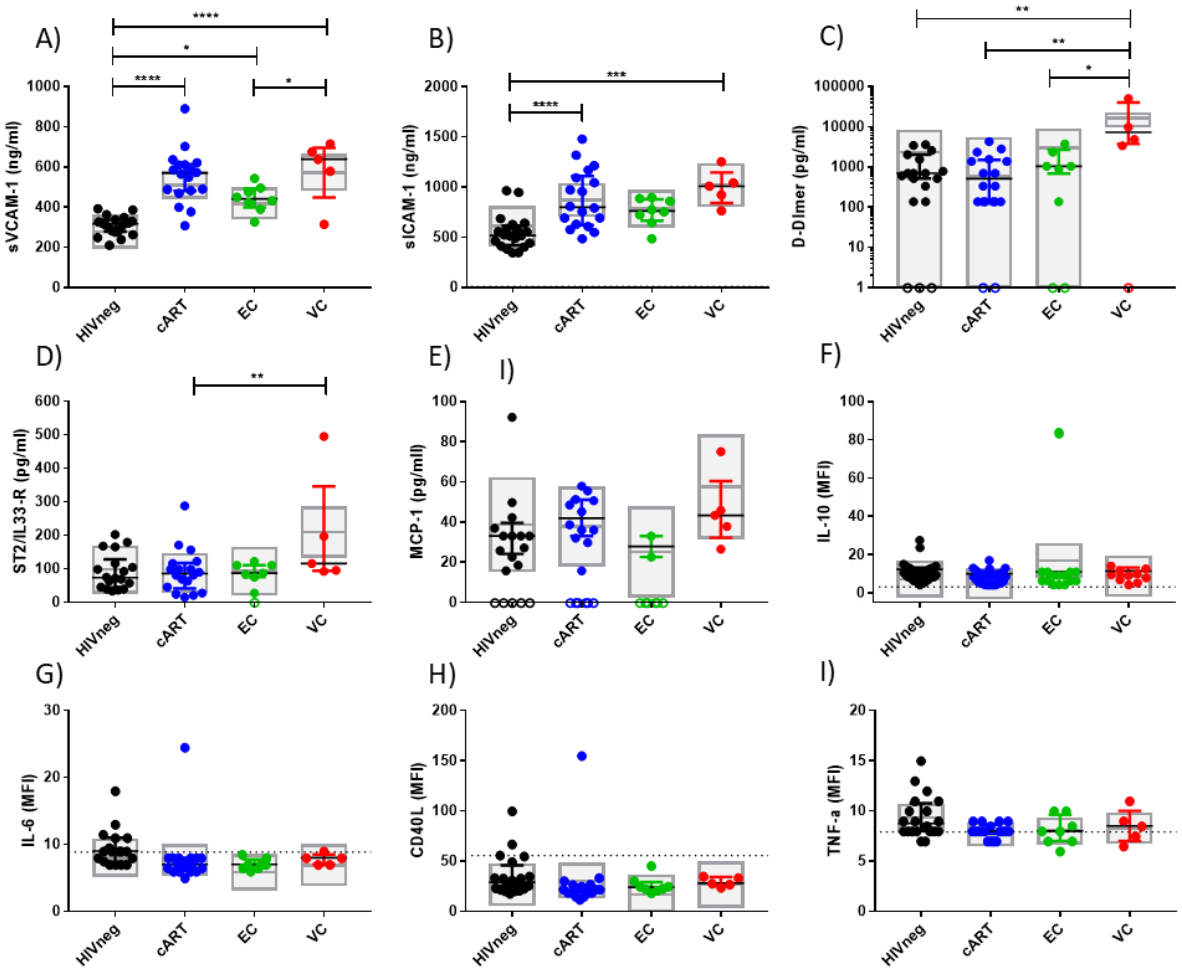

Figura 20- Níveis séricos de marcadores inflamatórios em HICs e grupos controle.
*Os gráficos representam os valores obtidos através de ensaio de imunofluorescência para: (A) sVCAM-1; (B) sICAM-1; (C)D-dímero; (D)MCP-1; (E) ST2/IL-33R; (F) TNF-α; (G) IL-10; (H) IL-6; (I) CD40L; Para os marcadores representados nos gráficos F a I, valores de MFI estão representados e a linha tracejada representa o valor de fluorescência de background. Barras horizontais coloridas representam o IQR e medianas amostrais, enquanto boxplots cinza representam as mediana marginais estimadas ajustadas para viés amostral e seus intervalos de 95% de confiança. Comparações das mediaans marginais entre os grupos foram realizadas através de testes T de contrastes obtidas a partir de modelos lineares multivariados ajustados por regressões de mínimos quadrados. P-valores foram corrigidos pelo método de Tukey Honest Significant Difference (HSD), sendo representados como: * p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.*

Para o marcador D-dímero (Figura 20-C), VCs apresentaram maiores valores em comparação com todos os outros grupos após correção estatística (p<0,01 vs HIVneg; p<0,002 vs cART; p<0,03 vs EC). Níveis aumentados de ST2 também foram observados em VCs comparados com o grupo cART após a correção (p<0,03 - Figura 20-E).

Para MCP-1, níveis aumentados para VCs em comparação com HIVneg (p<0,03) e diminuídos em ECs em comparação com todos os outros grupos (p<0,03 vs HIVneg e cART; p<0,002 vs VC; Figura 20-D) foram observados nas análises preliminares. Após correção estatística para os fatores de risco citados anteriomente,

nenhuma diferença estatística entre os grupos se manteve. Para os marcadores CD40L, IL-6, IL-10 e TNF-α, os valores quantificados para a maioria dos pacientes foram próximos ou abaixo do limite de detecção e, por isso, não foram observadas diferenças relevantes .

Os marcadores ICAM-1 (Figura 22) e VCAM-1 (Figura 21) e se correlacionaram um com o outro e simultaneamente com os dois modelos de contagem de linfócitos T $CD8^+$, risco Framingham, VLDL, Colesterol LDL, Triglicerideos, razão CD4/CD8, frequência de monócitos clássicos e não clássicos e AST. VCAM-1 ainda correlacionou independentemente com colesterol total, enquanto ICAM-1 correlacionou independentemente com a contagem de plaquetas, colesterol HDL e concentração de glicose no sangue.

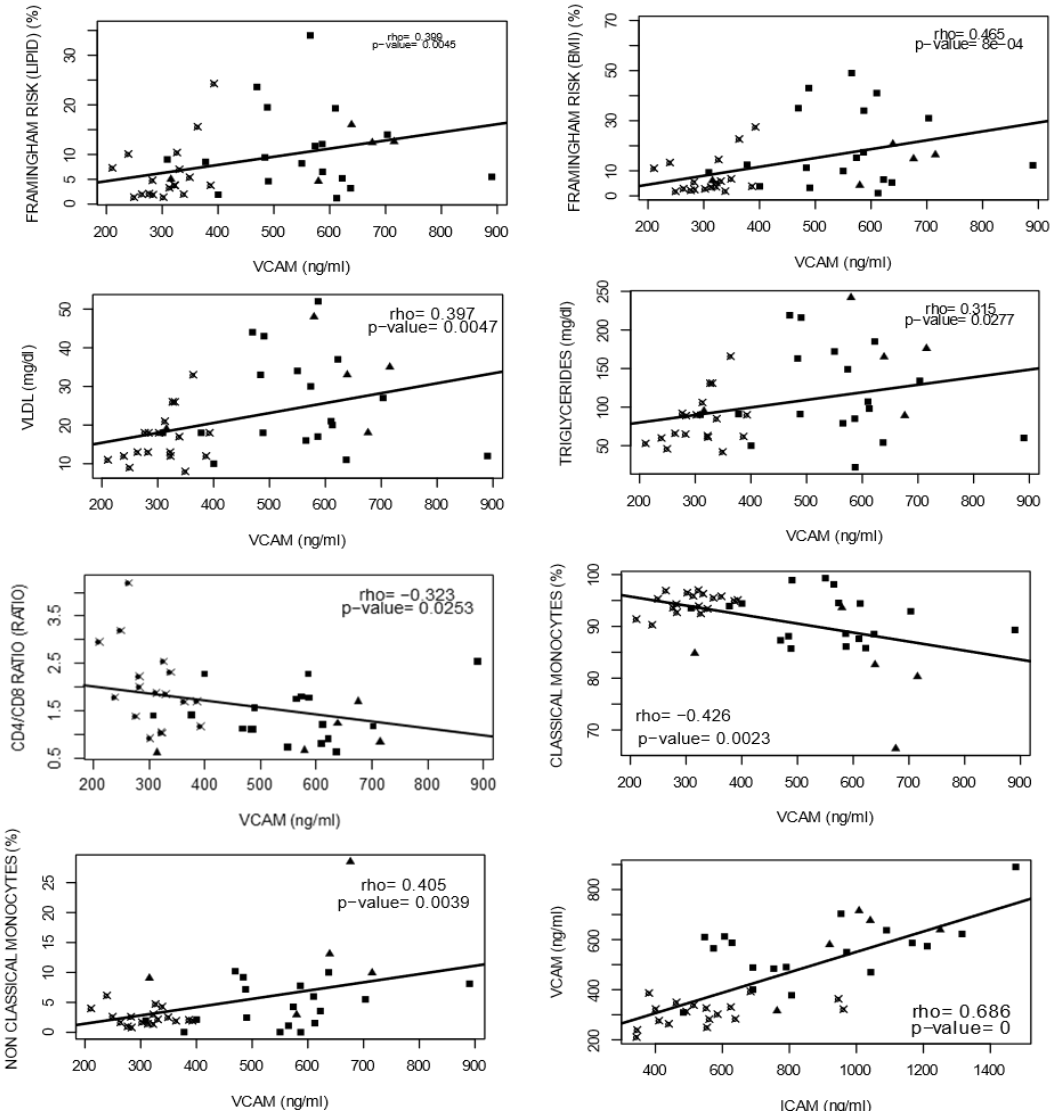

Figura 21- Correlações entre VCAM-1 e os diferentes parâmetros avaliados para o subgrupo B.
Correlações foram obtidas por meio de regressão de Spearman realizada através do pacote R e os valores rho e p estão demonstrados em cada correlação.

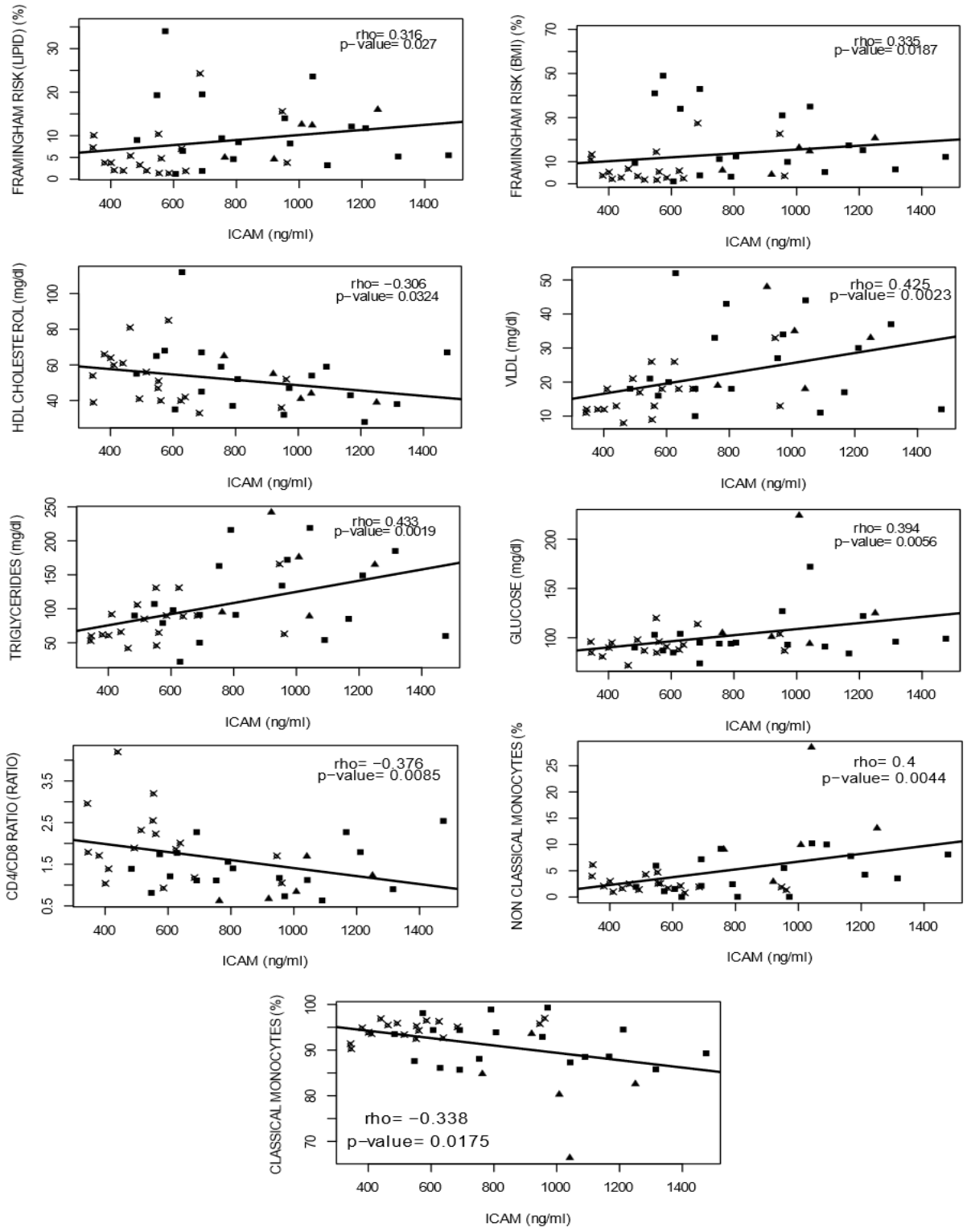

Figura 22- Correlações entre ICAM-1 e os diferentes parâmetros avaliados para o subgrupo B. *Correlações foram obtidas por meio de regressão de Spearman e os valores rho e p estão demonstrados em cada correlação.*

Para os outros marcadores, correlações foram observadas para ST2 (vs MFI de CD62P, AST e Colesterol HDL), MCP-1 (vs MFI de CD62P) e D-dímero (vs AST e monócitos não clássicos) (Figura 23).

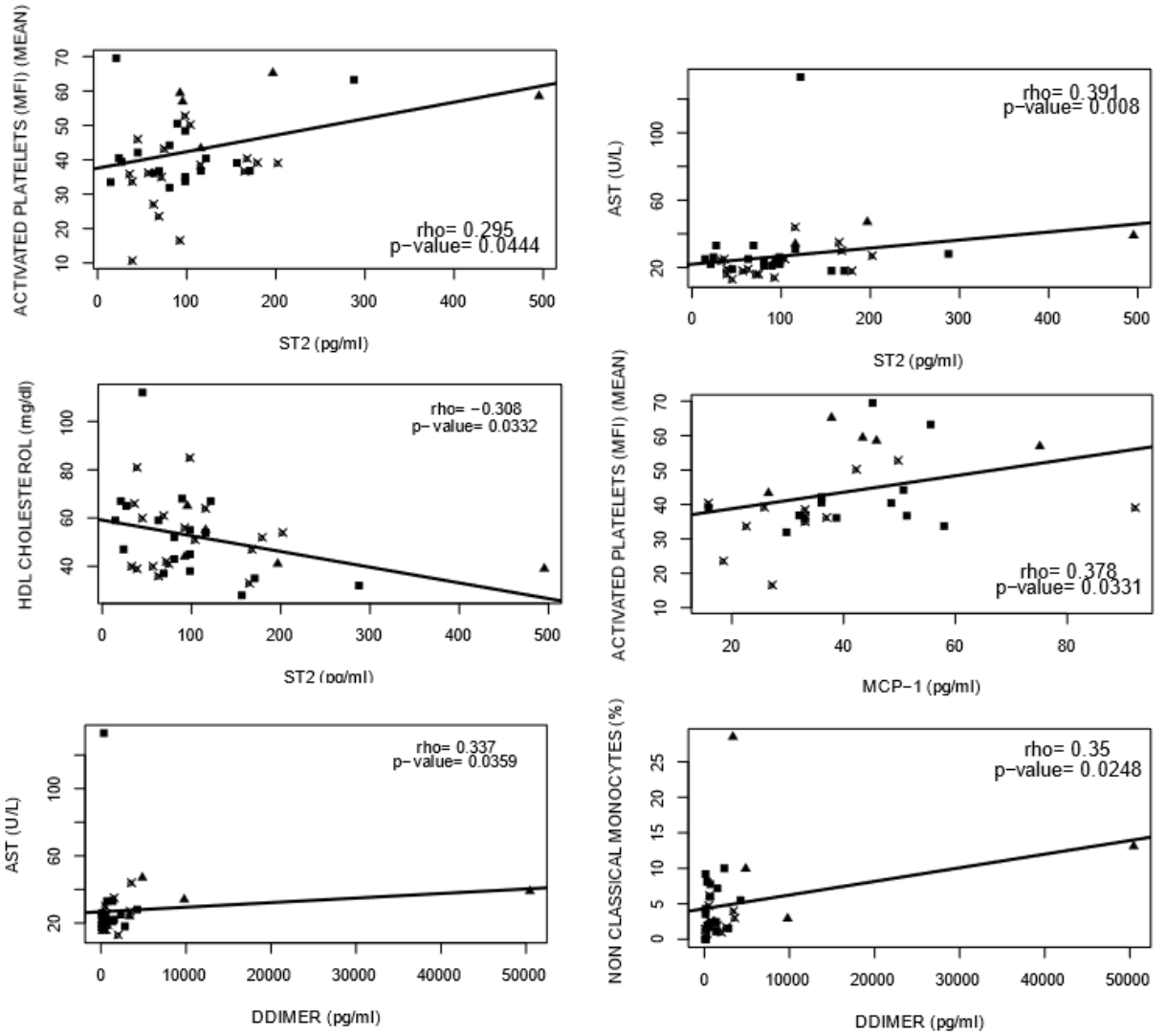

Figura 23- Correlações entre ST2, MCP-1 e D-dímero e os diferentes parâmetros avaliados para o subgrupo B.
Correlações foram obtidas por meio de regressão de Spearman realizada através do pacote R e os valores rho e p estão demonstrados em cada correlação.

No conjunto, estes dados apontam para a correlação entre a inflamação e os diversos marcadores avaliados. Para ECs, níveis elevados de VCAM-1 e ICAM-1 se somam a elevação da frequência de plaquetas ativadas como sinais de inflamação persistente, apesar dos níveis de ativação imune e frequência de subpopulações de células T e monócitos similares ao dos indivíduos não infectados pelo HIV. Para VCs, a elevação de marcadores como D-dímero e ST2, assim como as frequências alteradas de monócitos e plaquetas ativadas, sinalizam para um perfil inflamatório ainda mais intenso.

6. DISCUSSÃO

A infecção pelo HIV-1 é caracterizada por uma série de fenômenos complexos que culminam em uma desregulação do sistema imune. Embora a relação de causa e consequência entre o tropismo viral para linfócitos T $CD4^+$, a depleção gradual dessas células ao longo da fase crônica e a imunodeficiência característica da fase de aids indique uma aparente simplicidade na imunopatogênese da infecção, essas manifestações são causadas e alimentadas pela desregulação de diversos processos imunológicos que são essenciais para a manutenção da homeostase. A ativação imune e a inflamação, por exemplo, são processos chaves para a mobilização das células imunes para o combate de infecções. Por sua vez, mecanismos de regulação negativa, como a exaustão e senescência, possuem funções positivas, atuando, respectivamente, na modulação da autoimunidade e na renovação de tecidos.

Na infecção pelo HIV-1, no entanto, tais mecanismos são subvertidos e a hiperestimulação deles acaba por desregular o sistema imune como um todo, afetando não só os processos de combate ao HIV-1, mas também os de combate a outras infecções e os de manutenção da homeostase. Paradoxalmente, o sistema imune se torna responsável pela sua própria degradação devido a atividade intensa e desregulada.

Este desequilíbrio é influenciado pela replicação viral, porém permanece mesmo em condições de supressão viral mediada pelo tratamento antirretroviral. Ainda que o controle da viremia seja essencial para impedir a progressão para a aids, a cART não é completamente capaz de reconduzir o sistema imune ao estado de normalidade, influenciando assim, na susceptibilidade a uma série de comorbidades.

O aumento da expectativa de vida decorrente do aprimoramento dos regimes antirretrovirais revelaram que PVHIV, mesmo quando tratadas, possuem risco aumentado para o desenvolvimento de doenças não relacionadas a aids, como síndromes metabólicas e cardiopatias (Naidu et al. 2017; Nansseu et al. 2018; Dominick et al. 2020). Inicialmente, a influência de alguns medicamentos antirretrovirais na indução de dislipidemia, principalmente inibidores de protease, sugeriu que o tratamento contra o HIV-1 pudesse ser responsável pelo risco aumentado (Maggi et al. 2017a). Posteriormente, o desenvolvimento de medicamentos menos tóxicos e o melhor manejo dos efeitos colaterais associados ao tratamento revelaram que a gênese dessas comorbidades está principalmente

associada a processos decorrentes da ativação imune e inflamação persistentes durante a infecção (Duprez et al. 2012; Deeks et al. 2013; Maggi et al. 2017a, 2017b; Vachiat et al. 2017; Peterson and Baker 2018; Hsue 2019; Dominick et al. 2020).

Apesar dessa associação ser amplamente aceita para PVHIV sob tratamento antirretroviral, o mesmo não se pode dizer sobre os casos em que o controle da infecção é alcançado espontaneamente. HICs e LTNPs são uma parcela rara da população e tanto os mecanismos responsáveis por esse fenótipo, como a extensão e eficiência do controle da infecção, ainda não foram completamente elucidados. Dessa forma, relatos inconsistentes ou contraditórios acerca da persistência da ativação imune e inflamação em HICs mantém a dúvida sobre o quão em risco de desenvolvimento de tais comorbidades estão esses indivíduos.

Nesse sentido, o presente trabalho visou avaliar os níveis de inflamação e ativação celular em HICs e descobrir se existem indícios de que esses indivíduos estariam sob risco de desenvolverem comorbidades cardíacas e vasculares mesmo controlando a infecção. Para tal, diferentes marcadores de ativação imune e de inflamação, muitos dos quais também se encontram aumentados em processos patológicos de desenvolvimento de doenças cardiovasculares, foram analisados em uma coorte de HICs e comparados com os níveis observados em indivíduos não infectados pelo HIV-1 e indivíduos tratados com cART com longo tempo de supressão viral.

6.1. Controladores como modelo de cura funcional

Os indivíduos estudados aqui representam uma coorte de HICs que vem sendo acompanhada há cerca de 20 anos em uma colaboração entre o nosso grupo e o Instituto Nacional de Infectologia Evandro Chagas. Embora o número amostral possa parecer baixo para os dois subgrupos analisados, cabe ressaltar que estes representam uma população extremamente rara. Controladores de elite, por exemplo, são estimados em menos de 1% da população total de indivíduos infectados pelo HIV-1. Dessa forma, um número pequeno de indivíduos é esperado em estudos com HICs e tais estudos ainda seguem sendo relevantes, visto que estes são modelos de cura funcional e que a adoção de políticas de tratamento imediato após o diagnóstico da infecção tendem a impossibilitar a identificação de novos HIC.

O longo tempo de acompanhamento dos indivíduos estudados aqui também é um fator positivo para o presente estudo. Devido à dificuldade de identificação desse perfil de controle, uma ampla gama de critérios de classificação de HICs são

observados na literatura, o que pode gerar problemas na identificação desses indivíduos. A coorte francesa ANRS CO21, por exemplo, utiliza um corte de carga viral de até 400 cópias/mL para identificar HICs (Sáez-Cirión and Pancino 2013), agrupando, de acordo com os parâmetros do presente estudo, pacientes EC e VC. A coorte do Estudo de História Natural do HIV-1 do Departamento de Defesa dos EUA, por sua vez, classifica VCs e ECs com apenas um ano de acompanhamento (Okulicz et al. 2009), possibilitando a inclusão de pacientes que possam estar experimentando apenas um período transitório de controle virêmico. No presente estudo foram utilizadas amostras apenas de indivíduos com pelo menos cinco anos de acompanhamento e manutenção de cargas virais consistentemente abaixo do limite de detecção no caso dos ECs. As altas medianas de tempo de diagnóstico de infecção e a estabilidade nas contagens de células T $CD4^+$ também indicam que a maioria dos pacientes já alcançou critérios suficientes para serem classificados também como LTNPs, o que garante maior confiabilidade acerca da eficiência de controle da infecção nesses indivíduos.

O longo tempo de acompanhamento também permitiu uma extensa caracterização desses indivíduos. Ao longo dos anos, diferentes subgrupos dessa coorte possibilitaram verificar padrões de evolução viral intra hospedeiro (Bello et al. 2007; de Azevedo et al. 2017; Caetano et al. 2018), a ocorrência de mutantes virais de escape da resposta imune (Caetano et al. 2018), dinâmicas relacionadas a superinfecção (de Azevedo et al. 2018; Caetano et al. 2019), presença de determinantes genéticos associados ao controle da progressão (Passaes et al. 2014; Teixeira et al. 2014) e a caracterização de diversos aspectos da resposta imune humoral e celular nesses indivíduos (Bello et al. 2009; Côrtes et al. 2015, 2018). Essa possibilidade de utilizar amostras de alguns mesmos indivíduos ao longo de tantos estudos com diferentes perspectivas, também é um ponto positivo e possibilita um maior e melhor conhecimento acerca dos diferentes aspectos relacionados ao perfil de controle espontâneo da infecção.

O presente estudo também visa contribuir com informações que possam subsidiar um melhor manejo clínico desses indivíduos. A identificação de que o início precoce da terapia gera melhores respostas imunológicas (Ding et al. 2015; Okulicz et al. 2015) e a supressão da viremia impede a transmissão (Cohen et al. 2011; Rodger et al. 2019) gerou uma revolução nos protocolos de manejo clínico da infecção, favorecendo o surgimento de políticas de tratamento universal para

indivíduos infectados. No Brasil, a oferta do tratamento gratuito com antirretrovirais foi ampliada desde 2013 a todos os indivíduos infectados, independente dos níveis de linfócitos T CD4 (Ministério da Saúde 2014). Para HICs, no entanto, não está claro se a terapia apresentará efeitos benéficos o suficiente para ser indicada, visto que os relatos existentes até o momento são inconsistentes (Promer and Karris 2018) .

Em anos anteriores a essas políticas, os desfechos de aumento da carga viral ou queda na contagem de linfócitos T $CD4^+$ consistentes durante períodos de seis meses a um ano foram utilizados como um indicativo para o início da terapia antirretroviral nos indivíduos de nossa coorte, visto que elevações crescentes da viremia costumam ser precedentes de perda do controle (Lefrère et al. 1997; Toro et al. 2004; Madec et al. 2013; Noel et al. 2015; Leon et al. 2016; Yang et al. 2017). Com as novas políticas, no entanto, a terapia passou a ser oferecida a grande parte dos indivíduos da nossa coorte, independente do seus status atual de controle. Esta mudança de paradigma se reflete nas diferenças de número amostral entre os subgrupos A e B, principalmente para os pacientes com perfil VC, onde um número maior de indivíduos iniciou o tratamento antirretroviral. Para a segunda parte do estudo, a necessidade de coleta de material recente para as análises de plaquetas impossibilitou o recrutamento de muitos indivíduos que haviam aceitado iniciar a terapia.

Apesar do oferecimento por médicos, o início da terapia precisa ter o consentimento dos pacientes e, por isso, muitos indivíduos com perfil CE não iniciaram a terapia. Na literatura, alguns estudos que realizaram a avaliação dos efeitos da cART em HICs observaram efeitos positivos relacionados a contagem de linfócitos T $CD4^+$ (Okulicz et al. 2009; Boufassa et al. 2014), a carga viral plasmática e proviral (Chun et al. 2013; Hatano et al. 2013b; Li et al. 2019) e na ativação celular (Hatano et al. 2013b; Li et al. 2019). Para marcadores inflamatórios, no entanto, diferenças não foram observadas pós tratamento (Hatano et al. 2013b; Kim et al. 2014). Além disso, muitos dos efeitos positivos observados foram sutis, com pouco declínio dos marcadores virais e ganho pequeno de células T $CD4^+$, e assim, é preciso uma avaliação mais profunda sobre risco e benefício da cART nesses pacientes, considerando os efeitos colaterais da cART e as dificuldades de aderência ao tratamento. Dessa forma, os resultados obtidos aqui para diversos parâmetros relacionados à ativação imune e inflamação poderão vir a somar ao conhecimento, direcionando o manejo clínico de coortes de HICs.

6.2. Ativação imune em HICs

Na primeira parte do presente estudo, o objetivo foi avaliar a ativação imune em HICs e grupos controle do subgrupo A, não só do ponto de vista da frequência de linfócitos T ativados, mas também do balanço de subpopulações de linfócitos que possuem participação e são impactadas por esses processos de ativação imune persistente. A ativação imune durante a fase crônica da infecção pelo HIV-1 é um dos principais problemas associados com a progressão da doença, levando a depleção de linfócitos T $CD4^+$, aumento da replicação viral, exaustão e senescência de linfócitos (Moir et al. 2011). Apesar de significar um aumento da atividade imunológica, esta ativação persistente é prejudicial a longo prazo, como evidenciado pela associação de marcadores de ativação séricos e celulares com a progressão para a aids (Giorgi et al. 1993, 1999; Liu et al. 1997; Zangerle et al. 1998; Mildvan et al. 2005) e com a aterosclerose e o desenvolvimento de doença cardiovascular na população geral (Weiss et al. 1994; Fernandez et al. 2019) e na população HIV-1 positiva (Kaplan et al. 2011; Fitch et al. 2013; Longenecker et al. 2013; Siedner et al. 2016). Para HICs, estudos prévios demonstraram que alguns indivíduos ainda apresentam altos níveis de ativação imune apesar do controle da viremia (Hunt et al. 2008; Pereyra et al. 2012; Krishnan et al. 2014; Côrtes et al. 2015, 2018).

Ao avaliar a frequência de coexpressão de CD38 e HLA-DR entre linfócitos T $CD4^+$ e $CD8^+$, nós identificamos altos níveis de células T ativadas em VCs quando comparados com os outros grupos estudados, indicando a contribuição da replicação viral para o aumento na ativação imune, mesmo em indivíduos com viremia baixa detectável. Somando-se a isso, um aumento na diferenciação de células T evidenciado pelas altas frequências de linfócitos terminalmente efetores (TEFF) em ambos os compartimentos $CD4^+$ e $CD8^+$ também foram observados para esses indivíduos. Estes dados ressaltam a necessidade de acompanhamento clínico e uma vigilância mais cautelosa para indivíduos com baixa viremia, visto que indicam que mesmo baixos níveis de estimulação antigênica persistente levam a desregulação do sistema imunológico.

Menores níveis de ativação imune foram observados nos indivíduos tratados com cART, em comparação com VCs, ressaltando o efeito positivo da terapia antirretroviral no manejo da infecção pelo HIV-1 e indicando que a mesma poderia aprimorar a saúde imunológica de controladores virêmicos ao diminuir a ativação imune. No

entanto, os níveis de ativação em indivíduos tratados foram maiores do que os observados para indivíduos HIV-1 negativos, assim como observado em estudos prévios que indicam que a cART sozinha não é capaz de diminuir a ativação de células T a níveis similares aos observados em indivíduos não infectados pelo HIV-1 (Almeida et al. 2002; Hunt et al. 2003; French et al. 2009; Piconi et al. 2010; Lederman et al. 2011). Estes resultados reforçam a ideia de que, embora seja deflagrada pela infecção viral, a ativação imune é potencializada por fatores que vão além dos efeitos diretos da replicação viral. A circulação aumentada de citocinas pro-inflamatórias, por exemplo, induz a ativação de linfócitos T $CD8^+$ não específicos para o HIV-1 (Doisne et al. 2004; Biancotto et al. 2007; Jiang et al. 2014), enquanto a translocação microbiana decorrente da perda da integridade da mucosa intestinal, incluindo a massiva depleção de linfócitos T $CD4^+$, é considerada um dos principais mecanismos que promovem a ativação imune (Brenchley et al. 2006b; Luo et al. 2019; Younas et al. 2019). Além disso, a baixa permeabilidade dos medicamentos que acarreta em dosagem subótima dos antirretrovirais em sítios anatômicos como o sistema nervoso central, o GALT e os centros germinativos dos linfonodos é associada com a persistência da replicação viral nesses tecidos apesar de cargas virais plasmáticas abaixo do limite de detecção (Popovic et al. 2005; Chun et al. 2008; Cohen 2011; Fletcher et al. 2014; Anderson et al. 2017; Asahchop et al. 2017; Thompson et al. 2019).

Diferente do observado para VCs e indivíduos tratados, níveis de ativação imune similares em ECs e indivíduos não infectados pelo HIV-1 foram observados. Embora esses resultados contrastem com outros estudos que observaram maior ativação de células em ECs (Hunt et al. 2008; Pereyra et al. 2012; Krishnan et al. 2014), frequências similares as de indivíduos não infectados pelo HIV-1 observadas aqui para indivíduos com controle da infecção de longo termo são um sinal de manutenção do equilíbrio do sistema imune em uma magnitude que não é obtida nem mesmo com o auxílio da terapia antirretroviral. A maior frequência de linfócitos TCM $CD8^+$ encontrada nesses indivíduos também reforçam esse cenário de preservação, visto que outros estudos demonstraram a importância de linfócitos TCM $CD4^+$ para manutenção da resposta imune (Potter et al. 2007; Elrefaei et al. 2014). Em associação com a frequência maior de células TCM $CD8^+$, foi possível observar também uma maior frequência da subpopulação de células TSCM em ECs, quando comparados com HIVneg. Linfócitos TSCM são células de memória caracterizadas

por uma expressão aumentada de marcadores naive, alta capacidade proliferativa e potencial de auto renovação (Gattinoni et al. 2011). A proporção de TSCM $CD8^+$ já foi inversamente correlacionada com a replicação viral e a ativação imune (Ribeiro et al. 2014) e a preservação da população TSCM $CD4^+$ foi associada com um melhor prognóstico nas infecções por HIV-1 e por SIV (Klatt et al. 2014; Ribeiro et al. 2014). Os dados aqui apresentados também sugerem a associação entre a manutenção dessas populações e o melhor controle da infecção.

Além de alteração nas subpopulações naive, de memória e efetora, o presente trabalho também investigou as frequências de linfócitos Th17 e Treg nos HICs, visto que essas células são capazes de controlar algumas dinâmicas de outras células do sistema imune através de atividade supressora, no caso das Tregs, ou pró inflamatória, no caso das Th17. Nesse estudo, os dois grupos de HICs apresentaram baixas frequências de Tregs totais em comparação com os dois grupos controle. Indícios da capacidade imunossupressora dessas células podem ser observados na correlação negativa obtida entre a frequência de Tregs totais e a ativação de linfócitos T $CD8^+$, corroborando outros estudos que observaram a relação entre a ativação imune e as Tregs (Kinter et al. 2007; Jiao et al. 2009; Zelinskyy et al. 2009). Enquanto essa associação indica um efeito positivo para maiores frequências dessa subpopulação atuando no controle da ativação imune exacerbada associada a infecção pelo HIV-1 (Jiao et al. 2009; Schulze Zur Wiesch et al. 2011; Angin et al. 2012), diversos estudos observaram uma correlação direta entre frequências de Treg com progressão para aids e com maiores cargas virais plasmáticas (Andersson et al. 2005; Tsunemi et al. 2005; Nilsson et al. 2006; Baker et al. 2007; Chase et al. 2008; Bi et al. 2009; Suchard et al. 2010; Hunt et al. 2011b; Nikolova et al. 2011; Schulze Zur Wiesch et al. 2011).

Embora alguns estudos tenham observado maiores frequências ou contagens absolutas de Tregs em HICs quando comparados com indivíduos não infectados pelo HIV-1 (Brandt et al. 2011; Card et al. 2012), a maioria mostrou frequências menores (Hunt et al. 2011b; Simonetta et al. 2012) ou similares (Owen et al. 2010; Shaw et al. 2011; Angin et al. 2012; Jenabian et al. 2013; Falivene et al. 2015) de Tregs entre esses dois grupos. Nesse contexto, baixas frequências de Tregs em HICs, como observado nesse estudo, apontam mais uma vez para a preservação da capacidade de resposta imunológica em controladores do HIV-1. Com base na expressão diferencial de CD45RA e Foxp3, as Tregs podem ser classificadas em três diferentes

subpopulações: Tregs ativadas, em repouso e não supressoras. Apesar da menor frequência de Tregs totais, a frequência de Tregs ativadas observada estava aumentada em ECs quando comparados com os dois grupos controle. Estes resultados estão de acordo com observações prévias de Gaardbo e colaboradores, em 2014, e indicam que o balanço entre as diferentes subpopulações de Tregs pode ter um papel importante na patogênese do HIV-1 e que a influência deste tipo de linfócito na progressão da doença vai além da correlação simples entre aumento na frequência e progressão da doença.

Evidências de um sistema imune mais equilibrado também foram observados em relação a linfócitos Th17. Nesse estudo, altas frequências de células Th17 no sangue periférico dos dois grupos de HICs foram observadas em comparação com os dois grupos controle. Células Th17 são importantes no contexto da infecção pelo HIV-1 devido a sua participação em processos de defesa do hospedeiro contra diversos patógenos na mucosa intestinal. Além disso, células Th17 também contribuem para a regeneração da mucosa através da indução de mecanismos de proliferação e sobrevivência epitelial associados a IL-22 (Pickert et al. 2009; Brockmann et al. 2017), auxiliando na manutenção da integridade física da barreira da mucosa intestinal. O GALT é um dos principais sítios de replicação do HIV-1 e sofre uma depleção massiva de linfócitos T $CD4^+$, incluindo células Th17, ainda nos estágios iniciais da infecção (Veazey et al. 1998; Brenchley et al. 2004b), o que induz o estabelecimento de um estado pró inflamatório que leva a perda da integridade da barreira intestinal e potencializa a translocação microbiana. O aumento da translocação microbiana, como mencionado acima, é discutida como uma das principais causas da ativação imune observada em indivíduos infectados pelo HIV-1 (Brenchley et al. 2006b; Luo et al. 2019; Younas et al. 2019), indicando uma correlação entre células Th17 e a ativação imune.

Em geral, as frequências de linfócitos Th17 correlacionam negativamente com a carga viral plasmática e positivamente com as contagens de linfócitos T $CD4^+$. Apesar de declínios serem mais perceptíveis em regiões da mucosa intestinal (Cecchinato et al. 2008; Macal et al. 2008; Ciccone et al. 2011), Baixas contagens dessa subpopulação no sangue periférico foram observadas em indivíduos infectados pelo HIV-1 em progressão da doença (Brenchley et al. 2008; Macal et al. 2008; Ndhlovu et al. 2008; Prendergast et al. 2010; Salgado et al. 2011; Falivene et al. 2015). Esses resultados indicam que a depleção dessas células associada a infecção pelo HIV

ocorre no organismo como um todo, embora esse efeito seja mais perceptível na mucosa intestinal, podendo contribuir para a perda de do equilíbrio imunológico nesse local. Falivene e colaboradores demonstraram o valor prognóstico da frequência de células Th17, mostrando que baixas frequências desta população e altas frequências de células ativadas foram observadas na fase aguda de indivíduos que progridem mais rapidamente para a aids (Falivene et al. 2015). Em contraste, frequências maiores durante a fase aguda são associadas com o aprimoramento da resposta T HIV-1-específica (Falivene et al. 2015). Entre HICs ou LTNPs, as frequências de células Th17 no sangue periférico costumam ser similares às observadas em indivíduos não infectados pelo HIV-1 (Ciccone et al. 2011; Salgado et al. 2011; Jenabian et al. 2013; Falivene et al. 2015). No presente estudo, ECs, mas não VCs, tiveram maiores frequências de células Th17 comparado com os grupos controle, corroborando um papel protetivo desses linfócitos durante a infecção pelo HIV-1.

Além da dinâmica individual dos linfócitos Th17 e Treg, também foi observada uma correlação inversa entre a frequência das duas subpopulações, conforme esperado, visto que essas células compartilham etapas importantes de seus processos ontogênicos (Bettelli et al. 2006). Os dados respectivos a razão Th17/Treg nos dois grupos HIC concordam com as observações prévias que encontraram maiores razões em indivíduos com controle natural da infecção em comparação com progressores típicos ou indivíduos não infectados pelo HIV-1 (Brandt et al. 2011; Jenabian et al. 2013; Falivene et al. 2015). No geral, esta observação se soma as outras que indicam a manutenção da integridade e do equilíbrio da resposta imune em ECs.

As análises realizadas com o subgrupo A e os resultados discutidos nesta sessão foram compilados no artigo científico “HIV-1 elite controllers present a high frequency of activated regulatory T and Th17 cells”, publicado na revista PLoS One em 2020 e disponível na íntegra como Anexo 9.3.1.

6.3. Inflamação em HICs

Para a segunda parte do estudo, o objetivo foi complementar as observações verificadas para a ativação de células T com o estudo de uma série de processos associados a inflamação. A inflamação apresenta um caráter mais amplo e engloba uma série de processos imunológicos que visam principalmente o recrutamento de células para o sítio de infecção ou lesão tecidual. Mesmo sendo essenciais para a homeostase, a exacerbação desses mecanismos gera uma série de fenômenos que

ajudam a explicar a maior susceptibilidade a comorbidades em PVHIV. Em indivíduos infectados pelo HIV-1, o aumento de marcadores inflamatórios, como IL-6, (Kuller et al. 2008; Boulware et al. 2011; Sandler et al. 2011; Duprez et al. 2012; Hunt et al. 2014; Tenorio et al. 2014; Grund et al. 2016; Baker et al. 2017b; Carvalho et al. 2018; Peterson et al. 2018), sinais de hipercoagulabilidade (Karmochkine et al. 1998; Mayne et al. 2012; O'Brien et al. 2013; Nkambule et al. 2015b, 2015a, 2015c; O'Halloran et al. 2015; Mesquita et al. 2018) e alterações na frequência de subpopulações de monócitos (Abel et al. 1992; Thieblemont et al. 1995; Pulliam et al. 1997; Amirayan-Chevillard et al. 2000; Han et al. 2009; Funderburg et al. 2012; Liang et al. 2015; Chen et al. 2017; Luo et al. 2018) são observados, ao mesmo tempo que correlacionam com o risco para o desenvolvimento de doenças cardiovasculares. Dessa forma, o presente trabalho utilizou amostras dos indivíduos do subgrupo B para caracterizar o estado de ativação de plaquetas, as dinâmicas de ativação e de subpopulações de monócitos e a concentração de marcadores inflamatórios nas populações de HICs na tentativa de observar se estes indivíduos apresentam sinais de inflamação persistente que caracterize risco aumentado para o desenvolvimento de comorbidades.

Considerando que a inflamação é um processo normal do organismo que pode ser deflagrado por diversas razões e que o risco para comorbidades está associado a fatores como a idade e sexo, o recrutamento dos indivíduos dos grupos controle cART e HIVneg para a segunda parte do estudo foi realizado de modo a obter grupos pareados, buscando minimizar diferenças em relação a prevalência de fatores que poderiam ser confundidores. Dessa forma, os quatro grupos estudados no subgrupo B não apresentaram diferença estatística para a maioria dos possíveis fatores de risco para o desenvolvimento de doenças de cardiovasculares, como idade, hipertensão, diabetes e fumo. Diferenças significativas foram encontradas na prevalência de dislipidemia entre os grupos, porém todos os indivíduos com essa condição realizavam tratamento com estatinas e mantinham níveis controlados de colesterol. Estes indivíduos inclusive apresentaram níveis menores de lipídios do que os indivíduos não dislipidêmicos. Diferenças estatísticas também não foram observadas para as seguintes análises: frequência de exercício físico e abuso de álcool, ou parâmetros antropométricos, como valores de pressão arterial IMC e circunferência abdominal. Posteriormente, as análises estatísticas comparando os diferentes parâmetros analisados foram corrigidas por idade, sexo, hábito de fumo, IMC, ocorrência de hipertensão, dislipidemia e diabetes e valores de colesterol total e LDL,

de modo a confirmar que as diferenças observadas entre os grupos eram relacionadas a infecção pelo HIV-1 e não a outros fatores confundidores.

Um dos efeitos da inflamação na infecção pelo HIV-1 é a indução de um estado de hipercoagulação que se associa diretamente ao risco aumentado para eventos trombóticos em indivíduos infectados (Luz et al. 2014; Diaz et al. 2016; Rokx et al. 2020). No presente estudo, os maiores níveis de ativação plaquetária foram observados para o grupo VC, com esses indivíduos apresentando maiores frequências de plaquetas expressando P-selectina e maiores concentrações de D-dímero. Esses resultados concordam com estudos anteriores que demonstraram indução de um estado de hiperativação de plaquetas na infecção pelo HIV-1 (Karmochkine et al. 1998; Mayne et al. 2012; O'Brien et al. 2013; Nkambule et al. 2015b, 2015a, 2015c; O'Halloran et al. 2015; Mesquita et al. 2018) e que observaram que o nível de plaquetas ativadas ou de marcadores associados a hipercoagulabilidade correlacionam com a carga viral plasmática (Karmochkine et al. 1998; Mayne et al. 2012; Nkambule et al. 2015c).

Entre os indivíduos com viremia suprimida, dois padrões foram observados em relação a expressão de P-selectina. Apesar de outros estudos terem observado que a terapia antirretroviral não normaliza os níveis de ativação plaquetária (O'Brien et al. 2013; Mesquita et al. 2018), não observamos diferenças significativas na frequência de plaquetas ativadas ou no MFI de P-selectina entre o grupo cART e o grupo HIVneg. Vale ressaltar, no entanto, que os trabalhos citados utilizaram indivíduos mais jovens no grupo controle não infectado pelo HIV-1, o que pode ter impactado nas diferenças. No trabalho de O'Halloran e colaboradores, em 2015, os autores observaram normalização das concentrações séricas de P-selectina após 12 meses de terapia, comparando grupos sem diferença de idade, embora mais novos do que nos dois trabalhos anteriores e no presente estudo.

Em ECs, por sua vez, os valores de MFI para P-selectina foram significativamente mais altos quando comparados com HIVneg e cART, mostrando que controladores de elite mantém um estado de ativação plaquetária maior que o observado para indivíduos tratados. Até o momento, estas observações configuram o primeiro relato para HICs e dão indícios de um estado persistente de inflamação que pode contribuir para o desenvolvimento de complicações isquêmicas e trombóticas em indivíduos com controle natural da viremia.

Outra perspectiva para a ativação plaquetária, com resultados também inéditos para HICs, diz respeito a proporção de agregados monócitos plaquetas (MPAs). Estes agregados são formados pela interação entre a P-selectina (CD62P) de plaquetas e a glicoproteína ligante de P-selectina 1 (PSGL-1) de monócitos e intensificam o perfil inflamatório (Thomas and Storey 2015), aumentam a adesão ao endotélio (Martins et al. 2006) e estimulam a secreção de MCP-1 e TNF-α por monócitos (Weyrich et al. 1995). Estes agregados são observados em frequências aumentadas na infecção pelo HIV-1 (Singh et al. 2012; Tunjungputri et al. 2014; Liang et al. 2015; Nkambule et al. 2015b; van der Heijden et al. 2018) e durante o desenvolvimento de doenças cardiovasculares (Furman et al. 2001; Michelson et al. 2001; Htun et al. 2006; Smout et al. 2009; Czepluch et al. 2014), porém frequências distintas de MPAs não foram observadas no presente estudo, nem mesmo para o grupo VC.

De maneira independente, monócitos são células que apresentam grande importância no processo inflamatório. No presente trabalho, pacientes infectados pelo HIV-1 apresentaram maiores frequências de monócitos não clássicos concomitantes com menores frequências de monócitos clássicos. Estas diferentes populações são caracterizadas pelo nível de expressão de CD14 e CD16 e apresentam funções e características distintas no processo inflamatório. Monócitos clássicos ($CD14^{++}CD16^{-}$) englobam a maioria dos monócitos no organismo e são recrutados para os tecidos logo no início de processos inflamatórios, possuindo grande capacidade de resposta contra patógenos. Monócitos não clássicos ($CD14^{+}CD16^{++}$), por sua vez, apresentam função de patrulhamento endotelial e vascular e migram para os tecidos em momentos mais tardios do processo inflamatório, possuindo um caráter anti-inflamatório e participando de processos de reparo tecidual e controle da inflamação. Monócitos intermediários ($CD14^{++}CD16^{+}$) são caracterizados como um fenótipo intermediário entre os dois anteriores e apresentam alta capacidade de resposta, apresentação de antígenos e resposta pró inflamatória (Ziegler-Heitbrock 2006, 2015; Sprangers et al. 2016; Patel et al. 2017).

O balanço observado aqui em relação as populações de monócitos concordam com as observações prévias de que a infecção pelo HIV-1 favorece um incremento de populações intermediárias e não clássicas (Abel et al. 1992; Thieblemont et al. 1995; Pulliam et al. 1997; Amirayan-Chevillard et al. 2000; Han et al. 2009; Funderburg et al. 2012; Liang et al. 2015; Chen et al. 2017; Luo et al. 2018). O aumento maior observado em VCs em comparação com os grupos com viremia suprimida mostra, assim como

observado para todos os outros parâmetros desse estudo, a influência da replicação viral na indução de perfis imunes alterados e se conecta diretamente a estudos prévios que observaram correlações entre populações $CD16^+$ e a carga viral plasmática e a progressão para a doença (Han et al. 2009; Liang et al. 2015; Chen et al. 2017).

Para indivíduos em tratamento antirretroviral, diferenças significativas na proporção de monócitos clássicos e não clássicos em comparação com indivíduos HIVneg só foram observadas para os dados não ajustados. Esta observação demonstra que, embora alguns estudos prévios demonstrem que a cART não é capaz de normalizar as proporções das subpopulações de monócitos (Amirayan-Chevillard et al. 2000; Funderburg et al. 2012; Castley et al. 2014; Chen et al. 2017), a terapia é capaz de gerar resultados positivos, aumentando a proporção de monócitos clássicos e diminuindo a de não clássicos, e reestabelecer parte da dinâmica dessas células. Em indivíduos infectados pelo HIV em tratamento, a supressão da viremia induz um estado de equilíbrio imunológico parcial, de modo que a influência da infecção pelo HIV-1 na indução de um estado de inflamação persistente se confunde com o estado inflamatório associado a outras morbidades, como hipertensão, diabetes e dislipidemia. De maneira complexa, estas outras patologias influenciam e são influenciadas pela resposta inflamatória. A persistência da resposta inflamatória, por exemplo, leva a um desbalanço no metabolismo lipídico que aumenta os níveis de colesterol, caracterizando a dislipidemia (Esteve et al. 2005). Por sua vez, o aumento de lipídios circulantes e a ativação do endotélio decorrente da inflamação favorecem a formação de placas ateroscleróticas, associadas a hipertensão. Por fim, o desequilíbrio nutricional gerado pelo metabolismo lipídico alterado e o acúmulo de tecido adiposo associado são um dos mecanismos associados a resistência insulínica e o desenvolvimento de diabetes. Considerando que a migração de monócitos é favorecida em condições de ativação endotelial e que essas células são centrais no processo de aterogênese, a relação implícita entre monócitos e essas morbidades são de extrema importância. Estas relações complexas citadas anteriormente ficam claras nas correlações entre monócitos clássicos e não clássicos com os dois modelos de risco Framingham utilizados no presente estudo. De maneira independente, monócitos clássicos ainda correlacionaram negativamente com os valores de glicose sanguínea (marcador clássico para diabetes), enquanto monócitos não clássicos correlacionaram positivamente com valores de pressão arterial (marcador clássico de hipertensão).

Em soma aos indícios de inflamação associada a ativação plaquetária, ECs também apresentaram níveis anormais de monócitos não clássicos, concordando com o observado em estudos prévios com HICs (Spivak et al. 2011; Krishnan et al. 2014; Prabhu et al. 2019). A diferença entre os dois grupos permaneceu significativa mesmo após ajuste para fatores confundidores. As frequências dessas células, no entanto, foram próximas as observadas para muitos dos indivíduos tratados, indicando que ECs mantém a frequência dessa subpopulação de monócitos em níveis semelhantes aos alcançados através da cART.

No presente estudo não foram observadas diferenças entre os grupos para a proporção de monócitos intermediários, nem mesmo em VCs. Apesar dessa ser uma população alterada na infecção pelo HIV-1, junto com a população de monócitos não clássicos (Han et al. 2009, 2015; Funderburg et al. 2012; Castley et al. 2014; Chen et al. 2017), sua identificação é prejudicada em estudos de citometria de fluxo e dificultada pela imprecisão em se definir os limites entre a população clássica e não clássica (Ziegler-Heitbrock and Hofer 2013). Amostras não marcadas, amostras FMO e amostras marcadas com isotipos foram utilizadas neste trabalho na tentativa de minimizar erros na identificação das populações, mas a não detecção de diferenças entre os grupos podem transparecer as dificuldades de definição dos limites da população de monócitos intermediários, principalmente em indivíduos com viremia suprimida.

Monócitos não clássicos, apesar de serem classicamente associados com um perfil anti-inflamatório, adquirem perfil de expressão de citocinas pró-inflamatórias mediante estímulos específicos virais ou bacterianos (Belge et al. 2002; Cros et al. 2010; Mukherjee et al. 2015), o que tem grande influência na infecção pelo HIV-1. Apesar disso, a participação de monócitos clássicos não pode ser excluída ao se considerar o processo inflamatório. Em condições de inflamação aguda, essa é a população que migra mais rapidamente para os tecidos (Patel et al. 2017) e que possui os maiores potenciais de produção de TNF-α e IL-1β deflagrada pela ativação através de alguns receptores Toll Like (TLR) (Boyette et al. 2017). Além disso, por representarem a subpopulação mais frequente de monócitos, o fenótipo clássico é a população que mais pode contribuir para o aumento de marcadores de ativação de monócitos observado em processo inflamatórios.

Somando-se a alteração na frequência das subpopulações de monócitos, a infecção pelo HIV-1 também é caracterizado pelo aumento nos níveis séricos de

diversos marcadores de ativação dessas células, como sCD14 e sCD163 (Kuller et al. 2008; Burdo et al. 2011; Pereyra et al. 2012; Kelesidis et al. 2012; Fitch et al. 2013; Masiá et al. 2013; Castley et al. 2014; Westhorpe et al. 2014; Li et al. 2015; Liang et al. 2015; Chen et al. 2017; de Paula et al. 2018). Esses marcadores de ativação também são importantes indicadores da participação de monócitos na gênese de patologias cardiovasculares, visto que também correlacionam com esses processos (Aristoteli et al. 2006; Wildgruber et al. 2009; Sandler et al. 2011; Poitou et al. 2011; Pereyra et al. 2012; Rogacev et al. 2012; Fitch et al. 2013; McKibben et al. 2015; Chow et al. 2016; Hanna et al. 2017; Luo et al. 2018; SahBandar et al. 2019; Subramanya et al. 2019; Höpfner et al. 2019). No presente trabalho, estes marcadores não foram avaliados, porém estudos prévios com nossa coorte não foram observadas diferenças para sCD14 e sCD163 entre ECs, VCs e os grupos controle (Côrtes et al. 2018).

Além desses, o TF também é considerado um marcador de ativação de monócitos importante e que estabelece uma ponte entre a hipercoagulabilidade na infecção pelo HIV-1, a inflamação persistente e atividade de monócitos na aterogênese. Em condições normais, o TF é principalmente expresso por células perivasculares, mas em condições inflamatórias ou mediante estímulo com LPS, monócitos passam a produzir essa molécula. No presente trabalho, as análises da expressão de TF foram dificultadas por indícios de baixa expressão de TF nos pacientes analisados, tornando imprecisa a identificação dos limites entre a população positiva e negativa para este marcador. Os resultados obtidos foram semelhantes aos observados por Schecter e colaboradores, em 2017, que mostraram que a expressão de TF *in vivo* por monócitos não estimulados é muito baixa (Schechter et al. 2017). Análises futuras, testando a expressão basal de monócitos a partir de PBMCs criopreservados e a resposta dessas células mediante estímulo com LPS serão realizadas para melhor caracterizar a expressão deste marcador nos HICs estudados.

A ativação de monócitos também sofre grande influência da ativação de células do endotélio, um processo que é deflagrado em grande parte pela resposta inflamatória e leva a aumento da permeabilidade vascular, expressão de moléculas de adesão e migração de células do sistema imune da corrente sanguínea para os tecidos inflamados (Szmitko et al. 2003). No presente trabalho, foram avaliados nos grupos estudados os níveis séricos de MCP-1, o qual atua como um ativador do endotélio e fator quimiotático para a migração de células imunes, CD40L, um ativador do endotélio importante no processo de aterogênese, e VCAM-1 e ICAM-1, moléculas de adesão

superexpressas em endotélios ativados e que mediam a aderência de monócitos (Szmitko et al. 2003). Para MCP-1, nenhuma diferença estatística entre os grupos foi observada, embora VCs tivessem apresentado valores elevados em comparação com HIVneg nas análises não corrigidas. Os valores de MCP-1, no entanto, correlacionaram positivamente com o nível de expressão de CD62P em plaquetas, o que pode indicar uma ligação entre o processo inflamatório e a ativação plaquetária.

Correlações também foram observadas entre a expressão de VCAM-1 e ICAM-1 e as frequências de subpopulações de monócitos. Para monócitos clássicos, correlações negativas foram observadas, enquanto para monócitos não clássicos as correlações foram positivas, evidenciando a relação entre uma maior ativação do endotélio e o desbalanço nas proporções normais das subpopulações clássica e não clássica de monócitos com o processo inflamatório, o qual é intensificado e intensifica os dois processos.

Em relação aos marcadores de ativação endotelial, os níveis de CD40L foram indetectáveis através da metodologia utilizada para a maioria dos indivíduos estudados. No entanto, os níveis séricos de VCAM-1 e ICAM-1 diferiram significativamente entre os grupos e estavam aumentados nos grupos de indivíduos infectados pelo HIV-1 em comparação com indivíduos HIVneg, corroborando outros estudos que observaram aumento da expressão dessas moléculas na infecção pelo HIV-1 (Wolf et al. 2002; Papasavvas et al. 2008; Calza et al. 2009; Fourie et al. 2011; Graham et al. 2013; O'Halloran et al. 2015; Baker et al. 2017a; Mosepele et al. 2018; Kamtchum-Tatuene et al. 2019; Sereti et al. 2019; Angelovich et al. 2020). Os níveis observados para os dois marcadores foram similares entre cART e VC, apesar de outros estudos indicarem que a terapia antirretroviral diminui os níveis de expressão dessas moléculas (Wolf et al. 2002; Papasavvas et al. 2008; O'Halloran et al. 2015). Estudo recente de Sereti e colaboradores, em 2019, realizou a comparação entre os níveis pré e pós tratamento de VCAM-1 e ICAM-1 de indivíduos com viremia <3000 cópias/ml e observou pequenas quedas na concentração desses marcadores como efeito dos 8 meses de tratamento. No conjunto, estes resultados indicam que a terapia antirretroviral não é capaz de normalizar o estado de ativação do endotélio e as alterações na expressão de vCAM-1 e ICAM-1 permanecem mesmo após a supressão da viremia através da terapia antirretroviral.

Outro resultado que indica que níveis elevados de VCAM-1 e ICAM-1 ocorrem mesmo na ausência de viremia é a persistência de níveis anormais em controladores

de elite. Apesar dos menores níveis observados em comparação com VC e cART e da perda de significância estatística para ICAM-1 após ajuste para fatores de risco, ECs apresentaram níveis acima dos observados em HIVneg para os dois marcadores. No estudo de Sereti e colaboradores, em 2019, os autores também analisam os marcadores de ativação endotelial em um grupo de indivíduos com viremia <50 cópias/ml e observam que a terapia antirretroviral não tem efeito nos níveis desses marcadores. A manutenção de menores níveis de ICAM-1 e VCAM-1 que os observados para indivíduos tratados se assemelha ao observado para a ativação de células T na primeira parte desse estudo e é mais um indicativo de que ECs apresentam manutenção do equilíbrio e preservação do sistema imune em níveis melhores do que os possibilitados pela terapia antirretroviral. Porém, em contraste com a ativação de linfócitos, a ativação endotelial nesses indivíduos não é normalizada.

Outra observação realizada na análise de ICAM-1 e VCAM-1foi de que os p-valores corrigidos e não corrigidos diferiram muito para ICAM-1, mas não para VCAM-1. Isto pode significar que a inflamação mediada pelo HIV-1 leve a ativação de mecanismos independentes que culminem em maior expressão de VCAM comparado com ICAM-1. A expressão de ICAM-1, dessa forma, seria mais influenciada por outros fatores do que VCAM-1. Corroborando essa hipótese, estudo com camundongos como modelo de aterosclerose observou que a quantidade de colesterol na dieta dos animais influencia os níveis de expressão de ICAM-1, mas não de VCAM-1 na aorta. Indícios de vias de ativação de ICAM de maneira independente e a influência da dieta puderam ser observadas nas análises de correlação, as quais demonstraram que ICAM correlaciona com os níveis de colesterol HDL e Glicose, mas não VCAM.

Além desses marcadores, também foram observadas maiores frequências de ST2 em VCs quando comparados com indivíduos não infectados. O ST2 é um receptor de IL-33R que possui uma forma solúvel cuja concentração sanguínea é aumentada em resposta ao estiramento do miocárdio, funcionando como um biomarcador para estresse cardíaco e um marcador independente de mortalidade (Ky et al. 2011; Kohli et al. 2012; Braunwald 2013). Na infecção pelo HIV-1, estudos observaram maiores níveis em indivíduos infectados (Miyagaki et al. 2011; Mehraj et al. 2016; Younas et al. 2017) e que esses níveis eram preditores de mortalidade para todas as causas analisadas (Secemsky et al. 2015; Thiébaut et al. 2017). Os níveis aumentados de ST2 observados aqui nos VCs se juntam as observações que indicam maiores níveis

de ativação imune e inflamação nesses indivíduos, mesmo com o controle parcial da viremia denotam que estes indivíduos podem possuir um risco em potencial para o desenvolvimento de doenças cardiovasculares associado diretamente ao HIV-1.

A inexistência de um grupo de indivíduos infectados pelo HIV-1 sem tratamento e sem controle espontâneo da viremia, além de uma maior prevalência de indivíduos com CVD, não nos permite saber a magnitude comparativa desse risco em VCs. Além disso, o baixo N amostral decorrente da raridade dos HICs e de alguns terem iniciado a terapia, impossibilitando a inclusão no subgrupo B, também se configuram como fatore limitantes que atrapalham a confirmação de algumas correlações buscadas aqui. Somam-se a estas limitações as dificuldades técnicas de se acessar e obter amostras de tecidos que poderiam melhor evidenciar as dinâmicas de algumas células, como as Th17 que são mais prevalentes na mucosa.

Os dados observados, no entanto, servem de indicativo de que o risco para o desenvolvimento dessas comorbidades em ECs é, no máximo, similar ao risco existente para indivíduos em cART. Apesar de estudos prévios terem observado alterações no metabolismo lipídico (Vidal et al. 2012; Tort et al. 2018), maiores valores de espessura da camada intima media da carótida (Hsue et al. 2006; Pereyra et al. 2012; Brusca et al. 2020) e maior taxa de hospitalizações em ECs (Crowell et al. 2015), o único marcador associado a um pior prognóstico e que estava aumentado em relação a indivíduos tratados no nosso estudo foi a expressão de P-selectina em plaquetas. No conjunto, controladores de elite apresentaram melhor equilíbrio do sistema imune ou similares aos induzidos pela terapia antirretroviral, como uma menor frequência de linfócitos T ativados, subsidiando estudos que não observaram risco aumentado para o desenvolvimento de comorbidades nesses indivíduos comparados com indivíduos tratados (Lucero et al. 2013; Crowell et al. 2016; Noël et al. 2019a). A persistência de níveis alterados de alguns marcadores importantes, como as moléculas de adesão VCAM-1 e ICAM-1, indicam que o uso de terapias alternativas à cART, que visem diminuir os níveis persistentes de inflamação podem ser mais adequados para esses indivíduos.

7. CONCLUSÃO

No conjunto, diversos sinais de preservação do sistema imune são observados em controladores de elite na ausência da terapia antirretroviral enquanto controladores virêmicos mantém a persistência de diversos marcadores indicadores de desequilíbrio imunológicos e associados com a progressão para a aids. VCs apresentaram maior frequência de linfócitos ativados e efetores, maiores níveis de ativação plaquetária, alteração na proporção das diferentes subpopulações de monócitos e na expressão de marcadores inflamatórios relacionados a coagulação e ativação endotelial, indicando que a carga viral baixa característica desses indivíduos induz níveis de ativação imune e inflamação elevados que geram risco para piores prognósticos associados a infecção pelo HIV-1 e a outras comorbidades. Os menores níveis desses marcadores em indivíduos tratados com a terapia antirretroviral em comparação com VCs mostra que indivíduos com esse perfil podem se beneficiar do tratamento de maneira significativa.

ECs, por outro lado, apresentaram baixos níveis de células T ativadas e de células com perfil efetor e níveis aumentados de células T de memória importantes para a integridade do sistema imunológico, como TCM e TSCM. Em associação, maiores frequências de Tregs ativadas nesses indivíduos podem auxiliar no controle da ativação imune e dos desequilíbrios associados a este fenômeno, enquanto a preservação de populações Th17 no sangue periférico pode ser indicativo de preservação dessas células em regiões de mucosa e da preservação da resposta imunológica nesses sítios.

Apesar desses resultados, ECs ainda mantém níveis alterados de marcadores associados a inflamação. Dentre esses, destacam-se níveis aumentados de ativação plaquetária, tendências de diminuição na frequência de monócitos clássicos e aumento de frequência não clássicos, assim como concentrações aumentadas de VCAM-1/ICAM-1. Os níveis alterados, no entanto, foram menores do que os observados para VCs e similares aos observados para os indivíduos tratados, indicando que controladores de elite são capazes de manter espontaneamente níveis de inflamação similares aos alcançados em resposta a terapia antirretroviral. Assim, a utilização de terapias alternativas à cART que visem diminuir a inflamação

persistente em ECs, pode ser vantajosa ao controlar parâmetros relacionados ao risco para o desenvolvimento de comorbidades.

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9. ANEXOS

9.1. Lista de anticorpos e painéis de marcação utilizados no estudo

Tabela Suplementar 1. Painéis de marcação por citometria de fluxo utilizados no estudo

PAINEL	ANTICORPO	FABRICANTE	CATÁLOGO	Título
Ativação e Subpopulações de Linfócitos T	FVS 450	BD Biosciences	562247	0,125/100
	CD3 APC-H7		560176	0,5/100
	CD4 PECF594		562281	2/100
	CD8 APC		555369	4/100
	CD45RA PE-Cy7		561216	
	CD27 BV510		563092	1/100
	CCR7 Alexa Fluor 700		561143	1/100
	CD95 PERCP-cy5.5		561655	1/100
	HLA-DR PE		555812	3/100
	CD38 BB515		564498	1/100
Treg e Th17	FVS 450	BD Biosciences	562247	0,125/100
	CD3 APC-H7		560176	0,5/100
	CD45RA PE-Cy7		561216	
	Human Th17/Treg Phenotyping Kit		560762	20/100
	CD25 BB515		564467	
	CD8 BV510		563256	
Plaquetas	CD41 APC	BD Biosciences	559777	2/100
	CD62 PE		550888	4/100
MPA	CD3 FITC	BD Biosciences	555339	5/100
	CD41 APC		559777	5/100
	CD14 PERCP-Cy 5.5		561116	3/100
Monócitos	CD3 FITC	BD Biosciences	555339	3/100
	CD14 PERCP-Cy 5.5		561116	2/100
	CD16 APC		561304	2/100
	CD142 PE		550312	10/100

9.2. Esquemas Terapêuticos dos indivíduos cART do subgrupo B

Tabela suplementar 2. Esquemas terapêuticos e os respectivos medicamentos utilizados entre os indivíduos do Subgrupo B

INDIVÍDUO	ESQUEMA TERAPÊUTICO	MEDICAMENTOS
cART01	2ITRN+1ITRNN	AZT/3TC/EFV
cART02	2ITRN+ 1ITRNN	AZT/3TC/EFV
cART03	1ITRN+1INI+2IP	3TC/DGV/DRV/RTV
cART04	INI+2IP	DTG/DRV/RTV
cART05	2ITRN+1INI	TDF/3TC/DTG
cART06	2ITRN+1ITRNN	TDF/3TC/EFV
cART07	2ITRN+1ITRNN	TDF/3TC/EFV
cART08	2ITRN+1ITRNN	TDF/3TC/EFV
cART09	2ITRN+2IP	TDF/3TC/LPV/RTV
cART10	2ITRN+1ITRNN	TDF/3TC/EFV
cART11	2ITRN+1ITRNN	TDF/3TC/EFV
cART12	2ITRN+2IP	TDF/3TC/ATV/RTV
cART13	2ITRN+2IP	TDF/3TC/DRV/RTV
cART14	2ITRN+2IP	ABC/3TC/DRV/RTV
cART15	2ITRN+1IP	TDF/3TC/DRV
cART16	2ITRN+1ITRNN	TDF/3TC/NPV
cART17	2ITRN+1INI	TDF/3TC/DTG
cART18	2ITRN+1INI	TDF/3TC/DTG

ITRN: Inibidor da transcriptase reversa nucleosídeo; ITRNN: Inibidor da transcriptase reversa não nucleosídeo; IP: Inibidor da protease; INI: Inibidor da integrase; AZT: Zidovudina; 3TC: Lamivudina; EFV: Efavirenz; DGV: Dolutegravir; DRV: Darunavir; RTV: Ritonavir; LPV: Lopinavir; ATV: Atazanavir; NPV: Nevirapina

9.3. Documentos diretamente relacionados a tese

9.3.1. **Caetano DG**, de Paula HHS, Bello G, Hoagland B, Villela LM, Grinsztejn B, et al. HIV-1 elite controllers present a high frequency of activated regulatory T and Th17 cells. PLoS One. 2020

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RESEARCH ARTICLE

HIV-1 elite controllers present a high frequency of activated regulatory T and Th17 cells

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Abstract

HIV-1 infection is characterized by generalized deregulation of the immune system, resulting in increased chronic immune activation. However, some individuals called HIV controllers (HICs) present spontaneous control of viral replication and have a more preserved immune system. Among HICs, discordant results have been observed regarding immune activation and the frequency of different T cell subsets, including Treg and Th17 cells. We evaluated T cell immune activation, differentiation and regulatory profiles in two groups of HICs—elite controllers (ECs) and viremic controllers (VCs)—and compared them to those of cART-treated individuals (cART) and HIV-1-negative (HIV-neg) individuals. ECs demonstrated similar levels of activated $CD4^+$ and $CD8^+$ T cells in comparison to HIV-neg, while cART and VCs showed elevated T cell activation. $CD4^+$ T cell subset analyses showed differences only for transitional memory T cell frequency between the EC and HIV-neg groups. However, VC individuals showed higher frequencies of terminally differentiated, naïve, and stem cell memory T cells and lower frequencies of transitional memory and central memory T cells compared to the HIV-neg group. Among $CD8^+$ T cell subsets, ECs presented higher frequencies of stem cell memory T cells, while VCs presented higher frequencies of terminally differentiated T cells compared to the HIV-neg group. HICs showed lower frequencies of total Treg cells compared to the HIV-neg and cART groups. ECs also presented higher frequencies of activated and a lower frequency of resting Treg cells than the HIV-neg and cART groups. Furthermore, we observed a high frequency of Th17 cells in ECs and high Th17/Treg ratios in both HIC groups. Our data showed that ECs had low levels of activated T cells and a high frequency of activated Treg and Th17 cells, which could restrict chronic immune activation and be indicative of a preserved mucosal response in these individuals.

analysis, decision to publish, or preparation of the manuscript.

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Introduction

HIV-1 controllers (HICs) are a rare group of HIV-1-infected individuals able to spontaneously control viral replication in the absence of combined antiretroviral therapy (cART). Classically, these individuals are divided into two groups: Elite controllers (ECs), who are able to keep plasma viral loads below the detection limit of clinical assays (currently < 40 HIV-1 RNA copies/ml), and viremic controllers (VCs), who present plasma viral loads < 2,000 HIV-1 RNA copies/ml [1].

HIV-1 infection is characterized by generalized deregulation of the immune system, resulting in high levels of chronic immune activation [2,3], which has been described as a state of increased cellular turnover, cell cycle deregulation and establishment of an inflammatory setting [2,4] that is not fully normalized even after initiation of cART [5–8]. Moreover, alterations in the frequency of different T cell subsets, leading to an increase in effector or fully differentiated T cells [2,4,9–11] and a decrease in naïve T cells [2,10,12,13], have also been observed as a consequence of the chronic immune activation. Despite the viremia control, some HICs present higher levels of immune activation and inflammation than HIV-1-uninfected individuals [14–16], mainly the VC individuals [17,18].

In addition to alterations in the frequency of naïve, effector and memory T cells, the chronic phase of HIV infection has been associated with an increased frequency of regulatory T cells (Treg) [19–28], which are a subset of $CD4^+$ T cells that regulate the immune response and the proliferation of effector T cells [29–31]. In the context of HIV-1 infection, the immunosuppressive function of Treg cells has been described to have both detrimental and protective effects on disease progression. Higher frequencies of Treg cells correlate with high plasma viral load and progression to AIDS [19–28], while lower frequencies have been observed for HICs/long-term nonprogressors (LTNPs) [32–35] and cART-treated patients [25,26,28,35,36] and are associated with an increase in viral-specific $CD8^+$ T cell response [37–41]. On the other hand, higher frequencies of Treg cells are associated with a decrease in the systemic immune activation [28,35,42].

Another T cell subset affected during HIV-1 infection is Th17 cells. These cells are enriched in the mucosal tissues and classically produce a set of proinflammatory cytokines (e.g., IL-17, IL-22, IL-21) [43–45] that enhance the expression of antimicrobial peptides [46], recruit neutrophils [47,48] and induce epithelial regeneration [49], thus playing an essential role in the host defense against microbial pathogens and maintenance of epithelial integrity at mucosal sites. Th17 cells are preferentially depleted during the acute phase in pathogenic SIV models [50–52] but preserved in nonpathogenic infection [51,53], and a lower frequency of these cells is observed during the chronic phase in HIV-infected patients with progressive disease [53–56].

Despite their opposite functions, both the Treg and Th17 subsets are derived from a common progenitor cell, with their formation determined by the expression levels of IL-6 and TGF-β [57]. Thus, inverse and reciprocal alterations in both subsets have been observed in the context of HIV-1 infection, and the loss of the balance between these two populations has been associated with disease progression [32–34,51]. In contrast, higher Th17/Treg ratios have been observed in ECs compared to typical progressors [32–34].

In the present study, we aimed to evaluate parameters related to the immune activation, memory T cells, and regulatory T cells in HICs and the distribution of different T cell subsets involved in the immune response. Beyond the frequencies of activated T cells, we evaluated the frequencies of naïve, stem cell memory, central memory, transitional memory, effector memory and terminally differentiated T cells in both ECs and VCs, comparing with the frequencies observed for HIV-negative individuals and cART-treated individuals. We also evaluated the

frequencies of total Tregs and their different subsets, as well as the frequencies of Th17 cells to assess the Th17/Treg balance. Our data showed that ECs had low levels of activated T cells and a high frequency of activated Treg cells, which could contribute to lower immune activation in these individuals. Additionally, a higher frequency of Th17 cells in ECs might be indicative of preserved mucosal response resulting in low microbial translocation and immune activation.

Materials and methods

Study population and ethical statement

Twenty-seven HICs were selected from the Instituto Nacional de Infectologia Evandro Chagas/Fiocruz (INI-Fiocruz) HIV-1 cohort for this study and were classified into two groups: (1) ECs (n = 14) if the plasma viral load (VL) measurements were below the lower detection limit (<LDL) depending on the commercial method available during the clinical and laboratory follow-up (< 50–80 copies/ml) and (2) VCs (n = 13), if most (≥ 70%) VL measurements were >LDL and <2,000 copies/ml. Occasional VL measurements above the upper limits were accepted during the follow-up of the EC and VC groups. A group of HIV-1-infected individuals on cART with a suppressed VL for at least two years (cART; n = 18) and a group of HIV-1-uninfected individuals (HIV-neg; n = 18) were also included as controls. All participants provided written informed consent, and both the INI-Fiocruz Ethical Committee Board and the Brazilian National Human Research Ethics Committee (CONEP 840/2008) approved the study.

Sample preparation

Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Histopaque-1077 (Sigma-Aldrich, USA) density gradient centrifugation and stored in liquid nitrogen until use.

$CD4^+$ and $CD8^+$ T cell count and plasma VL determination

Absolute $CD4^+$ and $CD8^+$ T cell counts were obtained from whole blood using the MultiTest TruCount-kit and the MultiSet software on a FACSCalibur flow cytometer (BD Biosciences, USA). Plasma HIV-1 viral loads of the samples corresponding to the time points analyzed in the present study were measured using the Abbott RealTime HIV-1 assay (Abbott Laboratories, Germany), with LDL of 40 copies/ml.

Flow cytometry

For each patient, vials of $1x10^7$ cryopreserved PBMCs with viability >85% were thawed and rested overnight in RPMI 1640 (Sigma-Aldrich) supplemented with 10% fetal bovine serum (FBS, Gibco—Thermo Fisher Scientific, USA) at 37˚C with 5% of CO_2 and controlled humidity. For naïve, memory, effector and activated $CD4^+$ and $CD8^+$ T cell subsets, detailed below, PBMCs were stained with FVS450 (BD Biosciences, USA) for dead cells exclusion, and with anti-CD3 APC-H7, anti-CD4 PE-CF594, anti-CD8 APC, anti-CD45RA PE-Cy7, anti-CD27 BV510, anti-CCR7 Alexa Fluor 700, anti-CD95 PerCP-Cy5.5, anti- HLA-DR PE and anti-CD38 BB515 (all from BD Biosciences, USA). The T cell activation status was evaluated based on the analysis of CD38 and HLA-DR coexpression, while T cell subsets were classified as follows: naïve (TN: $CD45RA^+CCR7^+CD27^+CD95^-$), stem cell memory (TSCM: $CD45RA^+CCR7^+CD27^+CD95^+$), central memory (TCM: $CD45RA^-CCR7^+CD27^+$), transitional memory (TTM: $CD45RA^-CCR7^-CD27^+$), effector memory (TEM: $CD45RA^-CCR7^-CD27^-$), and effector or terminally differentiated (TEFF: $CD45RA^+CCR7^-CD27^-$).

FMO controls were used to properly identify the CD45RA+, CCR7+,CD27+, CD38+ and HLA-DR+ populations.

For Treg and Th17 cell frequencies determination, PBMCs were stimulated with PMA and ionomycin (50 ng/ml and 1 μg/ml, respectively; Sigma-Aldrich, USA) in the presence of Golgi Stop (Human Th17/Treg Phenotyping Kit; BD Biosciences, USA) according to the manufacturer's instructions, for five hours. The cells were stained with FVS450, anti-CD25-BB515, and anti-CD8-BV510 (all from BD Biosciences, USA). After, the cells were washed with staining buffer (2% of FBS in PBS) and fixed using the Human FoxP3 Buffer A (Human Th17/Treg Phenotyping Kit; BD Biosciences, USA). Subsequently, the cells were washed and incubated with a staining buffer at 4˚C overnight. Then, the cells were permeabilized using Human FoxP3 Buffer C (Human Th17/Treg Phenotyping Kit; BD Biosciences, USA) and stained with anti-CD3-APC-H7, anti-CD45RA-PeCy7 and Human FoxP3 cocktail (Human Th17/Treg Phenotyping Kit; BD Biosciences, USA). Samples were acquired on the same day using a BD FACSAria™ IIu flow cytometer (BD Biosciences, USA), and analyses were performed with FlowJo software v.10.0.7 (Tree Star, USA). Th17 cells were defined as $CD4^{+}IL17^{+}$ T cells, while Treg cells were defined as $CD4^{+}CD25^{high}Foxp3^{+}$ T cells, with the Treg subsets classified as follows: activated Treg ($CD45RA^{-}Foxp3^{high}$), non-suppressive Treg ($CD45RA^{-}Foxp3^{low}$) and resting Treg ($CD45RA^{+}Foxp3^{low}$). FMO controls were used to properly identify the $CD45RA^{+}$, $CD25^{+}$, $IL17^{+}$, $Foxp3^{+}$ populations.

Statistics

Mann-Whitney tests were used to compare the frequencies of the above-cited T cell subsets among the studied groups. Correlations were calculated using Spearman regression. P-values < 0.05 were considered significant. All analyses were carried out using GraphPad Prism v.7.

Results

Clinical and demographic characteristics

The clinical and demographic characteristics of the studied groups are shown in Table 1. No significant difference in age was found between the groups, but ECs had a significantly higher frequency of women than VCs and cART (79% vs. 31% vs. 39%). The plasma VL had a median of 450 copies/ml in VCs, but undetectable levels were found in all ECs and cART. Higher

Table 1. Demographic and clinical characteristics of study participants.

	HIV-neg (n = 18)	cART (n = 18)	EC (n = 14)	VC (n = 13)
Age, median [IQR]	37.1 [29.80–49.55]	44.5 [38.28–50.05]	42.8 [37.60–58.8]	42.7 [37.60–47.05]
Gender (%M)	50	61	21	69
Viral load (copies/ml), median [IQR]	N/A	<40 [<40]	<40 [<40–87]	450 [224.0–881.5]
CD4+ T cells count (cells/mm^3), median [IQR]	831 [741.80–1227]	853[a] [745–1006]	1165 [888–1486]	830 [605–1365]
Years since HIV-1 diagnosis, median [IQR]	N/A	10.9 [8.7–15.1]	8.5 [4.0–15.4]	10.4 [5.4–14.55]

HIV-neg: HIV-1-uninfected individuals; EC: Elite controllers; VC: viremic controllers; cART: Chronic HIV-1 infected individual under cART and at least two years of VL below limit. N/A: not applicable. P-value was obtained using the Mann-Whitney test.

[a] p = 0.0079, comparing EC with cART group.

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$CD4^+$ T cell counts were observed in ECs compared with cART ($p = 0.0079$). Detailed CD4+ T cells/mm3 and VL profiles of the ECs and VCs during the long-term follow-up were previously described [58,59]. ECs and VCs had medians of 8.5 and 10.4 years of HIV diagnosis time, respectively.

When evaluating the level of activation in $CD4^+$ T cells ($CD4^+CD38^+HLA\text{-}DR^+$), ECs showed similar frequencies of activated cells as those observed for the HIV-neg group. On the other hand, the VC and cART groups presented a higher frequency of these activated cells when compared to the EC ($p < 0.0001$ for both groups) and HIV-neg ($p < 0.0001$ and $p = 0.0003$, respectively) groups (Fig 1A). In relation to activated $CD8^+$ T cells ($CD8^+CD38^+HLA\text{-}DR^+$), VCs presented higher levels of activation in comparison with all other groups ($p < 0.0001$ for ECs and HIV-neg; $p = 0.0002$ for cART) (Fig 1B). The cART group presented higher frequencies of activated CD8+ T cells only when compared with HIV-neg individuals ($p = 0.0003$). As observed for the activated $CD4^+$ T cell subset, similar frequencies of activated $CD8^+$ T cells were found in the EC and HIV-neg groups.

Frequency of naïve, memory and effector $CD4^+$ and $CD8^+$ T cell subsets

Phenotypic analyses were performed to compare the frequencies of distinct T cell subsets (TN, TSCM, TCM, TTM, TEM, and TEFF) among the studied groups for both the $CD4^+$ and $CD8^+$

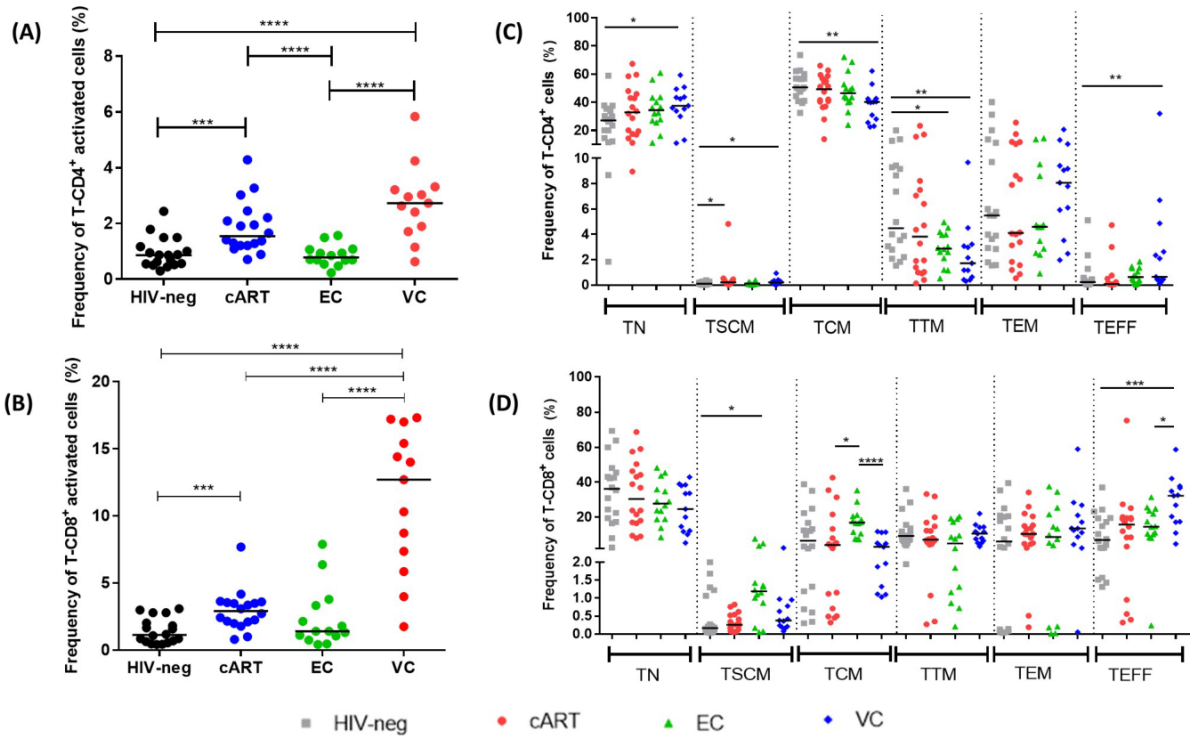

Fig 1. T cell activation levels and subset profiles in HICs and control groups. (A) Frequencies of activated $CD4^+$ ($CD38^+HLA\text{-}DR^+$) T cells. (B) Frequencies of activated $CD8^+$ T cells. (C) Frequencies of naïve (TN; $CD45RA^+CCR7^+CD27^+CD95^-$), stem memory (TSCM; $CD45RA^+CCR7^+CD27^+CD95^+$), central memory (TCM; $CD45RA^-CCR7^+CD27^+$), transitional memory (TTM; $CD45RA^-CCR7^-CD27^+$), effector memory (TEM; $CD45RA^-CCR7^-CD27^-$), and effector (TEFF; $CD45RA^+CCR7^-CD27^-$) $CD4^+$ T cells. (D) Frequencies of TN, TSCM, TCM, TTM, TEM and TEFF $CD8^+$ T cells. For panels C and D, gray squares represent HIV-neg, red circles represent cART, green triangles represent ECs and blue diamonds represent VCs; the horizontal line represents the median for the group; P-values were calculated using the Mann-Whitney test in GraphPad Prism and are represented as follows: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

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T cell compartments. When we evaluated $CD4^+$ T cell subsets (Fig 1C), ECs presented similar levels of all subsets compared to HIV-neg, except for TTM cells, for which a significantly lower level was observed (p = 0.0304). VCs presented higher frequencies of TEFF (p = 0.0062), TN (p = 0.0111) and TSCM cells (p = 0.0315), but lower frequencies of TTM (p = 0.0032) and TCM cells (p = 0.0020) when compared to the HIV-neg group.

Among the $CD8^+$ T cell subsets (Fig 1D), ECs presented higher frequencies of long-lived TSCM cells (p = 0.0139) than HIV-neg, while VCs presented a higher frequency of TEFF cells (p = 0.0007) in comparison to HIV-neg.

Frequency of total Treg cells and Treg subsets

We evaluated the frequency of total Treg cells, and their activated, resting, and non-suppressive subsets (Fig 2). Both ECs and VCs presented lower levels of total Treg cells compared to HIV-neg (p = 0.0018 and p = 0.0001, respectively) and cART groups (p = 0.032 and p = 0.006, respectively) (Fig 2A). When analyzing the Treg subsets, ECs presented higher frequencies of activated Treg cells than the cART (p = 0.037) or HIV-neg (p = 0.003) groups (Fig 2B) and, inversely, a lower frequency of resting Treg cells than the cART (p = 0.008) or HIV-neg (p = 0.036) groups (Fig 2C). Similar frequencies of non-suppressive Treg cells were observed for all groups (Fig 2D). We observed an inverse correlation between total Treg and $CD8^+$ T cell activation ($r = -0.3607$; $p < 0.004$), but we did not detect correlations between Treg subsets and $CD4^+$ T cell activation (S1 Fig).

Frequency of Th17 cells and Th17/Treg ratio

We also analyzed the frequencies of Th17 cells and the Th17/Treg ratio among the studied groups (Fig 3). ECs presented higher levels of Th17 cells and Th17/Treg ratios when compared to the HIV-neg (p = 0.048 and p = 0.002) and cART (p = 0.009 and p = 0.007) groups. Although no statistically significant differences were observed when VCs were compared to the other groups, VCs presented higher Th17/Treg ratios than the cART (p = 0.004) and HIV-neg groups (p = 0.001). We observed an inverse correlation between Th17 frequencies and total Tregs ($r = -0.2515$; p = 0.04, S1 Fig).

Discussion

In the present study, we evaluated parameters related to the immune activation state of T cells and the balance of Th17/Treg cells in HICs with different levels of viral replication control to evaluate immunologic factors related to the better infection control. Although most HIV-infected individuals present an immunological dysregulation characterized by alterations in the frequency of T cell subsets, excessive and systemic immune activation/inflammation and changes in the intestinal mucosa [2,4,60], HICs have a more preserved immunological system and represent a model of spontaneous infection control [61–63].

By evaluating the frequency of $CD38^+HLA\text{-}DR^+$ cells in both $CD4^+$ and T $CD8^+$ T cells, we identified higher levels of activated T cells in VCs compared to the other studied groups, indicating the contribution of viral replication to the increase in immune activation even among individuals with low but detectable viremia. Immune activation during chronic HIV infection is one of the major issues associated with viral persistence and disease progression, leading to CD4 T cell depletion, enhancement of viral replication, and exhaustion and senescence of T cells [2]. This setting results in an impairment of the immune response, despite the increased activity, as shown by the use of serum and cellular activation markers as predictors of AIDS [64–68]. We also observed higher frequencies of $CD4^+$ and $CD8^+$ TEFF cells in VCs, indicating an increase in T cell differentiation. These data highlight the need for increased care and

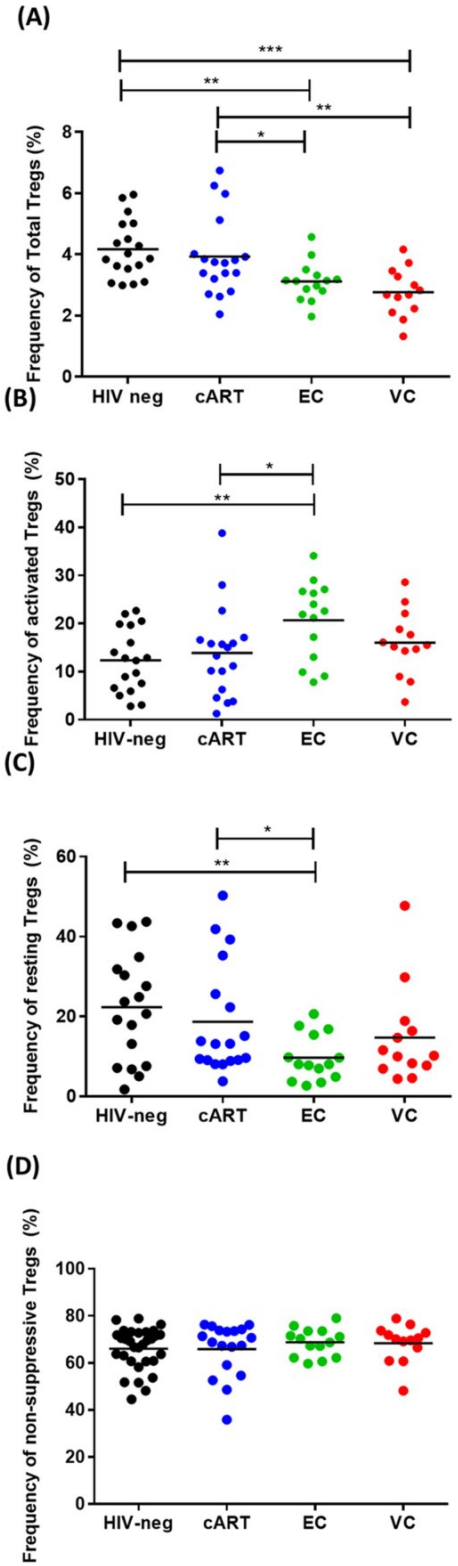
(A)
Frequency of Total Tregs (%)
HIV neg
cART
EC
VC
(B)
Frequency of activated Tregs (%)
HIV-neg
cART
EC
VC
(C)
Frequency of resting Tregs (%)
HIV-neg
cART
EC
VC
(D)
Frequency of non-suppressive Tregs (%)
HIV-neg
cART
EC
VC

Fig 2. Treg cells subset profiles in HICs and control groups. (A) Frequencies of total Treg cells (CD4+CD25highFoxp3+). (B) Frequencies of activated ($CD45RA^-Foxp3^{high}$) Treg cells. (C) Frequencies of resting ($CD45RA^+Foxp3^{low}$) Treg cells; (D) Frequencies of non-suppressive ($CD45RA^-Foxp3^{low}$) Treg cells. The frequencies of activated, resting and non-suppressive Treg cells are relative to those of total Treg cells. P-values were calculated using the Mann-Whitney test in GraphPad Prism and are represented as follows: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

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surveillance of individuals with low-level viremia since even lower levels of antigenic stimulation have a negative effect on the immunological system.

Activation levels were lower among cART-treated patients than VCs, pointing to the undeniable positive effect of cART. In the last decades, drugs with higher genetic barriers and new regimens have been developed [69], bypassing drug resistance issues, improving the survival and quality of life of infected individuals [70–72] and decreasing transmission rates [73,74], which supports the expansion of cART coverage and early initiation. In this context, antiretroviral therapy could improve immunological health in viremic controllers, lowering activation levels as observed in cART individuals. On the other hand, activation levels in cART individuals were higher than those observed in HIV-negative individuals, consistent with previous studies that indicate that cART alone cannot normalize T cell activation [5–8,75]. These data reinforce the idea that, although driven by HIV infection, immune activation is boosted by factors that go beyond the direct effects of viral replication. Bystander activation of $CD8^+$ T cells in HIV infection has been observed to be associated with the reactivation of other viruses [76,77] and with the circulation of proinflammatory cytokines [78,79], while microbial translocation due to $CD4^+$ T cell depletion in the gut mucosa is considered one of the major mechanisms driving immune activation [80–82]. Besides, suboptimal penetration of drugs in anatomical sites such as the central nervous system, GALT, and lymph nodes is associated with persistence of viral replication in those tissues despite plasma viral load <LDL [83–86].

Moreover, our study showed that ECs had low levels of activated T cells, similar to those observed for HIV-negative individuals. Although these results contrast with other studies that showed higher T cells activation in ECs [14–16], the normalized frequencies observed here in patients with long-term control of infection are a signal of immune preservation at a magnitude that is not achieved even with antiretroviral therapy, as most of our studied individuals had long-term HIV infection. The higher frequencies of $CD8^+$ TCM cells in ECs found in our study also point towards this hypothesis, as others have shown the importance of this population to the maintenance of the immune response [87,88]. Also, lower activation levels may not impair the immune response against HIV as other studies have shown that, despite the activation levels, ECs present efficient cytotoxic and HIV-specific response [89]. Together, these data suggest a better immune response in ECs related more to increased efficiency than to increased magnitude.

In addition to the increase in $CD8^+$ TCM frequency when compared with that in cART, we also detected an increased frequency of TSCM cells in ECs compared with HIV-neg. TSCM cells were identified as memory T cells characterized by the increased expression of naïve markers and presenting an increased proliferative capacity and self-renewal potential [90]. Despite the susceptibility of TSCM cells to HIV-1 infection [91], the proportion of $CD8^+$ TSCM cells has been previously inversely correlated to viral replication, and immune activation [92], which is in agreement with our study, and the preservation of the $CD4^+$ TSCM population was associated with a better prognosis in both HIV-1 and SIV infection [92,93]. Our data here also support the association between the maintenance of $CD4^+$ TSCM cells at normal levels with better control of infection.

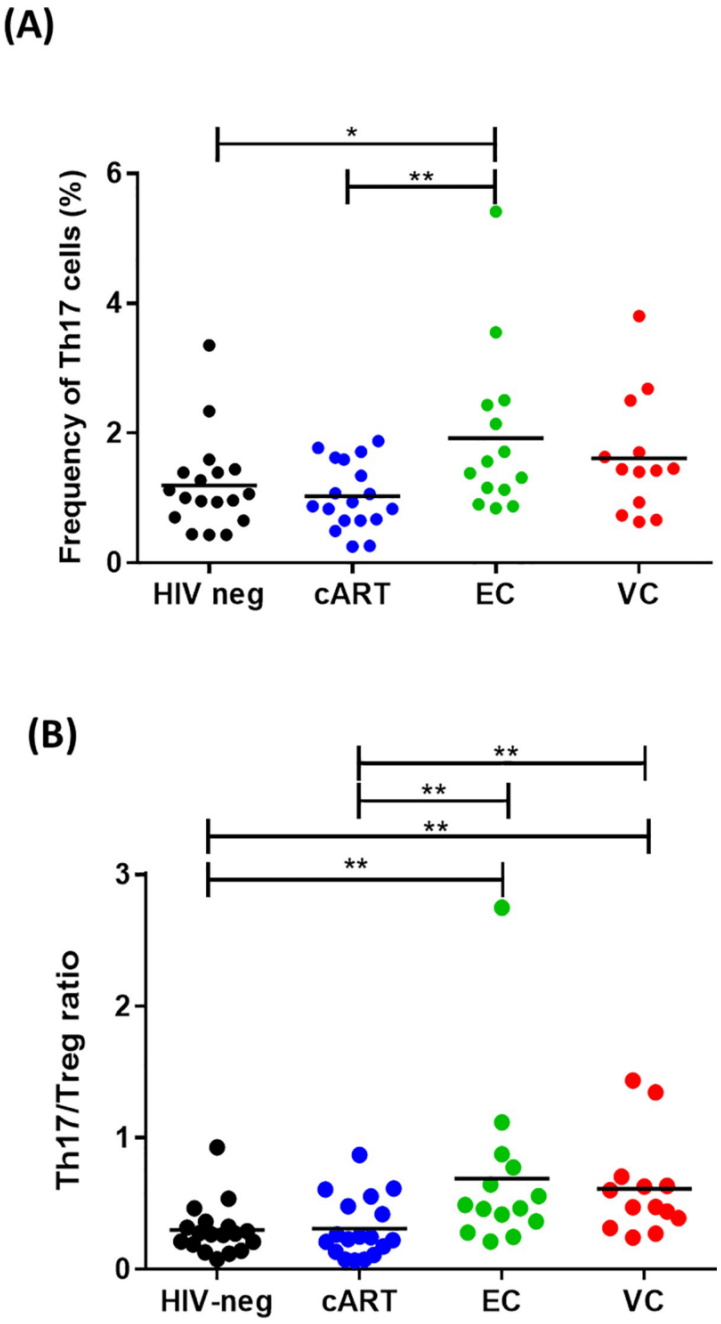

Fig 3. Th17 cell frequencies and Th17/Treg ratio in HICs and control groups. Frequencies of Th17 cells ($CD4^{+}IL17^{+}$) among the studied groups are shown in the graph. (B) Th17/Treg ratios are shown in the graph. The Th17/Treg ratio was calculated by using the frequencies of both populations concerning the CD4+ compartment. P-values were calculated using the Mann-Whitney test in GraphPad Prism and are represented as follows: $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$.

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In addition to alterations in classical naïve, memory and effector T cell subsets, we also investigated the frequencies of both Th17 and Treg cells, as these cells influence the activation of effector T cell profiles in different settings. In our study, we observed lower frequencies of total Treg cells in both HIC groups when compared to the cART and the HIV-neg groups. We also observed a negative correlation between the frequency of total Tregs and activated $CD8^+$ T cells, highlighting the immunosuppressive function of these cells. While this association indicates a positive effect for the increase in the frequency of these cells to control the exacerbated immune activation due to HIV infection [28,35,42], several studies have shown a correlation between higher Treg frequencies and increased viral load and progression to AIDS [19–28].

Although this duality indicates negative effects in the long term in the context of HIV infection, the relationship between Tregs and immune activation could be a useful tool for the development of alternative strategies aiming at reservoir elimination [94]. The depletion of Tregs could be used as a latency reversal strategy to induce HIV replication from reservoirs, contributing to the "shock" needed in "shock and kill" strategies. For example, Treg depletion in HIV-infected humanized mice led to viremia rebound under cART followed by a reservoir decrease in lymphoid tissue [95], while Treg depletion in the NHP model lead to viral rebound and increase in the SIV-specific response [96].

Although some studies observed higher Treg frequencies or absolute counts in HICs compared to HIV-negative individuals [32,97], the majority showed lower [98,99] or similar [33,34,42,89,100] levels of Treg cells among HICs vs. healthy subjects. In this context, the low frequencies of Treg cells in HICs observed in this study and others point towards the preservation of immune responsiveness in these individuals.

Based on CD45RA and Foxp3 expression, Treg cells can be further separated into three different subsets: activated, resting, and non-suppressive Treg cells. Despite the lower frequencies of total Tregs, we observed an increased frequency of activated Tregs and a decrease in resting Tregs in ECs when compared to control groups, as it was observed by Gaardbo et al. [101]. Together, these results indicate that the balance between the different Treg subsets could have an important role in HIV pathogenesis and that the influence of Tregs on disease progression goes beyond the increase in the total Treg population.

Evidence of a preserved immune system in HICs was also observed for Th17 cells. Here, we observed higher frequencies of these cells in the peripheral blood in both ECs and VCs compared with both the HIV-neg and cART groups. Th17 cells are important in the context of HIV infection due to their participation in the host defense processes against several pathogens in the gut tissue. Besides, Th17 cells also induce epithelial regeneration [49], helping to maintain the physical integrity of the mucosal barrier. The GALT is a major site of HIV replication and suffers a massive depletion of $CD4^+$ T cells early in the infection [102,103]. This setting leads to a pro-inflammatory state that disrupts the gut mucosal barrier and enhances microbial translocation. The increase in microbial translocation, as previously stated, is believed to be one of the most significant causes of the increased immune activation observed in HIV-infected patients [80,81,104], highlighting the importance of Th17 cells for the control of immune activation in the context of HIV infection.

In general, frequencies of Th17 cells correlate negatively with the plasma viral load and positively with $CD4^+$ T cell counts, and low frequency of this subset has been observed in HIV-infected patients with progressive disease [33,53–56,105], indicating impairment of the gut immune response. Falivene et al. demonstrated the prognostic value of Th17 cell frequency, showing that lower frequencies of Th17 cells and higher frequencies of activated cells were observed in acutely infected individuals who progress faster to AIDS [33]. In contrast, higher baseline Th17 frequencies in individuals undergoing acute infection are associated with

enhancement of the HIV-specific T cell response [33]. Among HICs or LTNPs, frequencies of Th17 cells are normally similar to those observed in HIV-1-uninfected individuals [33,34,54,106]. In the present study ECs, but not VCs, showed higher frequencies of Th17 cells compared to the HIV-neg and cART-treated controls, indicating a protective role of Th17 cells in HIV-1 infection.

Beyond the individual dynamic of Th17 and Treg subsets, we also observed an inverse correlation between the frequency of Th17 and Treg cells, as expected, since these cells share development pathways [57]. Our data regarding the Th17/Treg ratio on both HIC groups agreed to previous observations that found higher ratios in individuals with natural control of infection in comparison to typical progressors or HIV-negative individuals [32–34]. Overall, this preservation of the Th17/Treg ratio in both HIC groups indicates the preservation of the immune response.

Conclusions

Our data showed that ECs have low levels of activated T cells and a high frequency of activated Treg cells, which can contribute to lower immune activation in these individuals. In addition, the high frequency of Th17 cells in ECs can be indicative of a preserved mucosal response.

Supporting information

S1 Fig. Significant correlations involving the frequency of total Tregs. (A) Correlation between the frequencies of total Tregs and activated CD8+ T cells. (B) Correlation between the frequencies of total Tregs and Th17 cells. R and p-values are shown for each correlation. Dots related to each studied group are coloured according to legend on Fig 1.
(TIF)

S1 Table. Raw data obtained at the study.
(XLSX)

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9.4. Outras produções científicas

9.4.1. **Caetano DG**, Côrtes FH, Bello G, Teixeira SLM, Hoagland B, Grinsztejn B, et al. Next-generation sequencing analyses of the emergence and maintenance of mutations in CTL epitopes in HIV controllers with differential viremia control. Retrovirology. 2018

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Next-generation sequencing analyses of the emergence and maintenance of mutations in CTL epitopes in HIV controllers with differential viremia control

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Abstract

Background: Despite the low level of viral replication in HIV controllers (HICs), studies have reported viral mutations related to escape from cytotoxic T-lymphocyte (CTL) response in HIV-1 plasma sequences. Thus, evaluating the dynamics of the emergence of CTL-escape mutants in HICs reservoirs is important for understanding viremia control. To analyze the HIV-1 mutational profile and dynamics of CTL-escape mutants in HICs, we selected 11 long-term non-progressor individuals and divided them into the following groups: (1) viremic controllers (VCs; n = 5) and (2) elite controllers (ECs; n = 6). For each individual, we used HIV-1 proviral DNA from PBMCs related to earliest (V_E) and latest (V_L) visits to obtain *gag* and *nef* sequences using the Illumina HiSeq system. The consensus of each mapped gene was used to assess viral divergence, and next-generation sequencing data were employed to identify SNPs and variations within and flanking CTL epitopes.

Results: Divergence analysis showed higher values for *nef* compared to *gag* among the HICs. EC and VC groups showed similar divergence rates for both genes. Analysis of the number of SNPs showed that VCs present more variability in both genes. Synonymous/non-synonymous mutation ratios were < 1 for *gag* among ECs and for *nef* among ECs and VCs, exhibiting a predominance of non-synonymous mutations. Such mutations were observed in regions encoding CTL-restricted epitopes in all individuals. All ECs presented non-synonymous mutations in CTL epitopes but generally at low frequency (< 1%); all VCs showed a high number of mutations, with significant frequency changes between V_E and V_L visits. A higher frequency of internal mutations was observed for *gag* epitopes, with significant changes across visits compared to *Nef* epitopes, indicating a pattern associated with differential genetic pressure.

Conclusions: The high genetic conservation of HIV-1 *gag* and *nef* among ECs indicates that the higher level of viremia control restricts the evolution of both genes. Although viral replication levels in HICs are low or undetectable, all individuals exhibited CTL epitope mutations in *proviral gag* and *nef* variants, indicating that potential CTL escape mutants are present in HIC reservoirs and that situations leading to a disequilibrium of the host-virus relationship can result in the spread of CTL-escape variants.

Keywords: HIV-1, HIV controller, CTL epitope, Single-nucleotide polymorphism, Escape mutant, Next-generation sequencing

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Background

One of the main characteristics of HIV-1 infection is the occurrence of clinical, but not virological, latency between the acute and AIDS phases over time, with variable duration among infected individuals that generates distinct progression profiles. Although the majority of individuals present high viral loads during the chronic infection phase, evolving to AIDS after 8–10 years of infection, a small fraction remains clinically asymptomatic for a long period. These individuals have normal $CD4^+$ T cell counts in the absence of antiretroviral treatment (ART) and are termed long-term non-progressors (LTNPs) [1]. Moreover, some individuals, called HIV controllers (HICs), exhibit spontaneous control of viral replication at different levels, maintaining low or undetectable viremia during infection [2].

A crucial characteristic of HIV-1 is the high genetic variability and elevated rate of intra-host viral evolution [3]. This higher rate of viral evolution favors the emergence of viral variants that are more cytopathic [4–6], resistant to ART [7] and/or constitute escape variants from the host immune response [8–11]. In addition to variants that show escape from neutralizing antibodies [12], CTL-escape mutations are important to HIV-1 pathogenesis, as much evidence points to the pivotal role of the $CD8^+$ T cell response in viral control [13–16] and as a continuous force driving viral selection [17]. These escape mutations have been characterized as amino acid changes occurring in central (impairing TCR recognition) and terminal (modifying anchor residues) regions of CTL epitopes or in their flanking regions, impairing epitope processing [18]. CTL-escape mutations begin to arise during the acute phase of infection [19–22] and can be identified even at human population levels based on HLA profiles [23–25]. Especially in individuals with protective HLA alleles, like HLA B*57 and B*27, those mutations arise faster in higher numbers [26] and preferentially, at anchor residues or multisite [27].

Although different studies have identified low HIV-1 evolutionary rates in LTNPs and HICs [28–30], immune-escape variants continue to arise in patients with these profiles, mainly when harboring protective HLA-B alleles [17, 31, 32]. Moreover, HICs present an efficient $CD8^+$ T cell response [33, 34] that can favor high selective pressure and the emergence of immune-escape variants [35]. In HICs, this phenomenon can generate new effective $CD8^+$ T cell responses after viral escape and maintain viremia control [36, 37] or can result in a loss of viremia control and disease progression [38–40]. Regardless, studies have rarely detected CTL-escape mutants in proviral sequences from HICs, even when emerging in plasma viral sequences.

Next-generation sequencing (NGS) is shown a useful tool for studying the dynamics of minority HIV-1 variations in infected individuals. NGS has been successfully applied to reveal hidden mutations conferring resistance to antiretroviral drugs in treated patients [41–43], to monitor viral tropism change dynamics [43, 44], and to assess the dynamics of immune-escape mutations in HIV-1-infected individuals [16, 19, 20]. However, this approach has not yet been employed to evaluate CTL-escape mutations in HICs.

Thus, the present study aimed to apply the NGS strategy to evaluate the overall genetic variability of *gag* and *nef* genes and to identify the emergence of potential CTL-escape mutations in proviral DNA sequences from 11 LTNP/HICs during long-term follow-up.

Methods

Study subjects

A cohort of 11 HICs with an LTNP profile, defined as subjects infected with HIV-1 for at least eight years and maintaining RNA viral loads lower than 2000 copies/ml and $CD4^+$ T cells counts higher than 500 cells/mm^3 without ART, were followed-up at the Instituto Nacional de Infectologia Evandro Chagas (INI), Rio de Janeiro, Brazil. These subjects were classified into the following groups according to plasma viral load (VL): (1) elite controllers (ECs) if most ($\geq$70%) plasma viral load determinations were below the limit of detection for clinically available assays ($<$50 or $<$80 copies/ml) (n = 6) and (2) viremic controllers (VCs) if most ($\geq$70%) VL determinations were between 80 and 2000 copies/ml (n = 5). Patients were seen at least once every 6-12 months to perform clinical monitoring tests, such as RNA viral load quantification and $CD4^+$ T cell count. At each visit, PBMCs were obtained as previously described [45] and stored in liquid nitrogen until use. The present work was approved by the Brazilian National Committee for Research Ethics, and all patients provided 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 MultiSet software with a FACSCalibur flow cytometer (BD Biosciences, California, USA). Plasma VL was measured using the Nuclisens HIV-1 RNA QT assay (Organon Teknika, North Carolina, USA; limit of detection: 80 copies/ml) from 1999 to 2008, the Versant HIV-1 3.0 RNA assay (bDNA 3.0, Siemens, New York, USA; limit of detection: 50 copies/mL) from 2008 to 2013, and the Abbott RealTime HIV-1 assay (Abbott Laboratories, Wiesbaden, Germany; limit of detection: 40 copies/mL) from 2013 to 2016.

Genomic DNA extraction and PCR

For each patient, PBMCs samples from the earliest (V_E) and latest (V_L) visits were used for DNA extraction. Thawed PBMCs ($\cong 1 \times 10^7$ cells for VCs and $\cong 2 \times 10^7$ cells for ECs) were suspended in 1 ml of DNAZOL (Invitrogen, Wisconsin, USA) and incubated for 72 h at 4 °C. Genomic DNA was further extracted as previously described [46] and used to amplify fragments related to regions 408-1844 and 8697-9639 of the HIV-1 Genome (in relation to HXB2), encompassing *gag* and *nef*, respectively, as described elsewhere [45]. The primer sets are described in Additional file 1: Table S1. The PCR reactions were carried out by using Platinum® Taq DNA Polymerase High Fidelity (Invitrogen) according to the manufacturer's protocol. To avoid using samples near or at the limit of detection, all samples were previously tested in triplicate via nested PCR, and only samples with at least 2 positive reactions of 3 total were included in the study (data not shown). The PCR products were purified using an Illustra GFX PCR DNA purification kit (GE Healthcare, Pennsylvania, USA) and quantified with Qubit dsDNA BR Assay Kit (Invitrogen) using a Qubit® 2.0 fluorometer (Invitrogen).

Library preparation and NGS

Purified *gag* and *nef* amplicons obtained for each patient visit were multiplexed in equimolar pools and used to construct an NGS genomic library with Nextera XT DNA Library kits (Illumina, California, USA) and Nextera XT Index Kit (Illumina), according to the manufacturer's instructions. The generated libraries were normalized and clustered using HiSeq SR Rapid Cluster V2 (Illumina). NGS was performed using HiSeq Rapid SBS v2 of 200 cycles (Illumina) with an Illumina HISEQ 2500 sequencer (Illumina).

Data analysis

The raw NGS data obtained were compiled into FastQ files, and the quality of the reads was assessed by using the software FastQC [47]. The tool Trimmomatic v0.32 [48] was used to trim adapter sequences, and the first 100 bp of the reads were obtained. Furthermore, Sickle [49] was employed to select only sequences with size > 150 bp and Q > 30. Reads were mapped separately to *gag* and *nef* sequences from HXB2 (GenBank accession K03455) with Geneious 9.0.5 [50] by using medium sensitivity and 5 iterations with the Geneious mapping algorithm. Consensus sequences with a 90% base threshold and a minimum coverage of 20X for each mapping were obtained through Geneious. Reads were then remapped using obtained consensus sequences as a reference to filter differences between HXB2 and individual HIV-1 quasispecies. Samtools [51] was utilized to obtain mapping coverage statistics. Genetic divergence calculations between the consensus for V_E and V_L and Neighbor-Joining (NJ) trees with 1000 bootstrap replicates were generated with Mega v.6 and by using the Tamura Nei substitution model, as recommended by jModel test [52]. For each mapping, variant call analysis was performed in Geneious software to assess single-nucleotide polymorphisms (SNPs); variation at Q > 30, coverage > 100X and frequency > 0.5% were used as NGS sequencing quality parameters. Relevant CTL epitopes, restricted by the HLA-B alleles carried by the individuals included in the study group, were selected from the epitope database of Los Alamos HIV Immunology Database [53] available September 2017 (Additional file 1: Table S2 and S3) and used to identify variations within or adjacent (3 amino acids flanking the sequence) to epitopes that may be related to the immune response.

Statistical analysis

GraphPad Prism 6 was used to plot graphs and to estimate the median values of divergence per year, number of variants, ratio of variable positions/total of positions and synonymous/non-synonymous mutation ratio. The Mann–Whitney U test was performed using R v3.4 to compare *gag* and *nef* genes from the HIV-1 EC and VC groups. p values < 0.05 were considered statistically significant.

Results

Clinical and demographic characteristics of the cohort

Table 1 describes the clinical and epidemiologic characteristics of a group of HIV-infected individuals from the INI cohort classified as LTNPs and HICs. The median age of the individuals was 48 years (IQR 45–51); most of them (67%) were women, and the heterosexual category of exposure was prevalent (58%). The median time of HIV-1 suppression was 15 years (IQR 14–18). The medians of $CD4^+$ and $CD8^+$ T cells counts were compatible with the values currently described for HIV-1-uninfected individuals [54]. Among the 11 individuals analyzed, all were infected with HIV-1 variants genotyped as subtype B, except for VC14, who was infected with subtype F1. Six of 9 patients carried HLA-B allele B*57 or B*52, associated with slow clinical progression [55–57]. Two individuals were heterozygotes for CCR5Δ32 deletion, also described as a protective factor [56]. EC17, VC14, and EC42 did not carry an HLA-B allele or exhibit a CCR5 genetic profile associated with the control of viral replication and/or non-progression to AIDS.

Table 1 Clinical and epidemiological characteristics of individuals in the study

Patient	Gender	Age (years)	Year of HIV diagnosis	Years of HIV suppression	Exposure category	Median $CD4^+$ T cells (cells/mm^3) (IQR)	Median $CD8^+$ T cells (cells/mm^3) (IQR)	Viral load frequency (%)		
								<80[a]	81–400[a]	401–5000[a]
EC02	Female	52	1997	15	HET	1229 (1088–1443)	1539 (1406–1741)	100		
EC17	Female	65	2000	15	NI	1771 (1505–2134)	836 (693–994)	90	10	
EC52	Female	43	1997	18	HET	1263 (1056–1420)	485 (423–540)	100		
EC11	Female	48	1995	20	HET	1078 (987–1218)	965 (806–1168)	92		8
EC18	Female	82	2001	9	HET	809 (675–940)	666 (594–761)	87	13	
EC42	Female	61	1993	22	HET	974.5 (871–1133)	734 (515–933)	74	26	
VC05	Male	51	1991	24	NI	1278 (1114–1461)	857 (645–1009)	16	47	17
VC06	Male	37	2000	11	MSM	1093 (929–1222)	1112 (870–1224)	42	50	8
VC14	Female	45	1999	16	HET	701.5 (652–767)	657 (585–749)	55	36	9
VC15	Female	41	2001	14	HET	703 (677–826)	894 (755–1002)			100
VC16	Male	48	1998	17	MSM	563 (528–637)	837 (719–958)	21	37	32

Patient	Genotype HLA-B	Genotype CCR5	Early visit date	Later visit date	Difference $V_E \times V_L$ (months)
EC02	B*48, *B*52*	WT/WT	NOV/08	AUG/12	45
EC17	B*07, B*40	WT/WT	SEP/09	OCT/13	49
EC52	B*45, *B*57*	WT/WT	FEB/09	AUG/13	54
EC11	B*49, B*81	*WT/Δ32*	DEC/09	APR/12	28
EC18	B*07, *B*52*	WT/WT	OCT/09	SEP/10	11
EC42	B*15, B*51	WT/WT	DEC/09	NOV/14	59
VC05	B*15, *B*52*	WT/WT	FEB/09	JUN/13	52
VC06	B*15, B*48	*WT/Δ32*	JAN/09	MAY/11	28
VC14	B*42, B*44	WT/WT	APR/09	NOV/14	67
VC15	B*56, *B*57*	WT/WT	AUG/09	FEB/13	42
VC16	B*14, *B*57*	WT/WT	SEP/09	SEP/14	60

[a] Copies/ml

HET heterosexual, *NI* non-Informed, *MSM* men who have sex with men, *IQR* interquartile range, *WT* wild-type, *HLA-B* protective alleles are in italic

NGS data yield and gene mapping

From the NGS, a general median number of 4,667,122 reads (IQR 5,491,122–3,688,230) of 35–200 bp were obtained per visit from each individual. Approximately 70% of reads (IQR 68–76%) were retained after selection by size and quality controls. Additional file 1: Table S4 shows the mapping coverage for both genes of each patient per visit. For *gag*, the median coverage was 212,674 reads/bp (IQR 173,132–261,207) per individual/per visit. The data generated allowed reconstruction of the consensus sequence for at least the first 1050 bp of *gag*. However, *gag* mapping was not successful for EC17 and EC42 samples at the V_E visit. With regard to the *nef* gene, the median coverage was 286,463 reads/bp (IQR 179,882–426,834) per individual/per visit. The generated data allowed reconstruction of the consensus sequence of 821 bp, covering full-length *nef*. Correct *nef* mapping was not successful for VC06 V_E and EC17 V_L visit samples.

Genetic diversity of *gag* and *nef* regions among HICs

To estimate evolution of the studied viral genes, we calculated the genetic divergence between the V_E and V_L

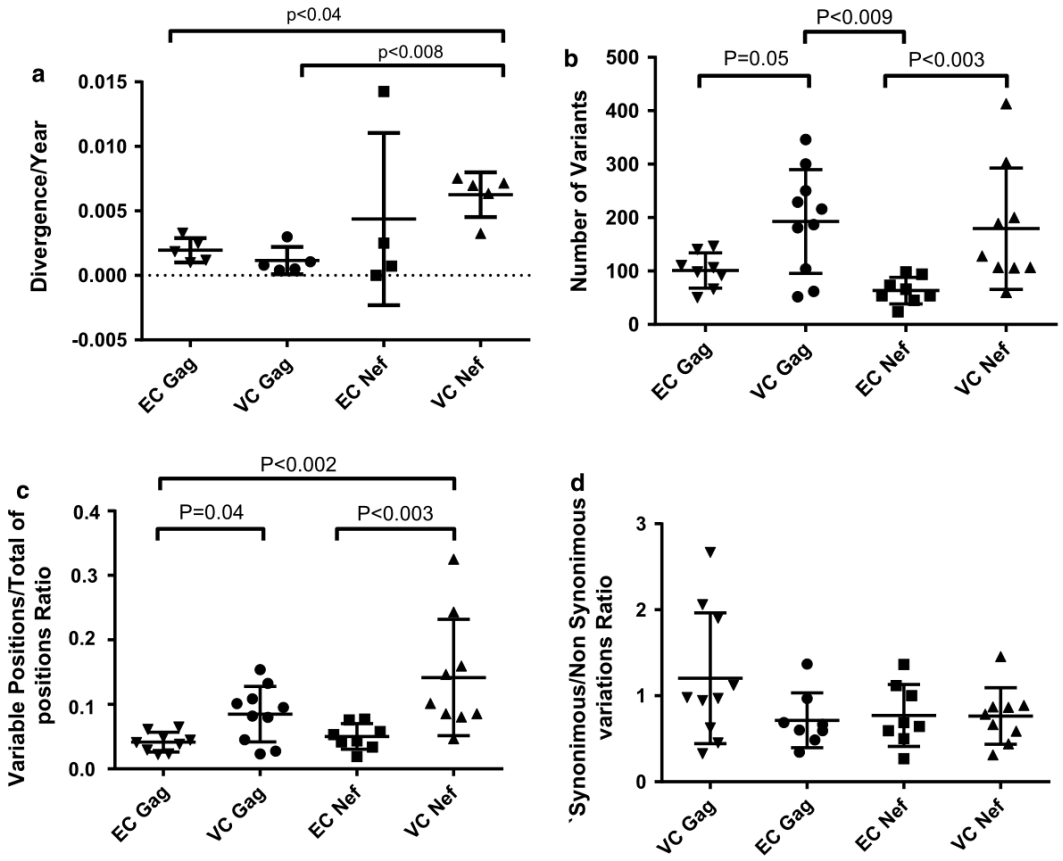

Fig. 1 Divergence/year (**a**), number of variants (**b**), variable positions/total positions ratio (**c**) and synonymous/non-synonymous variation ratio (**d**) for *gag* and *nef* of HIV-1 infected VC and EC groups. Divergence was calculated as the genetic distance between V_E and V_L of each patient and normalized by year. Values obtained by NGS data for each visit and each patient for parameters (**b**–**d**) were plotted as individual values. Median values are indicated in the figure. The Mann–Whitney U test was used to compare groups. A p value < 0.05 was considered significant

consensus sequences obtained from HICs, normalizing the values by year according to the follow-up time, and compared them between ECs and VCs (Fig. 1a). For *nef* sample VC06 V_E and *gag* samples EC17V_E/EC42 V_E, which were not successfully mapped by NGS, bulk sequences available from previous studies using conventional Sanger sequencing [58] were employed. Among the HICs, the median of viral divergence per year was significantly higher for *nef* compared to *gag* (0.6 vs 0.1%; $p < 0.03$). Similar divergence rates were observed comparing EC and VC groups for both genes. Compared with *gag* from the VC ($p < 0.008$) and EC ($p < 0.04$) groups, *nef* from the VC group showed significantly higher divergence rates.

We used variant call analysis to quantify and qualify SNPs and variations identified in the NGS data for each individual and visit. For both genes, VCs showed a higher number of variations (Fig. 1b) than did ECs ($p = 0.05$ for *gag* and $p < 0.003$ for *nef*). Comparison of the variable position ratio per total analyzed positions showed the same pattern (Fig. 1c; *gag* VCs vs ECs $p < 0.04$; *nef* VCs vs ECs $p < 0.03$). Otherwise, *gag* in VCs had a higher number of variants than did *nef* in ECs ($p < 0.009$), whereas *nef* from VCs showed higher ratios of variable positions than did *gag* from ECs ($p < 0.002$). Synonymous/non-synonymous mutation ratios were not significantly different between the HIC groups or the studied genes, even though the median ratio for VC *gag* was greater than one and the ratios for *gag* in ECs and *nef* in both groups were less than one (Fig. 1d).

Variability in CTL restricted epitopes in HICs

To assess the occurrence of potential Gag and Nef protein immune-escape mutations in the HICs included in this study, we identified for each individual the epitopes

Table 2 Gag mutations with significant frequency changes across V_E and V_L and their associated epitopes

Patient	Mutation	Frequency (%)		Epitope			
		V_E	V_L	HLA	Position	Sequence	Location
EC02	A83S	0.0	22.9	B52	Gag (74–82)	ELRSLYNTV	A
	S278C	0.0	22.8	B52	Gag (275–282)	RMYSPTSI	I
EC11	K26R	0.0	94.1	B81	Gag (19–27)	IRLRPGGKK	I
EC42	E40Q	E[a]	17.7	B51	Gag (36–44)	WASRELERF	I
	N126S	S[a]	79.9	B15	Gag (127–135)	QVSQNYPIV	A
	N126R	S[a]	21.0	B15	Gag (127–135)	QVSQNYPIV	A
EC18	K28Q	33.9	0.0	B07	Gag (22–30)	RPGGKKHYM	I
	K28R	65.9	99.6	B07	Gag (22–30)	RPGGKKHYM	I
	I34L	21.0	99.8	B52	Gag (34–44)	LVWASRELERF	I
	R39K	0.0	99.5	B52	Gag (34–44)	LVWASRELERF	I
	R43Q	17.2	0.0	B52	Gag (34–44)	LVWASRELERF	I
	R76K	20.2	0.0	B07	Gag (71–79)	GSEELRSLY	I
	T280A	79.7	0.0	B07/B52	Gag (274–282)/Gag (275–282)	VRMYSPVSI/RMYSPTSI	I
	T280V	19.9	0.0	B07/B52	Gag (274–282)/Gag (275–282)	VRMYSPVSI/RMYSPTSI	I
	T280S	0.0	99.7	B07/B52	Gag (274–282)/Gag (275–282)	VRMYSPVSI/RMYSPTSI	I
VC10	N126S	0.7	94.2	B15	Gag (127–135)	QVSQNYPIV	A
	N126S	0.0	5.6	B15	Gag (127–135)	QVSQNYPIV	A
	A146P	39.9	37.6	B15	Gag (144–152)/Gag (147–155)	HQAISPRTL/ISPRTLNAW	I
	A146S	59.3	62.4	B15	Gag (144–152)/Gag (147–155)	HQAISPRTL/ISPRTLNAW	I
	S173T	43.1	67.5	B15	Gag (168–175)	VIPMFSAL	I
	I223V	41.6	32.4	B15	Gag (226–236)	GQMREPRGSDI	A
	T280S	45.3	66.7	B15/B52	Gag (274–282)/Gag (275–282)	VRMYSPTSI/RMYSPTSI	I
	T280I	52.7	17.4	B15/B52	Gag (274–282)/Gag (275–282)	VRMYSPTSI/RMYSPTSI	I
VC14	V82I	99.7	77.9	B44	Gag (78–86)	LYNTVATLY	I
	C87Y	97.4	99.7	B44	Gag (78–86)	LYNTVATLY	A
	I147L	99.7	81.1	B42	Gag (144–152)	HQAISPRTL	I
	S310T	99.7	81.1	B44	Gag (306–316)	AEQASQDVKNW	I
VC15	V82I	43.4	84.9	B57	Gag (76–86)	RSLYNTVATLY	I
	D121A	30.6	81.8	B57	Gag (114–122)	KTQQAAADK	I
	T122A	49.2	82.1	B57	Gag (114–122)	KTQQAAADK	I
	H124N	59.7	0.0	B57	Gag (114–122)	KTQQAAADK	A
	N271T	0.0	24.7	B57	Gag (274–282)	VRMYSPVSI	A
	T280V	1.8	98.8	B57	Gag (274–282)	VRMYSPVSI	I

Table 2 (continued)

Patient	Mutation	Frequency (%)		Epitope			
		V_E	V_L	HLA	Position	Sequence	Location
VC16	I34L	32.2	0.0	B57	Gag (34–44)	LVWASRELERF	I
	V35I	32.6	0.0	B57	Gag (34–44)	LVWASRELERF	I
	V46I	29.3	0.0	B57	Gag (34–44)	LVWASRELERF	A
	V82L	25.9	0.0	B57	Gag (76–86)	RSLYNTVATLY	I
	A118P	24.9	0.0	B57	Gag (114–122)	KTQQAAADK	I
	A119T	24.9	0.0	B57	Gag (114–122)	KTQQAAADK	I
	T122A	4.8	71.6	B57	Gag (114–122)	KTQQAAADK	I
	G123K	24.7	0.0	B57	Gag (114–122)	KTQQAAADK	A
	H124N	99.1	64.4	B57/B14	Gag (114–122)/Gag (127–135)	KTQQAAADK/QVSQNYPIV	A
	H124S	0.7	35.3	B57/B14	Gag (114–122)/Gag (127–135)	KTQQAAADK/QVSQNYPIV	A
	S125R	30.9	0.0	B57/B14	Gag (114–122)/Gag (127–135)	KTQQAAADK/QVSQNYPIV	A
	Q127H	76.7	98.3	B14	Gag (127–135)	QVSQNYPIV	I
	I138L	75.5	94.1	B14	Gag (127–135)	QVSQNYPIV	A
	A146P	74.6	99.4	B57	Gag (145–155)	QAISPRTLNAW	I
	A163G	78.3	75.4	B14/B57	Gag (160–168)/Gag (162–172)	EEKAFSPEV/KAFSPEVIPMF	I
	S165N	77.6	75.0	B14/B57	Gag (160–168)/Gag (162–172)	EEKAFSPEV/KAFSPEVIPMF	I
	V168T	0.0	22.8	B14/B57	Gag (160–168)/Gag (162–172)	EEKAFSPEV/KAFSPEVIPMF	I
	S173T	37.7	74.1	B57	Gag (162–172)	KAFSPEVIPMF	A
	V191I	33.3	99.4	B14	Gag (183–191)	DLNMMLNIV	I
	T242N	78.4	99.5	B57	Gag (240–249)	TSTLQEQIGW	I
	D295N	0.0	9.9	B14	Gag (298–306)	DRFFKTLRA	A
	K335R	49.2	58.9	B14	Gag (329–337)	DCKTILKAL	I
	A340G	70.9	96.6	B14	Gag (329–337)	DCKTILKAL	A

[a] Consensus amino acid from the available bulk sequence shown instead of frequency; Location A—adjacent to epitope; Location I—within epitope

described in the literature as restricted by their HLA-B alleles. We further analyzed all non-synonymous SNPs associated with those epitope regions for each patient by comparing their frequencies between V_E and V_L. Tables 2 and 3 show HICs carrying epitope mutations with a frequency change > 10% between visits, as distributed according to the HLA-B allele. Full lists of Gag and Nef epitope mutations for all the individuals included in this study are available as Additional file 1: Tables S5 and S6.

Among HICs, EC individuals presented two main patterns of epitope mutation changes. For those individuals with none (EC02, EC52) or very few (EC17) viral blips during clinical follow-up, the majority of the epitope mutations detected were rare (< 2%) in both Gag and Nef, except for Gag epitope positions A83S (adjacent to EV9) and S278C (RI9), which each corresponded roughly to 23% in EC02 V_L.

In addition, we observed mutations with major frequency changes between visits for all ECs who had more frequent viral blips (< 30%) during clinical follow-up (Tables 2 and 3). For EC11, we detected main changes in the frequency of Nef epitope mutations V133P (from 70.7 to 99.4%) and F191 V (from 85.5 to 60.2%) and Gag epitope mutation K26R (from 0 to 94.1%) between V_E and V_L. For EC18, Nef epitope mutation K92R appeared only at V_L (68%); for Gag, epitope mutations K28R, I34L, R39 K and T280S became the majority at V_L, whereas R43Q and R76K mutations decreased from V_E (approximately 20%) to undetectable levels at V_L. EC13 showed the highest number of Nef changes in mutation frequency, such as SNPs V10 K, G12R, M79I, and G140R, which became predominant at V_L; in contrast, reversion to subtype B consensus Y and V residues were observed for Y135F and V148I. For Gag, NGS data for EC13 at V_L revealed E40Q and N126R mutations in approximately 20%, but with no significant changes in comparison to the corresponding bulk sequence available for this individual.

As expected, VCs had more mutations with frequencies above 1% than did ECs. In addition to the major frequency changes, some patients also showed a high number of mutations with equivalent frequency throughout the visits. VC06 presented dominant changes in Nef mutations V85I, L87I, R105K, and I114V, despite no significant change in Gag. VC10 also presented major changes in Nef mutations T15A and E182L; for Gag, main alterations were observed in N126S and in

Table 3 Nef mutations with significant frequency changes across V_E and V_L and their associated epitopes

Patient	Mutation	Frequency (%)		Epitope			
		V_E	V_L	HLA	Position	Sequence	Location
EC11	V133P	70.7	99.4	B49	Nef (136–145)	PLTFGWCYKL	A
	F191 V	85.5	60.2	B81	Nef (183–192)	WRFDSRLAFH	I
EC42	V10R	99.0	8.4	B15	Nef (13–20)	WPAIRERM	A
	V10 K	0.0	91.0	B15	Nef (13–20)	WPAIRERM	A
	G12R	0.0	90.4	B15	Nef (13–20)	WPAIRERM	A
	M79I	0.0	89.6	B51/B15	Nef (72–81)/Nef (75–82)	PQVPLRPMTY/PLRPMTYK	I
	N126S	99.2	0.0	B51	Nef (120–128)	YFPDWQNYT	I
	Y135F	99.2	10.4	B15	Nef (137–145)	LTFGWCFKL	A
	G140R	0.0	88.5	B15	Nef (137–145)	LTFGWCFKL	I
	V148I	99.2	0.7	B15	Nef (137–145)	LTFGWCFKL	A
EC18	K92R	0.0	68.5	B07	Nef (83–91)/Nef (90–97)	AAVDLSHFL/FLKEKGGL	A/I
VC06	V85I	V[a]	45.2	B15	Nef (84–92)	AVDLSHFLK	I
	V85L	V[a]	28.3	B15	Nef (84–92)	AVDLSHFLK	I
	L87I	L[a]	56.9	B15	Nef (84–92)	AVDLSHFLK	I
	R105 K	R[a]	74.7	B15	Nef (106–114)	RQDILDLWI	A
	I114 V	I[a]	60.3	B15	Nef (106–114)/Nef (116–124)	RQDILDLWI/HTQGYFPDW	I/A
VC10	T15A	59.2	98.5	B15	Nef (13–20)	WPTVRERM	I
	E182Q	99.9	17.8	B15	Nef (183–191)	WRFDSRLAF	A
	E182L	0.0	81.8	B15	Nef (183–191)	WRFDSRLAF	A
	R188G	99.8	90.9	B15/B52	Nef (183–191)/Nef (188–196)	WRFDSRLAF/RLAFHHVAR	I
VC14	T71R	94.5	3.3	B42	Nef (71–79)	RPQVPLRPM	I
	I101 V	5.2	96.8	B44	Nef (92–100)	KEKGGLEGL	A
	H102Y	4.9	97.1	B44	Nef (92–100)/Nef (105–115)	KEKGGLEGL/KRQEILDLWVY	A
	H102 N	2.0	0.0	B44	Nef (92–100)/Nef (105–115)	KEKGGLEGL/KRQEILDLWVY	A
	H116 N	94.5	2.4	B44	Nef (105–115)	KRQEILDLWVY	A
	P129Q	5.7	96.5	B42	Nef (128–137)	TPGPGVRYPL	I
	V133I	5.2	96.5	B42	Nef (128–137)	TPGPGVRYPL	I
VC15	V85L	66.8	99.5	B57	Nef (82–90)	KAAFDLSFF	I
	H102Y	68.4	99.7	B57	Nef (105–115)	KRQEILDLWVY	A
VC16	Y81F	54.3	47.6	B57	Nef (82–90)	KAAFDLSFF	A
	H89Y	0.0	29.3	B57	Nef (82–90)/Nef (90–97)	KAAFDLSFF/FLKEKGGL	I/A
	H102Y	99.9	48.6	B14/B57	Nef (105–113)/Nef (105–115)	QRQDILDLW/KRQEILDLWVY	A
	H116 N	63.4	63.1	B57	Nef (105–115)/Nef (116–124)	KRQEILDLWVY/HTQGYFPDW	A/I
	V133T	99.4	13.3	B57	Nef (127–135)	YTPGPGIRY	I
	V133I	0.0	74.3	B57	Nef (127–135)	YTPGPGIRY	I

[a] Consensus amino acid from the available bulk sequence shown instead of frequency; Location A—adjacent to epitope; Location I—within epitope

co-circulation of A146P/A146S, T280S/T280I, S173T/S173, and I223V/I223 variants at both visits. VC14 showed reversion of Nef T71R and H116N and the emergence of I101V, H102Y, P129Q, and V133I at V_L; for Gag, major changes were approximately 20% of the reversions for V82I, I147L, and S310T. Furthermore, VC15 presented main changes in Gag for V82I, D121A, T122A, and T280V, with reversion observed for H124N, whereas Nef V85L and H102Y increased by approximately 30%. VC16 presented a large number of amino acid changes, mainly in Gag epitopes, with reversion of approximately 30% for I34L, V35I, V46I, V82L, A118P, A119T, G123 K, and S125R mutations and an increase from 20 to 30% for H124S, Q1227H, I138L, A146P, V168T, T242 N and A340G from V_E to V_L. Major changes from approximately 40–70% were also found for T122A, S173T, and V191I. Co-circulation of subtype B consensus amino acids with mutations was found for K335R/K335. For Nef, we observed co-circulation of wild-type and Y81F and H116N mutations, reversion of H102Y and major

frequency changes of V133I (undetectable vs 74%) from V_E to V_L.

Discussion

HICs are a rare population of HIV-1-infected individuals who represent the best existing model of spontaneous viral control [2, 59, 60]. Although the mechanisms responsible for this control are not fully understood, studies show that these individuals have a differentiated and more effective CTL response [61], which should be then reflected in greater genetic pressure on their viral quasispecies, mainly in immunodominant regions. Moreover, the presence of protective HLA alleles related to clinical non-progression and/or viremia control is also associated with stronger selective pressure for virus diversity [17, 22, 26, 27, 31, 32, 35, 36, 62–66].

Although most HICs have plasma viral loads below the limit of detection of commercial assays, basal viral replication levels can be detected by ultrasensitive methods [59, 67, 68] and should favor viral evolution to some degree due to the characteristic high-genetic variability from HIV. Several studies have reported lower levels of viral diversity in HICs compared with the levels in typical progressors [28, 29, 32, 59, 69]. Gijsberg et al. estimated divergence rates of 0.9–1.9% over ten months for *gag* in HIV-1 samples from typical progressors [70]. In our study, a median of viral divergence of 0.1% per year was observed for *gag* gene from HICs, corroborating the low level of viral evolution found in HIV-1 samples from those individuals. A higher median of divergence was observed for *nef* (0.6% per year), suggesting that *nef* has a greater potential for viral diversity than does *gag,* in agreement with previous studies showing greater conservation of *gag* [71, 72]. Previous observations from our group of lower quasispecies diversity for the *env* gene from EC samples in comparison to VCs [73] and the low, but similar, median values of divergence in *gag* and *nef* for viral samples from ECs and VCs in the present study indicate that lower levels of viral replication restrict evolution.

To our knowledge, this is the first study employing NGS to analyze HIV-1 diversity in major CTL epitopes of HICs. Indeed, most similar studies were performed with SIV-infected primates with a viremia control profile [74, 75]. The use of the NGS platform allowed the estimation of variability in terms of SNP quantity and nature. Previously, Cale et al. [76] using 454 sequencing, showed that full coverage of 50,000 reads/bp was sufficient to detect variants with frequencies of 0.006%. In our work, we used a medium coverage of > 200,000 reads/bp to assess SNPs with frequencies higher than 0.5%, which should identify the most representative escape mutation variants while preventing analysis of data related to sequencing artifacts. With this approach, we showed higher levels of variants and variable positions in the *gag* and *nef* genes of viral samples from VCs compared to ECs, correlating with the higher variability expected for the first group. Although differences between the synonymous/non-synonymous mutation ratios of both groups and genes were not statistically significant, *gag* of ECs and *nef* of ECs and VCs displayed a predominance of non-synonymous mutations, in contrast with previous studies reporting that synonymous mutations are more significant to evolution of *gag* and *nef* [32, 77].

To characterize possible CTL-escape mutants, we performed analysis of variations in the epitopes restricted by each patient's HLA-B allele. Similar molecular analysis has been able to identify that most mutations arising in the first weeks of the acute phase are the result of CTL response selective pressure [13, 19–22]. Concerning HICs, Migueles et al. [78] showed a low frequency of CTL-escape mutations for the KF11 epitope, despite its high level of CTL recognition in individuals carrying the B57 allele. Additionally, a more in-depth description of *gag* and *nef* gene evolution in $B57^+$ elite suppressors showed that despite the predominance of immune-escape mutations in *gag* and *nef* quasispecies obtained from plasma viral RNA, these mutations are rare in proviral sequences [32, 65, 77, 79].

In our study, non-synonymous mutations were found in Gag and Nef CTL epitope regions in all HIV-controllers regardless of their rarity in the proviral compartment. Due to the low HIV proviral load inherent to ECs, a higher number of PBMCs was used for DNA extraction than for VCs ($\cong 2 \times 10^7$ cells for ECs vs $\cong 1 \times 10^7$ cells for VCs) in order to assure a proviral input in the nested-PCR sufficient to assess the viral variability in each sample. Moreover, all samples were tested in triplicate and only those with at least 2 out of three positive nested-PCR amplification were used to prepare the NGS amplicons. These strategies were employed to prevent low input of viral copies on PCR that could lead to template resampling. Moreover, the high number of sequences generated from each sample and the higher sensitivity of NGS to access minority viral variants, in contrast to techniques such as single-genome amplification (SGA) or cloning [19, 20], allowed the detection of those mutations. The occurrence of unique low-frequency mutations for both early and late visit samples from the same individual showed that in contrast to the results of Bailey et al. [65], possible escape mutants from the CTL response replicating in the plasma compartment can successfully integrate into host cells. Although this low frequency might appear to be insignificant, new CTL-escape mutants do not often arise in massive frequencies but can expand

from very low to predominant conditions, as previously exemplified for SIV [80] and observed in our work for the following mutations: Gag-N126S (0.7 –> 99.8%) for VC10 and Gag-T280V (1.8 –> 98.8%) for VC15.

Comparing Gag and Nef mutations with significant frequency changes revealed that mutations within the analyzed epitopes predominantly occurred in Gag, whereas no pattern of mutation was present in Nef epitopes, within or in adjacent regions. Although both types of mutations can generate CTL escape through different mechanisms, mutations within epitope are more easily associated with the escape profile, as it directly affects epitope anchoring and TCR recognition [18, 81]. This observation may be related to the predominance of the CTL response associated with Gag during the chronic phase of infection [13, 15, 22, 34], generating higher selective pressure in this gene and resulting in greater diversity of the epitopes.

In general, the low number of patients in each group is a limitation to identifying statistically significant associations between the level of viremia control and the emergence dynamics of mutations related to CTL epitopes. The low viral load observed for ECs was also a limitation to assessing escape mutations in the plasma compartment, which reflects the variants that are effectively replicating in the host. However, by evaluating the proviral reservoir, which represents the pool of viruses that can be a source of plasma viral particles, we were able to assess a greater number of mutations with significant frequency changes either in VCs or ECs. Although some ECs, such as EC08, did not present any significant change across visits, all VCs showed mutations that characterized variant replacement with regard to both Nef and Gag. In those patients, we were able to observe co-circulation of more than one mutation in the same position, which is indicative of greater dynamic quasispecies turnover. Reversion to wild-type amino acids, as based on the reference subtype, was also observed for VCs, and reversion of escape mutations has been extensively described in the literature as a common viral mechanism of evolution related to the CTL response [81–86].

For the individuals EC08, EC18, VC15 and VC16, who carried the protective HLA* B57 allele, analyses of IW9 (Gag 147-155), KF11 (Gag 162-172) and TW10 (Gag 240-249) epitopes indicated a low level of viral evolution, even in these HIV-1 CTL epitopes related to high selective pressure [22, 35, 36, 62–66]. Although EC08 and EC18 individuals presented wild-type amino acids at all positions of the epitopes analyzed, VC15 presented I147L, A146P, and T242 N mutations, and VC16 harbored A146P, A163G, and T242 N mutations. All of the mutations identified herein have been described in several studies as commonly arising in individuals carrying HLA-B*57 and B*58 alleles, despite resulting in a loss of viral fitness [18, 36, 65, 66].

Conclusion

Although none of the observed mutations could be confirmed as a CTL-escape mutation due to the lack of $CD8^+$ T cell functional analyses, the present study shows that despite low or undetectable levels of viral replication among HICs, genetic variability occurs in viral quasispecies in the proviral compartment. Amino acid substitutions across visits and the existence of low-frequency mutants, even in ECs, indicate that potential CTL-escape mutants exist and are present in those individual reservoirs. This fact implies that situations leading to a disequilibrium of the host-virus relationship can result in the spread of CTL-escape variants with pathological consequences. More studies are necessary to address why those adapted variants do not achieve replicative success in ECs.

Additional file

Additional file 1. Table S1. Primer set used in the present study. **Table S2.** Gag Epitopes selected for study according to the HLA-B alleles carried by the HICs. **Table S3**. Nef Epitopes selected for study according to the HLA-B alleles carried by the HICs. **Table S4.** NGS mapping and coverage statistics of *gag* and *nef* distributed according to the patients. **Table S5.** Full list of Gag mutations and associated epitopes recognized by HLA alleles carried by the HICs. **Table S6.** Full list of Nef mutations and associated epitopes recognized by HLA alleles carried by the HICs

Authors' contributions

DGC conducted most of the experiments and analyzed the data. MGM conceived the study design and supervised the project. SLT conducted the HLA-B genotyping experiments. GB, FHC, MGM, and MLG supervised the experiments and provided intellectual input. BH, BG, and VGV conducted the patient recruitment and follow-up. DGC, MGM, and FHC wrote the first draft. All authors read and approved the final manuscript.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The NGS dataset supporting the conclusions of this study is available in the NCBI SRA database through accession numbers SRX4105267–SRX4105305. Consensus sequences obtained from NGS data and bulk sequences from previous studies have been deposited in GenBank under the primary accession codes MH378285–MH378326.

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.

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CASE REPORT Open Access

A case report of HIV-1 superinfection in an HIV controller leading to loss of viremia control: a retrospective of 10 years of follow-up

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Abstract

Background: HIV controllers (HICs) are a rare group of HIV-1-infected individuals able to naturally control viral replication. Several studies have identified the occurrence of HIV dual infections in seropositive individuals leading to disease progression. In HICs, however, dual infections with divergent outcomes in pathogenesis have been described.

Case presentation: Here, we present a case report of a HIC diagnosed in late 1999 who displayed stable $CD4^+$ T cell levels and low plasmatic viral load across 12 years of follow-up. In early 2013, the patient started to present an increase in viral load, reaching a peak of 10,000 copies/ml in early 2014, followed by an oscillation of viremia at moderate levels in the following years. The genetic diversity of *env* proviral quasispecies from peripheral blood mononuclear cells (PBMCs) was studied by single genome amplification (SGA) at six timepoints across 2009–2017. Phylogenetic analyses of *env* sequences from 2009 and 2010 samples showed the presence of a single subtype B variant (called B_1). Analyses of sequences from 2011 and after revealed an additional subtype B variant (called B_2) and a subsequent dominance shift in the proviral quasispecies frequencies, with the B_2 variant becoming the most frequent from 2014 onwards. Latent syphilis related to unprotected sexual intercourse was diagnosed a year before the first detection of B_2, evidencing risk behavior and supporting the superinfection hypothesis. Immunologic analyses revealed an increase in $CD8^+$ and $CD4^+$ T cell immune activation following viremia increase and minor T cell subset alterations during follow-up. HIV-specific T cell responses remained low throughout the follow-up period.

Conclusions: Altogether, these results show that loss of viremia control in the HIC was associated with superinfection. These data alert to the negative consequences of reinfection on HIV pathogenesis, even in patients with a long history of viremia control and an absence of disease progression, reinforcing the need for continued use of adequate prevention strategies.

Keywords: HIV-1, HIV-controllers, LTNP, Superinfection, Dual infection

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Background

HIV controllers (HICs) are a rare group of HIV-1-infected individuals able to naturally control viral replication. A fraction of those individuals is also classified as long term nonprogressors (LTNP), as they maintain $CD4^+$ T cell counts > 500 cells/mm^3 during more than 10 years of infection without progressing to AIDS in the absence of antiretroviral treatment [1].

An aspect of HIV infection is the possibility of infections by two or more phylogenetically distinct and unrelated variants in a single individual, characterizing a dual infection (DI). More specifically, these DIs are classified as coinfections when all variants are concomitantly acquired in a single transmission event or superinfections (SIs) when the viruses originate from multiple subsequent transmission events [2]. HIV DI has been described in several studies with significant prevalence mainly among key populations [3–7] and is related to a faster progression to AIDS [8–10]. DIs were also observed among LTNPs and/or HICs [11–15], with variable consequences on HIV pathogenesis. While some individuals retain spontaneous disease control [11–13], others present loss of viremia control and experience disease progression [11, 14, 15].

Here, we report the case of an HIV-1 positive individual with natural control of viral replication and no progression to AIDS over more than 10 years of clinical follow-up who presented a partial loss of viremia control after an SI event.

Case presentation

Subject VC06 is a 40-year-old, transgender woman from Rio de Janeiro, Brazil, who was diagnosed with HIV-1 infection at the end of 1999 and has been seen for routine clinical follow-up at the Instituto Nacional de Infectologia Evandro Chagas (INI-Fiocruz), Rio de Janeiro, Brazil since 2005. In 2009, VC06 signed an informed consent and was enrolled in the INI-Fiocruz LTNP/HIC cohort study, approved by the Brazilian National Human Research Ethics Committee (CONEP 840/2008) and by the FIOCRUZ *Research Ethics* Committee (CEP 1717.0.000.009–07). Due to study enrollment, individual VC06 was followed at least once every 6–12 months to perform specific infection-monitoring tests (such as HIV-1 RNA viral load quantification and $CD4^+$ T cell counts) and routine clinical laboratory exams and to assess data related to clinical status and exposure to sexually transmitted infections. In addition, blood was collected at each visit to isolate plasma, whole blood and peripheral blood mononuclear cell (PBMC) samples for study. Subject VC06 was initially classified as an LTNP HIV viremic controller (< 2000 cp/ml dually infected with two HIV-1 subtype B viruses (de Azevedo et al. 2017) [16]. She carries a nonprotective HLA-B genotype (HLA-B*15:01/ B*48:02) but has heterozygosis for the CCR5-Δ32 mutation, which is considered a host-protective allele for disease infection and progression.

Subject VC06 displayed low-level viremia (< 500 copies/mL) in the absence of antiretroviral therapy until early 2013, when she started to show increases in the viral load, reaching approximately 10,000 copies/ml 1 year later (Fig. 1, $V9_{2014}$). The following months were associated with a spontaneous decrease in viral load, reaching 577 copies/ml in August 2015. Combination antiretroviral therapy (cART) with a scheme containing TDF, 3TC, and EFZ was prescribed in November 2015 but interrupted 1 month later by the patient due to intense dizziness related to the treatment. Side effects ceased, but the continuity of the therapy was refused by the patient in the following years. Transient recovery of viremia control was followed by intermittent viral loads above 2000 copies/ml and a new peak of approximately 8000 copies/ml in May 2017. This new peak of viremia was followed by a spontaneous decrease in viral load, reaching 1435 copies/ml in May 2018. The most recent available data indicated a viral load of approximately 3500 copies/ml at the end of 2018 (Fig. 1). Despite increasing viremia, $CD4^+$ T cell counts during the whole period were stable at high levels, suggesting no immunological commitment or disease progression.

In addition to the intermittence of the plasmatic viral load after a controller period, individual VC06 was diagnosed with latent syphilis in May 2010 based on positive VDRL results (1/32) in the absence of clinical signs or symptoms and a previous negative VDRL test in December 2009. The syphilis diagnosis coincided with unprotected sexual intercourse reported by the patient, and treatment with weekly benzathine benzylpenicillin 1,200, 000 IU intramuscular injections were administered for 3 weeks starting in November 2010. Late latent syphilis was further diagnosed again at two additional timepoints: first in September 2015, based on VDRL titer of 1/8; second in November 2017, based on TPHA positive and a VDRL titer of 1/512. Both cases were preceded by a VDRL titer of 1/1 6 months before and were treated, as described above, in November 2015 and May 2018, respectively. No clinical signs or symptoms associated with syphilis infection were observed during follow-up. Another clinical event during the follow-up period included the diagnosis of an anal fistula at the beginning of 2014, which was surgically treated in the same year.

To assess the patterns of intrahost viral evolution and to investigate the cause associated with the loss of viremia control, PBMCs (1×10^7 cells) from selected visits (Fig. 1) were thawed and used for genomic DNA extraction, as previously described [17]. The genomic DNA obtained was used for amplification by nested PCR single genome amplification

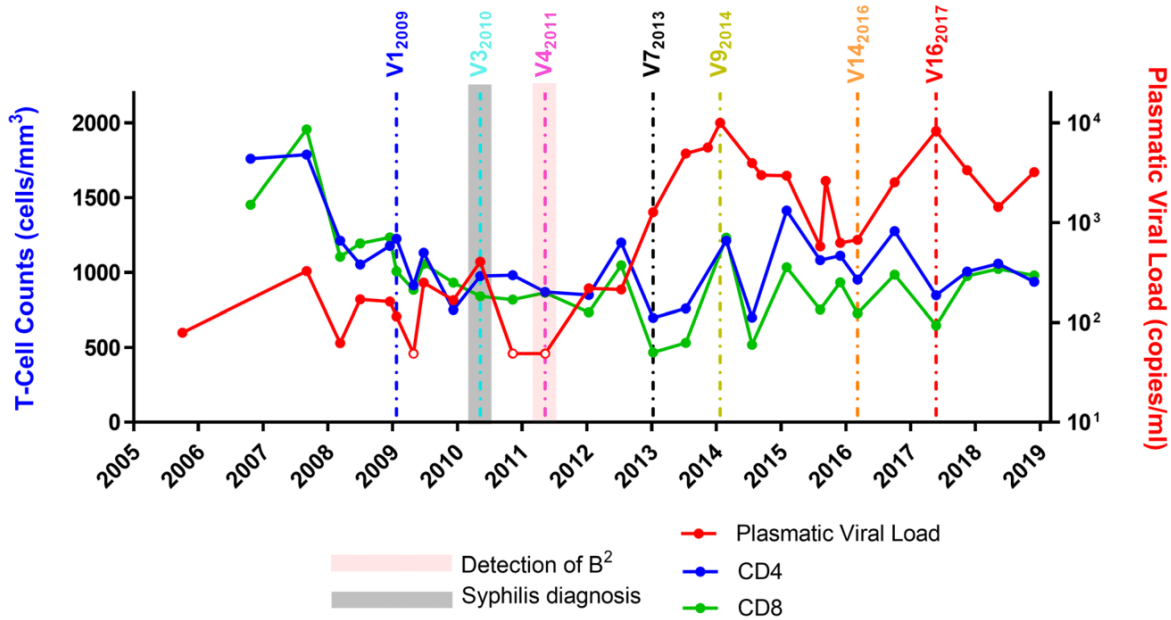

Fig. 1 T cell counts (cells/mm^3) and plasmatic viral load (copies/ml) of VC06. The first syphilis diagnosis point and the first point of detection of B_2 are indicated by shaded areas, while the visits used for experiments are indicated by dashed lines in the graph. Time points with plasmatic viral load < 50 copies/ml are indicated by open circles

(SGA) and sequencing of a ≈ 600 bp C2-C4 fragment of HIV-1 *env*, as previously described [18]. A neighbor-joining phylogenetic tree containing all sequences obtained from samples collected over time is shown in Fig. 2. *Env* sequences from 2009 ($V1_{2009}$; $n = 29$) and 2010 ($V3_{2010}$; $n = 43$) samples showed the presence of a single subtype B variant (called B_1). Analysis of *env* sequences from 2011 ($V4_{2011}$; $n = 32$), 1 year after the diagnosis of syphilis infection, showed the presence of a second subtype B variant (called B_2) in addition to the previous B_1 variant. These variants branched separately and displayed a mean *env* genetic distance of 16.8%. Tropism analyses, realized through Geno2pheno tool using a false-positive rate (FPR) of 10% [19], of *env* sequences, obtained at all time points, showed that both B_1 and B_2 variants correspond to R5-tropic viruses that present different predominant motifs at the top of the V3 loop (QPGR/QPGG for B_1 and GPGR for B_2). *Env* analyses of samples from subsequent time points revealed a shift in the proviral quasispecies proportion, with an increase of B_2 variant frequency from 16% in 2011 ($V4_{2011}$; $n = 32$) to 93% in 2014 ($V9_{2014}$; $n = 27$). The majority of the B_2 (93%) viral quasispecies was maintained even after the reduction of plasmatic viral load in 2016 ($V14_{2016}$; $n = 14$) as well as after a new peak of viremia (75%) in 2017 ($V16_{2017}$; $n = 12$) (Fig. 2). For B2 quasispecies from all timepoints ($n = 52$), 80% of the sequences obtained were classified as R5 with FPR values greater than 45%, while the remaining presented FPR values between 11.5% and 18,5%.

Plasma sequences were obtained from the $V9_{2014}$ and $V16_{2017}$ samples, as previously described [18], supporting that B_2 was the replicating variant accounting for the increase in viremia observed at both time points (Fig. 2). Moreover, the distribution of B_2 sequences on the *env* phylogenetic tree indicates a pattern of increasing divergence along the follow-up period, with the identification of two distinct clusters harboring most sequences for the $V9_{2014}$ and $V16_{2017}$ samples. In additional analyses, all B_2 sequences were used for estimation and reconstruction of the most recent common ancestor (MRCA), using the Phylip package [20]. The mean genetic distance between the MRCA and the B_2 sequences for each visit was calculated to determine B_2 viral divergence during follow-up. We observed increasing values of divergence during follow-up (0.9% for $V4_{2011}$, 1.7% for $V9_{2014}$; 3.5% for $V14_{2016}$; 4.4% for $V16_{2017}$), indicating a temporal evolution pattern. These results, in addition to the observation that sequences from the $V14_{2016}$ samples were distributed between the $V9_{2014}$ and $V16_{2017}$ clusters, with no clear predominance of any population, also indicate that different B2-related viral quasispecies accounted for the viral replication in each viremia peak.

To better characterize the SI and assess the divergence between B_1 and B_2 at a more conserved region of the

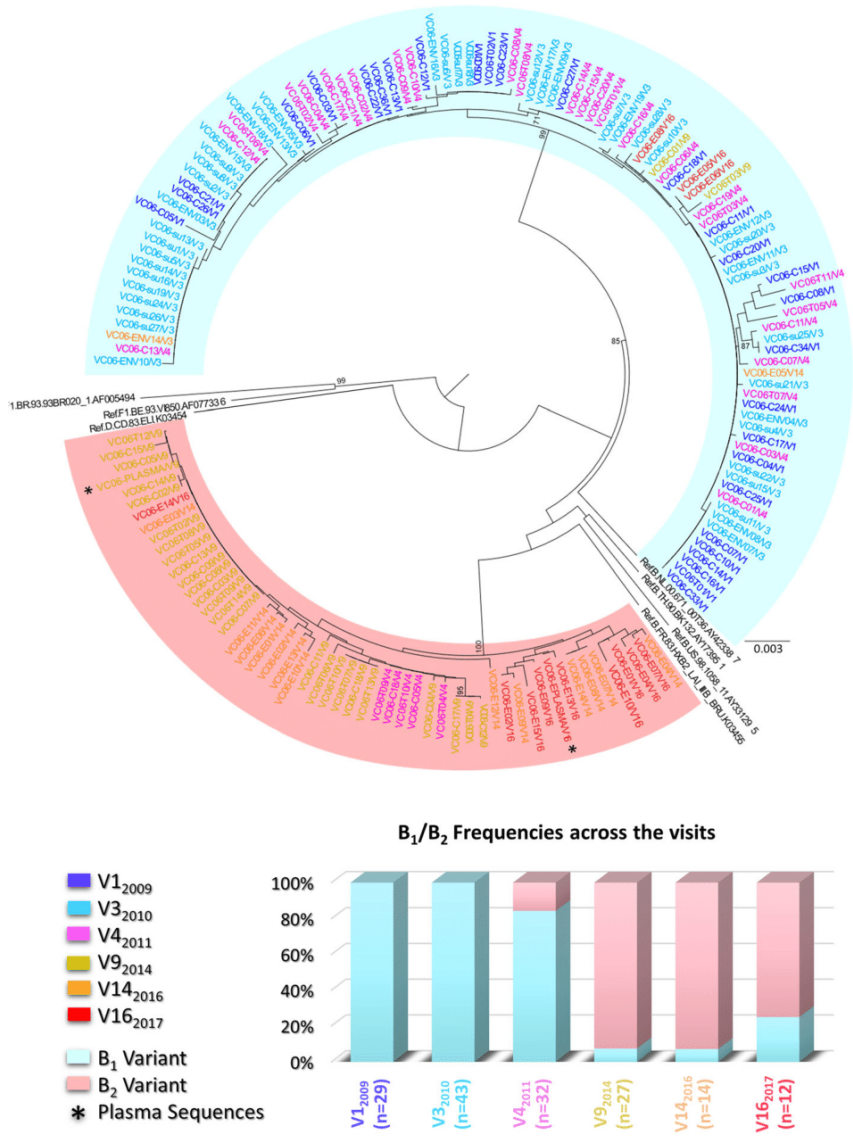

Fig. 2 Longitudinal analysis of proviral and plasma HIV-1 *env* sequences obtained from VC06 during 2009–2017. Sequence names are colored according to their respective visit. Clusters containing B_1 and B_2 sequences are shaded in blue and red, respectively. For each visit, the total number of sequences and proportion of B_1 and B_2 variants are indicated on the stacked bar graph and colored according to the legend. The tree was constructed using the Tamura Nei model and 1000 bootstrap replicates in MEGA 6 software. Bootstrap values lower than 75% are not shown. Reference sequences for HIV-1 subtypes D, F1, and B were used as an outgroup and are shown in black

viral genome, we conducted SGA of the *int* region from the $V4_{2011}$ sample, in which we first detected the B_2 variant, and from the $V9_{2014}$ sample, in which B_2 became the dominant variant. A neighbor-joining phylogenetic tree containing the *int* sequences is shown in Fig. 3. In the $V4_{2011}$ sample (obtained at the time of SI detection), a single *int* variant related to $B_1$was found despite the detection of two variants in the *env* analysis from the same time point. The absence of a second *int* variant is probably related to the low number of sequences obtained at this time point (12 sequences for *int* vs 32 sequences for *env*). In the $V9_{2014}$ sample, however, we identified two *int* variants with a mean genetic distance of 4.2%. Although these data indicate the presence of B_1 and B_2, the frequencies of both *int* variants differ from those observed for *env* (40% vs 7% for B_1; 60% vs 93% for B_2). Even though different fragments have distinct PCR efficiencies, which could introduce a bias factor, the

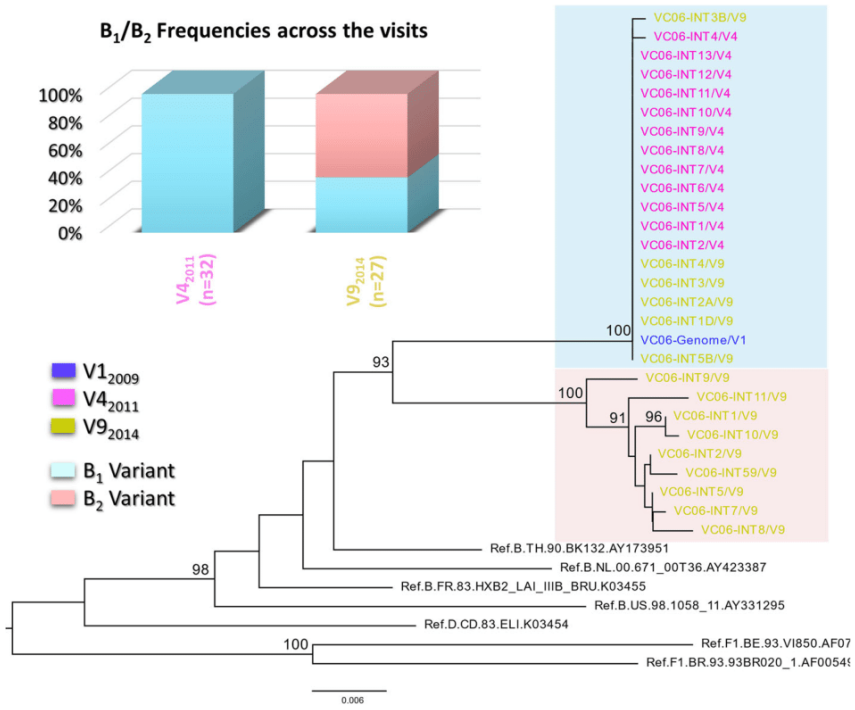

Fig. 3 Longitudinal analysis of proviral HIV-1 integrase sequences obtained from VC06 during 2011 and 2014. Sequence names are colored according to their respective visit, clusters containing B_1 and B_2 sequences are shaded in blue and red, respectively, and the total number of sequences and proportion of B_1 and B_2 variants are indicated on the stacked bar graph, as previously described. *Int* sequence obtained from B_1 full length genome is included to confirm B_1 cluster identity. The tree was constructed using the Tamura Nei model and 1000 bootstrap replicates in MEGA 6 software. Bootstrap values lower than 75% are not shown. Reference sequences for HIV-1 subtypes D, F1, and B were used as an outgroup and are shown in black

great divergence of representation could be indicative of recombination between *int* and *env* genes.

Finally, to evaluate viral integrity, we obtained the full-length HIV-1 genome from the $V1_{2009}$ sample, as previously described [21]. The overlapping fragment sequences obtained allowed the identification of the B_1 variant full-length genome and the absence of deletions or frame-shift alterations related to genetic defects, indicating that B_1 is a replication-competent virus. The full-length B_1 genome also confirmed that the *int* variants obtained via SGA of the $V4_{2011}$ sample are related to this variant (Fig. 3). Isolation of the full-length genome for the B_2 variant was not possible due to the equivalent frequencies of B_1 and B_2 in some genes, as demonstrated by *int* SGA, which could lead to the generation of PCR artifacts.

To understand the potential impact of the SI on the host immune system, we analyzed alterations during the follow-up period in the frequencies of the T cell subsets and immune response to HIV peptides in PBMC samples collected at $V3_{2010}$ (prior to SI), $V4_{2011}$ (at the moment of B2 *env* variant identification after SI onset), $V7_{2013}$ (prior to the first peak of viremia), $V9_{2014}$ (at the first peak of viremia and detection of B_1 and B_2 *env* and *int* variants), $V14_{2016}$ (after viremia control) and $V16_{2017}$ (at the second peak of viremia). Briefly, T cell activation was evaluated by multiparametric flow cytometry by staining the cells with anti-CD3, anti-CD4 or anti-CD8, anti-CD38 and anti-HLA-DR antibodies to determine the frequencies of $CD38^+HLA\text{-}DR^+$ cells in both $CD4^+$ and $CD8^+$ subsets, as previously described [22]. In addition, cells were also labeled with anti-CD45RA, anti-CD27 and anti-CD95 antibodies to evaluate the frequencies of naïve (TN; $CD45RA^+CD27^+CD95^-$), system memory (TSCM; $CD45RA^+CD27^+CD95^+$), central memory (TCM; $CD45RA^-CD27^+CD95^+$), effector memory (TEM; $CD45RA^-CD27^-CD95^+$) and effector (TEFF; $CD45RA^+CD27^-CD95^+$) T cell subsets.

An increase in the percentage of $CD8^+CD38^+HLA\text{-}DR^+$ T cells was observed in samples from $V3_{2010}$ (6.81%) to $V9_{2014}$ (14%), followed by a decrease at $V14_{2016}$ (6.76%) and a new peak at $V16_{2017}$ (14.40%) (Fig. 4a). This higher values in the $V4_{2011}$ sample than in the $V3_{2010}$ sample, despite the lower plasmatic viral load, could be suggestive of an association between the SI event and an increase in immune activation. After the

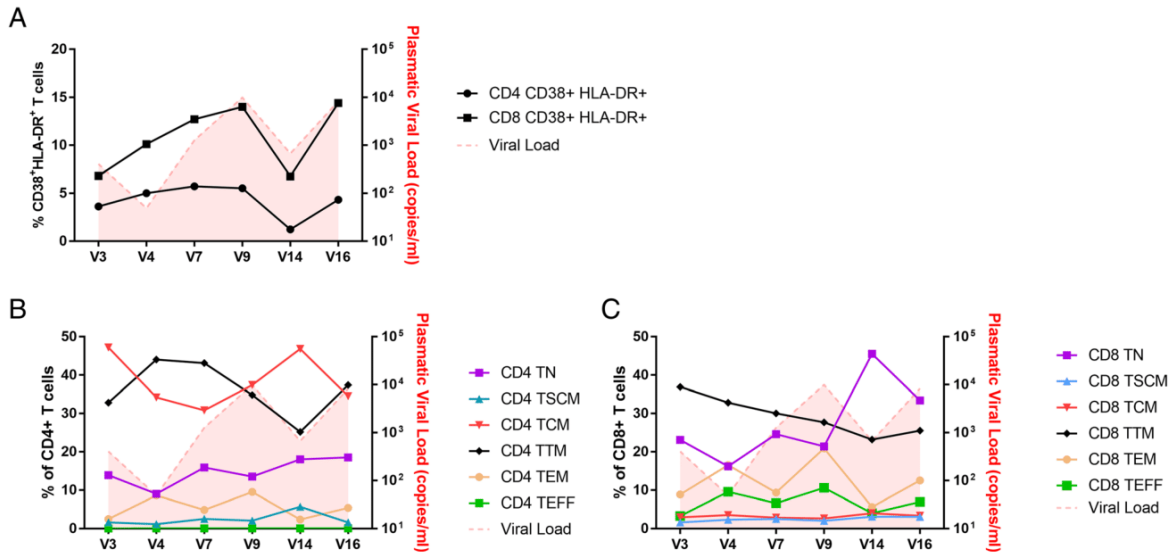

Fig. 4 T cell profile of VC06 during follow-up between 2010 and 2017. Frequencies of activated T cells ($CD38^+HLA\text{-}DR^+$) are shown in graph (**a**); frequencies of naïve (TN; $CD45RA^+CD27^+CD95^-$), stem-memory (TSCM; $CD45RA^+CD27^+CD95^+$), central-memory (TCM; $CD45RA^-CD27^+CD95^+$), effector-memory (TEM; $CD45RA^-CD27^-CD95^+$) and effector (TEFF; $CD45RA^+CD27^-CD95^+$) T cells are shown in graphs (**b**) and (**c**). For all graphs, red shaded areas indicate the plasmatic viral load for each visit analyzed

emergence of B_2, the percentage of $CD8^+CD38^+HLA\text{-}DR^+$ T cells followed plasmatic viral load levels, highlighting the relationship between the antigen viral load and $CD8^+$ T cell activation. Although at more discrete levels, the same trend was also observed for $CD4^+$ T cells (Fig. 4a). In relation to the $CD4^+$ T cell subsets (Fig. 4b), we observed a decrease in the frequency of TCM cells between the $V4_{2011}$ and $V14_{2016}$ samples, with the recovery of those cells in the $V16_{2017}$ sample and an inverse pattern observed for TTM cells. The frequency of $CD4^+$ TEM cells reached the highest levels at visits near the detection of the superinfection ($V4_{2011}$) and at both peaks of viremia ($V9_{2014}$ and $V16_{2017}$). For $CD8^+$ subsets (Fig. 4c), the frequency of TEM and TEFF cells followed the viral load dynamics, which was in contrast with the expected TN cells expansion after the first viral load peak. Despite these variations, no statistical correlations between the frequencies of the different T cell subsets and plasmatic viral load were found during the follow-up.

In addition, we used Gag and Nef HIV-1 peptides matching CTL epitopes, based on the VC06 HLA-B genotype, to evaluate the HIV-specific T cell response by IFN-γ ELISpot assay and intracellular cytokine and CD107 staining. In general, very low or undetectable HIV-1-specific responses were observed, with the exception of the $V9_{2014}$ sample, when approximately 1% of $CD107^+CD8^+$ T cells showed detectable Gag- or Nef-specific responses (data not shown). No pattern of increase/decrease in the cytokine response was observed in consequence of the antigenic stimulation related to the viral load peak.

Discussion and conclusions

Here, we report the case of a transgender HIC who experienced a partial loss of viremia control after HIV-1 intrasubtype SI with another subtype B variant. Through molecular analyses of the HIV-1 *env* gene during clinical follow-up, we were able to trace the SI to some time between mid-2010 and mid-2011. At the time, VC06 already had 10 years of diagnosed HIV-1 infection with consistently low viremia and high counts of $CD4^+$ T cells in the absence of antiretroviral therapy. This finding indicates that despite natural protection against disease progression, HICs can still be at risk for subsequent infections with new variants, reinforcing the need for continued use of prevention strategies.

The mechanisms underlying viremia and disease progression control phenotypes are not yet fully understood. Studies have shown that they could be associated with host genetic background [23–25], virological characteristics [26, 27], and low levels of immune activation and preservation of memory T cells, among other immunological aspects [28]. CCR5 genetic analysis of the VC06 patient identified heterozygosity for the Δ32 allele (CCR5wild type/CCR5Δ32), a mutated allele previously associated with protection against infection when presented in homozygosis and that has a partial protective role or delayed AIDS progression in heterozigosis [29–33]. However, the association of this genetic characteristic

with the of HIV pathogenesis and superinfection could not be established in the present case study.

Although the existence of X4-tropic variants after the superinfection could explain the loss of viremia control in this context, our tropism analyses identified only R5-tropic quasispecies. Despite phenotypic analyses for tropism characterization were not realized, the genotypic analysis in geno2pheno tool had been previously showed to be highly concordant when compared to in vitro assays [34–37]. Moreover, most of the sequences presented high FPR values and lower FPR values were found only in a few sequences representing minoritary variants that should not have a great impact on disease progression or viremia. In the whole, although we can not rule out completely, it is very unlikely that loss of control in VC06 might be associated with the onset of an X4-tropic virus after superinfection. In addition to the VC06 genetic background described in the present study, the diversity of protective elements described in the literature suggest that the control phenotype is not determined by a single factor but is rather probably a result of a set of host and virus characteristics acting synergistically.

Only a few studies are available in the literature with cases of SI in HICs or LTNPs. For some of those studies, SI was associated with disease progression in HICs soon after reinfection [14, 15, 38, 39], as previously observed for noncontrollers [8–10]. However, other studies showed that HICs are able to maintain high $CD4^+$ T cell counts and plasmatic viral load at low or undetectable levels after SI [11–13, 18, 40, 41]. This sustained progression control, however, seems to have some complexity, as some of those individuals maintain stable $CD4^+$ T cell counts despite experiencing transient viremia incompatible with a previous clinical history [11–13, 18, 40, 41]. The data presented in this study, along with those previously published, reaffirm the complexity of the control phenotype and show that a previous control profile of a single variant does not guarantee immediate and/or efficient control of subsequent infections.

Despite reinfection at the end of 2010, VC06 only started to present increasing viremia at the beginning of 2013, with a peak of 10,000 copies/ml 1 year later. In some cases, the HIC phenotype is the result of infection with defective or attenuated viral strains [42–44]. Previously, Braibant et al. [14] demonstrated the case of an elite controller previously infected with a defective virus who presented disease progression after SI with a competent HIV-1 variant. In our study, the lack of genetic defects in the B_1 complete genome sequence indicates that B_1 is a replication-competent virus, and the partial viremia control and the absence of disease progression after entry of the second variant pointed to an inherent and differential ability of the VC06 immune system to control HIV infection, compatible with the HIC phenotype.

The delay in viremia increase also indicates that the loss of viremia control was related to some evolutionary dynamics of both the B_1 and B_2 variants and not only to the entry of a new virus. Part of this dynamic could be related to recombination processes occurring between the B_1 and B_2 variants. Recombination is an important mechanism of diversity generation and immune escape [45] observed in many cases of SI [14, 38, 46–48], including in some HICs who developed disease progression [14, 38]. The frequency discrepancy for B_1-related *int* vs *env* sequences via SGA suggested the presence of recombinant B_1B_2. Although a PCR bias could also explain this difference, a variation of more than 30% in the representativity between the two fragments is less likely to occur due to PCR efficiency. In addition, the sample dilution prior to PCR, that is characteristic of the SGA methodology, should soften the template competition. Albeit this indicates that the differences observed are really due to a variation at the balance between B_1 and B_2 variants in *int* vs *env*, our analyses did not observed a recombination point in *int*.

Another sign of the importance of evolutionary dynamics for the clinical consequences of SI for VC06 was the observation of different B_2 *env* clusters associated with both the $V9_{2014}$ and $V16_{2017}$ samples plus the increase of viral divergence between 2011 and 2017. These data also support the hypothesis that partial loss of viremia control is related to the escape of specific viral populations from the immune response.

The pattern of increase or decrease in the percentage of $CD38^+HLA\text{-}DR^+$ T cells following alterations in the plasmatic viral load also points toward the participation of the immune response in the management of the infection during follow-up. T cell subset analyses showed an increase in the proportion of T cells with an effector phenotype (TEM and TEFF) at the timepoints close to SI and of increased viremia, suggesting a possible role for these cells in controlling viral replication. Although no expressive alterations in the Gag and Nef HIV-specific responses were observed during follow-up, this result did not exclude the presence of an HIV-specific immune response to regions other than those analyzed by us.

Finally, it is important to highlight that VC06 continued to maintain high and stable $CD4^+$ T cell counts despite the partial loss of viremia control. These data support that virological and immunological control are not necessarily concomitant. Together with other studies that described superinfected HICs with no alterations in $CD4^+$ T cell counts but a loss of viremia control [11–13, 18, 40, 41], our data indicate that moderate viremia, in some cases, is not able to impair immunological control.

Despite the partial loss of viremia control and the policy of the Brazilian Ministry of Health indicating antiretroviral treatment to all HIV-infected individuals, VC06 refused to initiate cART. The knowledge of her HIC status and the maintenance of immunological control together with side effects observed during a short period of cART were motivations for this refusal. This patient has been followed-up every 6 months to assess her immunological, clinical and virological status, and no signal of disease progression has been detected thus far. At all clinical visits, cART has again been offered.

Overall, this case raises awareness of the need for continued use of adequate preventive strategies after HIV-1 infection, even in patients with a long history of viremia control and an absence of disease progression. For HICs, our data demonstrate that natural control of HIV-1 replication can be a labile state since the underlying mechanisms associated with this phenotype do not guarantee unrestricted control of any other variant. More studies identifying the factors associated with control of multiple variants in HICs can be an important pathway to identify factors associated with natural control.

Abbreviations

cART: Combination antiretroviral therapy; CTL: Cytotoxic T Lymphocyte; DI: Dual-infection; *env*: env gene; HICs: HIV controllers; *int*: integrase gene; LTNP: Long-term non progressor; PBMC: Peripheral blood mononuclear cells; SGA: Single genome amplification; SI: Superinfection; TCM: Central-memory T cell; TEFF: Effector T cell; TEM: Effector-memory T cell; TN: Naive T cell; TPHA: Treponema pallidum hemagglutination assay; TSCM: Stem-memory T cell; VDRL: Venereal disease research laboratory test

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Authors' contributions

DGC conducted most of the experiments and analyzed the data. SSDdA conducted part of the SGA experiments and prepared plasma sequences. GB, FHC, MGM and MLG supervised the experiments and gave intellectual input. LMV, BH, BG, and VGV conducted patient recruitment and follow-up. DGC, MGM, and FHC wrote the first draft. All authors revised and approved the final manuscript.

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Availability of data and materials

HIV-1 *env* sequences generated from $V4_{2011}$ sample were previously deposited in GenBank under the accession numbers KY852775-KY852806. The remaining sequences generated during the current study were deposited in GenBank under the accession numbers MK757267-MK757421.

Ethics approval and consent to participate

The present work was approved by the Brazilian National Human Research Ethics Committee (CONEP 840/2008) and by the FIOCRUZ *Research Ethics* Committee (CEP 1717.0.000.009–07). The studied subject gave written informed consent for the use of biological samples.

Consent for publication

The studied subject gave written informed consent for publication of the results showed in this study.

Competing interests

The authors declare that they have no competing interests.

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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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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

controllers (VCs), individuals able to maintain the plasmatic VL below 2,000 RNA copies/mL (2). Despite the exceptional control of viremia, there are controversies as to whether EC might be considered a natural model of a functional cure.

Some ECs display a progressive loss of $CD4^+$ T cell counts and eventually progress to AIDS over time. The disease progression in those individuals appears to be mostly driven by persistent immune activation and inflammation likely associated with residual viremia (3–5). Consistent with this evidence, some studies suggest that the cART might lead to a marked decrease in immune activation and increased $CD4^+$ T cell counts in ECs, reducing the risk of non-AIDS-related events (6–8). Conversely, other studies describe that elevated levels of T cell activation and soluble inflammation markers are not associated with a faster rate of $CD4^+$ T cell decline in ECs (9, 10) or that ECs maintain absolute $CD4^+$ T cell counts and T cell activation levels within the normal range over time (10–12), thus concluding that these individuals may not have benefited from early cART initiation.

These observations confirm that ECs are heterogeneous with regard to both virologic (5, 11, 13, 14) and immunologic (2, 3, 10, 12, 15, 16) features. Such heterogeneity may result from the absence of a standardized classification of ECs mainly in relation to the cutoff level of viremia (≤50–500 copies/mL), minimum follow-up time (1–10 years), and/or presence of occasional blips (17). That heterogeneity may also reflect different underlying mechanisms of natural suppression of viremia across individuals.

The aim of this study was to evaluate different immunologic parameters in a group of ECs with stringent criteria for a definition of both the VL cutoff limit of detection (≤50 copies/mL) and the durability of viral suppression (>5 years) to identify those individuals who could represent the best model of a natural functional cure. To this end, we compared the plasma levels of inflammatory biomarkers and T cell activation between ECs with persistent undetectable viremia, ECs with occasional viral blips, VCs, cART-treated patients with undetectable viremia and HIV-uninfected controls. We also assessed the relationship between $CD4^+$ T cell counts, CD4%, CD4/CD8 ratio, levels of $CD8^+$ T cell activation, and levels of soluble markers of inflammation among HIV-1 controllers.

MATERIALS AND METHODS

Study Subjects and Ethical Issues

Twenty-eight HIV-1 controllers from the Instituto Nacional de Infectologia Evandro Chagas (INI) were selected for this study and divided into three groups as described previously in Ref. (14): (1) persistent elite controllers (PECs), if 100% of VL measures were below the limit of detection (<50–80 copies/mL) depending on the commercial method available, along with the clinical and laboratory follow-up ($n = 8$); (2) ebbing elite controllers (EECs), if subjects had occasional (<30% of frequency) episodes of transient low-level (51–400 copies/mL) viremia ($n = 7$); and (3) VCs, if most (≥70%) VL determinations were between 51 and 2,000 copies/mL ($n = 13$). Occasional VL measurements above the upper limits were accepted along with the follow-up for the EEC and VC groups. A group of HIV-1 infected individuals on cART with a VL suppressed for at least 2 years (cART; $n = 18$) and a group of HIV-1-uninfected individuals (HIV-neg; $n = 18$) were also included as controls. 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).

$CD4^+$ T Cell Count and VL Measurement

Absolute $CD4^+$ T and $CD8^+$ T cell counts were obtained using the Tritest or MultiTest TruCount kit and the MultiSet software on a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA). Plasma VL was measured using the Nuclisens HIV-1 RNA QT assay (Organon Teknika, Durham, NC, USA; limit of detection: 80 copies/mL) from 1999 to 2008, the Versant HIV-1 3.0 RNA assay (bDNA 3.0, Siemens, Tarrytown, NY, USA; limit of detection: 50 copies/mL) from 2008 to 2013, and the Abbott Real Time HIV-1 assay (Abbott Laboratories, Wiesbaden, Germany; limit of detection: 40 copies/mL) since 2013. When available, absolute $CD4^+$ T and $CD8^+$ T cell counts and VL data were collected from 1997 to 2017, depending on the HIC study entry.

Markers of T Cell Activation and Inflammation

Cryopreserved PBMCs were thawed in RPMI 1640 medium, GlutaMax supplemented (Gibco, Invitrogen, Carlsbad, CA, USA) containing 10% FBS (R10 medium) (Gibco, Invitrogen, Carlsbad, CA, USA), then washed using R10 medium and incubated overnight at 37°C, 5% CO_2 and controlled humidity. Afterward, the cells were washed and stained with FVS450 for viability evaluation (BD Biosciences, San Diego, CA, USA) and the following monoclonal antibodies: anti-CD3 APC-H7, anti-CD4 PECF594, anti-CD8 APC, anti-CD38 BB515, and anti-HLA-DR PE (BD Biosciences, San Jose, CA, USA). Then, the cells were washed, fixed with 1% PFA (Sigma, Germany), and acquired using a BD FACSAria IIu Flow Cytometer (BD Biosciences, San Jose, CA, USA). Flow cytometric analysis was performed with Flow Jo v.10.0.7 (Tree Star Inc., Ashland, OR, USA).

Plasmatic levels of IP-10, IL-18, sCD163, sCD14, CRP, and IL-6 were measured using commercial ELISA assays (R&D systems, USA), following the manufacturer's instructions.

Statistical Analysis

In the evaluation of the sociodemographic, clinical, and laboratorial features among the different groups of HICs, cART, and HIV-1-uninfected individuals, for continuous numerical variables, Kruskal–Wallis ANOVA by Ranks tests were used for assessing the hypothesis that the different samples in the comparison were drawn from the same distribution or from distributions with the same median. Likewise, for categorical nominal variables, Fisher's exact tests were used in the evaluation of frequencies among the different groups for assessing the hypothesis of independence between the groups of individuals and these variables. In addition, graphical exploratory analyses were performed for continuous numerical variables for dimension reduction and visualization by multivariate principal component analysis (PCA), and Spearman's rank correlation coefficient analyses were calculated for these variables. Pairwise comparisons of laboratory variables averaged among groups of interest were performed by contrasts obtained

after both bi- and multivariate-linear models fitted by ordinary least square regressions. *P*-values were corrected by the Tukey Honest Significant Difference (HSD) method (18). After all laboratory variable pairwise comparisons, we conducted a type I error adjustment for multiple comparisons following the Holm–Bonferroni method (19). Likewise, confounding variables were selected by bivariate linear models fitted by ordinary least square regressions and included in multivariate models if any adjusted-*P*-value <0.2 to eliminate sample bias. Box–Cox Power family of transformations (20) was used whenever necessary to normalize laboratory variables. Before modeling the CD4$^+$ T cell counts and the CD4/CD8 ratio kinetics of the HIC individuals, the variables were log- (base 10) and square-root transformed, respectively, and then nested linear mixed-effect models (21) were fit by maximum likelihood and had their deviance compared by *F*-test with Kenward–Roger approximation. Contrasts were obtained from the fitted model to compare both CD4$^+$ T cell counts and the CD4/CD8 ratio kinetics means among HIC groups of individuals. Degrees of freedom for adjusted effects were approximated by the Satterthwaite method (22). Again, *P*-values were corrected by the Tukey HSD method (18). All statistical analysis was performed in software R v. 3.4.3.

RESULTS

Characteristics of the Study Groups

Table 1 summarizes the characteristics of the five study groups at a time point selected for this study (highlighted in Figure S1 in Supplementary Material). We found no dependence among the observed frequencies of any of the nominal variables evaluated (age, gender, level of education, exposure category, and time since HIV diagnosis) and the study groups, with the exception of skin color. The median follow-up time of the HIC groups was 9.02 years (IQR = 6.46). Despite no global difference among the groups, the PEC group had the highest median CD4$^+$ T cell counts at the inflammation/immune activation assay date (1,243.5; IQR = 472.8). Some patients started cART due to the recommendation of the "Departamento de Vigilância, Prevenção, e Controle das IST, do HIV/Aids e das Hepatites Virais" from the Ministry of Health in Brazil, which offer cART to all PLWH.

Most ECs Have Levels of Inflammation and Immune Activation Similar to HIV-1-Uninfected Individuals

Chronic inflammation and elevated immune activation are associated with a faster progression to AIDS and CD4$^+$ T cell depletion (23, 24). Here, we evaluated the concentration of six plasmatic markers of inflammation/immune activation and frequency of activated CD8$^+$ T cells (**Figure 1**; Figure S2 in Supplementary Material). Among the HICs, the estimated mean levels of IL-18 and IP-10 increased as the level of viremia increased (PECs < EECs < VCs), although the difference between PECs and EECs was not significant (**Figures 1A,B**). The estimated mean levels [95% confidence intervals], after adjustment for gender, race, education, and age, of IL-18 and IP-10 in VC (524.1 pg/mL [432.4; 634.8]; 136.3 pg/mL [137.3; 225.7]) were significantly higher than that in HIV-neg (247.3 pg/mL [206.3; 296.2]; 80.7 [63.0; 102.7]), cART (318.6 pg/mL [273.7; 370.6]; 95.3 [77.7; 116.5]), and PEC (224.1 pg/mL [177.6; 282.0]; 73.1 [53.1; 99.5]) groups. Both EC groups had levels of IL-18 and IP-10 that were not different from the HIV-neg or cART groups. VCs also presented a higher mean level of CRP (4,103.8 ng/mL [2,319.2; 7,255.9 ng/mL]) than did the PEC group (725.1 ng/mL [367.1; 1,423.2 ng/mL]) (**Figure 1C**). The levels of IL-6, sCD14, and sCD163 were not different among the five groups of individuals (Figure S2 in Supplementary Material).

The expression of CD38 and HLA-DR on CD8$^+$ T cells has been broadly used to evaluate immune activation in HIV-1 infection (3, 10, 23, 25). The VC group presented a higher estimated mean proportion, 12.2% [10.0; 14.5], of activated CD8$^+$ T cells; this proportion was significantly higher than that of the other groups (**Figure 1D**). The PEC (3.0% [1.6; 4.7]) and EEC (1.0% [−0.4; 2.6]) groups displayed CD8$^+$ T cell estimated mean activation levels that

TABLE 1 | Individuals' characteristics.

	HIV-neg (*n* = 18)	Combined antiretroviral therapy (*n* = 18)	Persistent elite controller (*n* = 8)	Ebbing elite controller (*n* = 7)	Viremic controller (*n* = 13)	*P*-value
Age (years)	37.07 (IQR = 17.76)	44.22 (IQR = 9.81)	40.82 (IQR = 8.49)	45.6 (IQR = 18.63)	41.39 (IQR = 9.25)	0.4773
Gender; *n* (%)						0.2503
Female	9 (14.1)	7 (10.9)	5 (7.8)	6 (9.4)	5 (7.8)	
Male	9 (14.1)	11 (17.2)	3 (4.7)	1 (1.6)	8 (12.5)	
Skin color; *n* (%)						0.0259
Black	2 (3.1)	1 (1.6)	0 (0)	1 (1.6)	6 (9.4)	
Brown	3 (4.7)	9 (14.1)	6 (9.4)	2 (3.1)	3 (4.7)	
White	11 (17.2)	8 (12.5)	2 (3.1)	4 (6.2)	4 (6.2)	
Exposure category; *n* (%)	NA					0.2135
Het/Other	NA	15 (23.4)	6 (9.4)	7 (10.9)	8 (12.5)	
MSM	NA	3 (4.7)	2 (3.1)	0 (0)	5 (7.8)	
Number of blips [means (IQR)]	NA	0 (IQR = 0)	0 (IQR = 0)	3 (IQR = 1.5)	12 (IQR = 8)	<0.0001
CD4$^+$ T-cell count (cells/μL)	831 (IQR = 372.25)	853 (IQR = 221)	1,243.5 (IQR = 472.75)	1,027 (IQR = 505)	820 (IQR = 711)	0.1216
CD4/CD8 ratio	1.69 (IQR = 0.46)	0.95 (IQR = 0.69)	1.44 (IQR = 0.92)	1.17 (IQR = 0.41)	1.06 (IQR = 0.31)	<0.0001
CD4%	44 (IQR = 3.5)	32.95 (IQR = 7.04)	43.5 (IQR = 9.75)	44 (IQR = 11)	37 (IQR = 6)	<0.0001
Viral load (copies/mL)	NA (IQR = NA)	49 (IQR = 0)	49 (IQR = 0)	49 (IQR = 127.5)	450 (IQR = 549)	<0.0001
Time since HIV diagnosis (days)	NA	3,988 (IQR = 1,968.75)	1,694 (IQR = 4,348.25)	3,615 (IQR = 2,243.5)	4,162 (IQR = 2,970)	0.3893

Het, heterosexual; MSM, males who have sex with males; IQR, interquartile range; NA, not applicable.

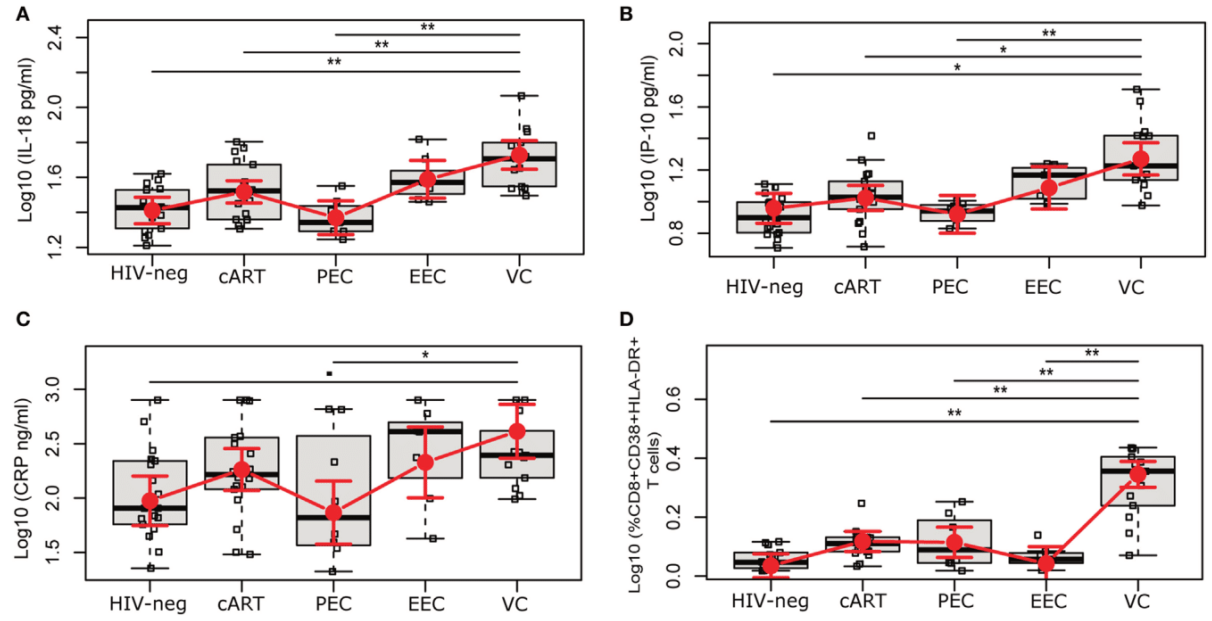

FIGURE 1 | HIV-1 elite controllers have normal levels of inflammation and CD8⁺ T cell activation. HIV controllers were divided according to the level of viral controller: persistent elite controller, if 100% of VL measures were below the limit of detection; ebbing elite controller, if subjects had occasional (<30% of frequency) episodes of transient, low-level (51–400 copies/mL) viremia; and viremic controller, if most (≥70%) VL determinations were between 51 and 2,000 copies/mL. The plasmatic levels of **(A)** IL-18, **(B)** IP-10, and **(C)** CRP were measured by the ELISA test, and **(D)** the frequency of %CD8⁺CD38⁺HLA⁺DR⁺ was evaluated by flow cytometry. The time point selected for this study is highlighted in Figure S1 in Supplementary Material. The results are expressed as Log10. Boxplots represent the IQR and sample median (central solid black line). Red dots and vertical bars represent linear model estimated adjusted means and 95% confidence intervals (CI 95%). Comparisons of means among groups were performed by contrasts/differences obtained after both bi- and multivariate-linear models fitted by ordinary least square regressions. *P*-values were corrected by the Tukey Honest Significant Difference *post hoc* method, and a Type I error adjustment was conducted for multiple comparisons following the Holm-Bonferroni method. *$P < 0.05$, **$P < 0.01$.

were not different from the HIV-1-uninfected (0.8% [−0.1; 1.9]) and cART (3.1% [2.1; 4.2]) groups.

ECs Maintain a Normal CD4/CD8 Ratio and CD4%

Studies have reported an association between CD4/CD8 ratio and T cell activation (26, 27). We observed no difference of CD4/CD8 ratio (**Figure 2A**) among the HICs or even with the cART and HIV-1-uninfected individuals. We also evaluated the CD4% (**Figure 2B**), as this marker is considered one of the best predictors of AIDS-associated events (28); a previous study (10) that divided ECs based on CD4% demonstrated different levels of immune activation among the groups. The cART group showed a lower mean estimated CD4% (34.3% [31.0; 37.5]) than did the HIV-1-uninfected (45.2% [41.3; 49.1]) and PEC (45.7% [40.7; 50.6]) groups. The VC group also presented a mean adjusted CD4% <40% (39.1% [34.9; 43.3]), although this was not significantly lower than the HIV-neg or EC groups.

No Evidence of CD4⁺ T Cell Decline Over Time in HICs

The CD4⁺ T cell dynamics is an important marker of disease progression, and different studies have correlated immune activation with CD4⁺ T cell depletion (24, 29). To analyze the trajectories of CD4⁺ T cell counts and the CD4/CD8 ratio, we fitted linear mixed-effects models (Tables S1 and S2 and Figure S3 in Supplementary Material). The mean CD4⁺ T cell count (log, base 10, transformed) trajectories were distinct among the groups, but without evidence of a decline in CD4⁺ T cell counts for any group in 5 years of follow-up intervals (**Figures 3A–C**; Table S3 in Supplementary Material). Despite that, the fitted mean $\log_{10}$-transformed CD4⁺ T cell counts of the PEC group presented a clear, continuously rising tendency after 5 years of follow-up, while for the EEC group, this rising tendency was limited to 12.5 years, after which there was a slightly descending fitted mean. Conversely, for the VC group, the fitted mean described a much more stable pattern, with a slight descent until 12.5 years of follow-up that was reversed in the other half period. The square root of CD4/CD8 ratios, $\sqrt{\text{CD4}/\text{CD8}}$ (Table S4 in Supplementary Material) was somewhat different from the fitted mean $\log_{10}$-transformed CD4⁺ T cell count dynamics (Figure S3A in Supplementary Material). Although there is also a clear, continuously rising tendency after 5 years of follow-up for the PEC group, for the fitted mean square root of CD4/CD8 ratios (Figure S3B in Supplementary Material), we observed a more linear tendency, i.e., straightness. For the EEC group, the rising tendency for the fitted mean square root of the CD4/CD8 ratio was shorter than that for the $\log_{10}$-transformed

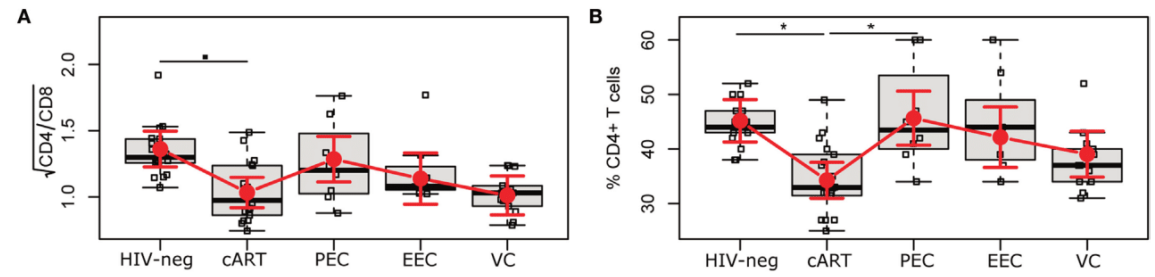

FIGURE 2 | HIV controllers have a normal CD4/CD8 ratio and CD4%. The CD4/CD8 ratio **(A)** and the CD4% **(B)** were obtained using the MultiTest TruCount kit. The time point selected for this study is highlighted in Figure S1 in Supplementary Material. Boxplots represent the IQR and sample median (central solid black line). Red dots and vertical bars represent linear model estimated adjusted means and 95% confidence intervals (CI 95%). The CD4/CD8 ratio was square-root transformed; comparisons of means among groups were performed by contrasts/differences obtained after both bi- and multivariate-linear models fitted by ordinary least square regressions. *P*-values were corrected by the Tukey Honest Significant Difference *post hoc* method, and a type I error adjustment was conducted for multiple comparisons following the Holm-Bonferroni method. •$P < 0.1$, *$P < 0.05$.

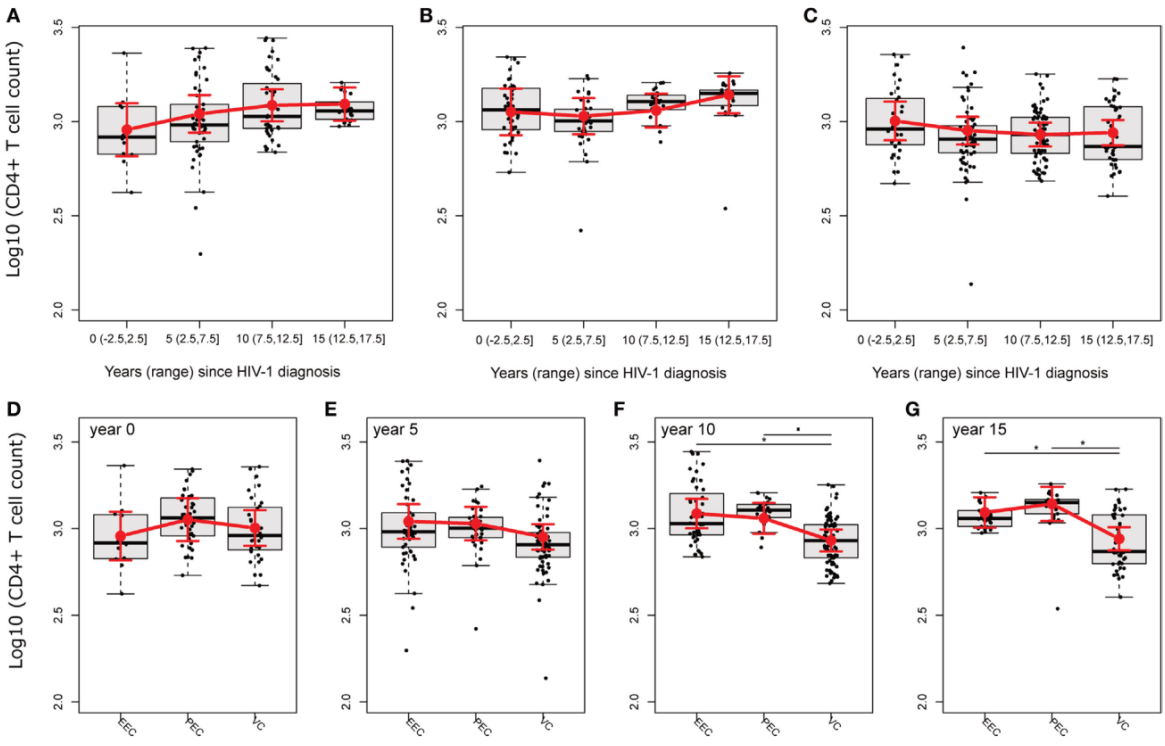

FIGURE 3 | HIV controllers (HICs) have no evidence of CD4⁺ T cell decline. Fitted (sample) instant in/decrement of $\log_{10}$-transformed CD4⁺ T cell counts at 5-year intervals (ranges) after HIV-1 diagnosis for HICs, ebbing elite controller **(A)**, persistent elite controller **(B)**, and viremic controller **(C)**, and the comparison of fitted mean $\log_{10}$-transformed CD4⁺ T cell counts among HICs at year 0 **(D)**, 5 **(E)**, 10 **(F)**, and 15 **(G)** after HIV-1 diagnosis. Analysis was performed by selecting the best nested linear mixed-effect models fitted by maximum likelihood. Deviance analysis was performed among nested models by *F*-test with Kenward–Roger approximation. Slopes/contrasts were obtained from the best fitted models to compare CD4⁺ T cell counts kinetic estimated means and CI 95% (red dots and vertical bars) among HIC groups of individuals. Degrees of freedom for estimated effects were approximated by the Satterthwaite method. Boxplots represent the IQR and sample median (central solid black line). Red dots and vertical bars represent linear model estimated adjusted means and 95% confidence intervals (CI 95%). Again, *P*-values were corrected by the Tukey Honest Significant Difference. •$P < 0.1$, *$P < 0.05$.

CD4⁺ T cell counts, stopping at 5 years of follow-up, and the slight descent observed for the $\log_{10}$-transformed CD4⁺ T cell counts was much more evident and continuous for at least the remaining 3/4 of follow-up time. The more distinct pattern among HIC groups was clearly the one for the VC group (Figure S3C in Supplementary Material). We saw a clear continuously descending

tendency for the fitted mean square root of the CD4/CD8 ratio that initiated right at the beginning of the observation and continued for at least 20 years. To show some of these differences along the follow-up time for both the fitted mean log_{10}-transformed mean $CD4^+$ T cell counts and square root of the CD4/CD8 ratio, and given the small sample size, we decided to test either if there were instantaneous in/decrements (slopes) within groups in 5-year intervals (Tables S3 and S4 in Supplementary Material), or if there were within-group differences between the beginning and 15 years of follow-up time (Tables S5 and S6 in Supplementary Material). In the former analysis, we found a significant increment of log_{10}-transformed mean $CD4^+$ T cell counts for the EEC group at 5 years of follow-up (log_{10}Count = 0.05 [0.012; 0.088]; Adj-*P*-Val = 0.047), which is equivalent of a mean increment of 12.24% [2.89%; 22.44%] $CD4^+$ T cells. No significant in/decrement was observed for the PEC or VC fitted log_{10}-transformed $CD4^+$ T cell count mean at 0, 5, 10, or 15 years of follow-up. Also, no significant in decrement was observed for any HIC group for the fitted mean square root of the CD4/CD8 ratio. In the second analysis, the EEC group had a tendency of increase of the log_{10}-transformed $CD4^+$ T cell count (**Figures 3A–C**; Table S5 in Supplementary Material) after 15 years of follow-up (log_{10}FC = 0.136 [−0.005; 0.277]; Adj-*P*-Val = 0.0584). No difference was observed for the PEC or VC fitted log_{10}-transformed $CD4^+$ T cell count mean after 15 years of follow-up. The PEC group showed a significant fitted mean square root of CD4/CD8 ratio increase after 15 years of follow-up (Figures S4A–C and Table S6 in Supplementary Material) (Diff. = 0.224 [0.026; 0.423]; Adj-*P*-value = 0.0289). No difference was observed for the EEC or VC fitted mean square root of the CD4/CD8 ratio after 15 years of follow-up (Figures S4A–C and Table S6 in Supplementary Material). We then decided to compare both the fitted mean log_{10}-transformed mean $CD4^+$ T cell counts and fitted mean square root of the CD4/CD8 ratio among groups (**Figures 3D–G**). In these analysis, we observed differences between PECs and VCs after 10 years of follow-up (log_{10}FC = 0.128 [0.014; 0.241]; Adj-*P*-Val = 0.0727) and EECs and VCs (log_{10}FC = 0.155 [0.044; 0.266]; Adj-*P*-Val = 0.021) (**Figure 3F**; Table S7 in Supplementary Material). Differences between both PECs and VCs (log_{10}FC = 0.201 [0.073; 0.329]; Adj-*P*-Val = 0.01) and EECs and VCs (log_{10}FC = 0.152 [0.033; 0.271]; Adj-*P*-Val = 0.0378) were also observed after 15 years of follow-up (**Figure 3G**; Table S7 in Supplementary Material). Once VCs had no evidence of $CD4^+$ T cell count decrease, these results were possibly due to an increase of $CD4^+$ T cell counts in EECs. Using resembling analyses (Figures S4D–G and Table S8 in Supplementary Material), we observed both a difference after 10 years of follow-up between the fitted mean square root of CD4/CD8 ratios of PECs and VCs (Figure S4F and Table S8 in Supplementary Material) (Diff. = 0.249 [0.054; 0.444]; Adj-*P*-Val = 0.036) and a borderline difference between EECs and VCs (Figure S4F and Table S8 in Supplementary Material) (Diff. = 0.217 [0.024; 0.41]; Adj-*P*-Val = 0.072). The difference between PECs and VCs was also observed after 15 years of follow-up (Figure S4G in Supplementary Material) (Diff. = 0.373 [0.134; 0.611]; Adj-*P*-Val = 0.009). No differences of the fitted mean square root of CD4/CD8 ratios among groups were observed at the beginning or after 5 years of follow-up.

IL-18 and IP-10 Correlate With Cumulative Viral Load (cVL), CD4/CD8 Ratio, and CD4%

We analyzed the correlation between cVL, CD4%, CD4/CD8 ratio, and markers of inflammation or immune activation. When considering only EECs and VCs, both IP-10 (ρ = 0.4830; *P*-value = 0.0327) and IL-18 (ρ = 0.5140; *P*-value = 0.0176) presented a moderate positive linear relationship with cVL (**Figures 4A,B**), and no correlation was observed between $CD8^+$ T cell activation and cVL. When considering all HICs, the HIV-neg group and cART groups, IL-18 and IP-10 correlated positively with $CD8^+$ T cell activation (ρ = 0.3310; *P*-value = 0.0075 and ρ = 0.2990; *P*-value = 0.0162, respectively) and negatively with CD4/CD8 (ρ = −0.2840; *P*-value = 0.00228 and ρ = −0.3240; *P*-value = 0.0094, respectively) (**Figures 4C,F**) and CD4% (ρ = −0.2600; *P*-value = 0.0381 and ρ = −0.3280; *P*-value = 0.0081, respectively) (**Figures 4D,E,G,H**). $CD8^+$ T cell activation presented a weak correlation with CD4/CD8 ratio and CD4% (ρ = −0.2950; *P*-value = 0.0179 and ρ = −0.2490; *P*-value = 0.0474, respectively) (Figures S5A,B in Supplementary Material). We also observed a correlation between IP-10 and IL-18 with $CD8^+$ T cell activation (Figures S5C,D in Supplementary Material).

Some ECs Displayed an Immune Profile Indistinguishable From HIV-1 Uninfected Individuals

Elite controllers are considered models of HIV control, and their immunological profiles could give insights to identify biomarkers of an HIV functional cure (30). Here, we performed PCA combining the different immunological characteristics analyzed among the five groups included in this study (**Figure 5**). The VC individuals are the more disperse among the groups and are totally separated from HIV-1-uninfected individuals and EECs, and they have almost no superposition with PECs. The cART individuals have a very limited superposition with HIV-1 negative individuals, reinforcing the idea that despite prolonged viral suppression, the most treated individuals do not achieve a normal immunological profile. Half of the PECs and two EECs grouped with HIV-negative individuals, highlighting the preserved immunological profile of these individuals. IL-18 and IP-10 concentration and $CD8^+$ T cell activation have the highest contribution to the formation of the first principal component, which is alone responsible for explaining 39.4% of the data covariance. The combined analysis of these inflammatory/activation markers suggests the existence of two subgroups of ECs. One subgroup (EC^{low}) displayed inflammatory and activation markers within the normal range, while the other subgroup (EC^{high}) displayed inflammatory and/or activation markers that were above the normal range (Table S9 in Supplementary Material).

DISCUSSION

In this study, we evaluated inflammation, $CD8^+$ T cell activation, CD4%, and $CD4^+/CD8^+$ T cell dynamics in HICs with long-term (>5 years) viral control at different levels. We observed that long-term ECs with undetectable viremia (≤50–80 copies/mL)

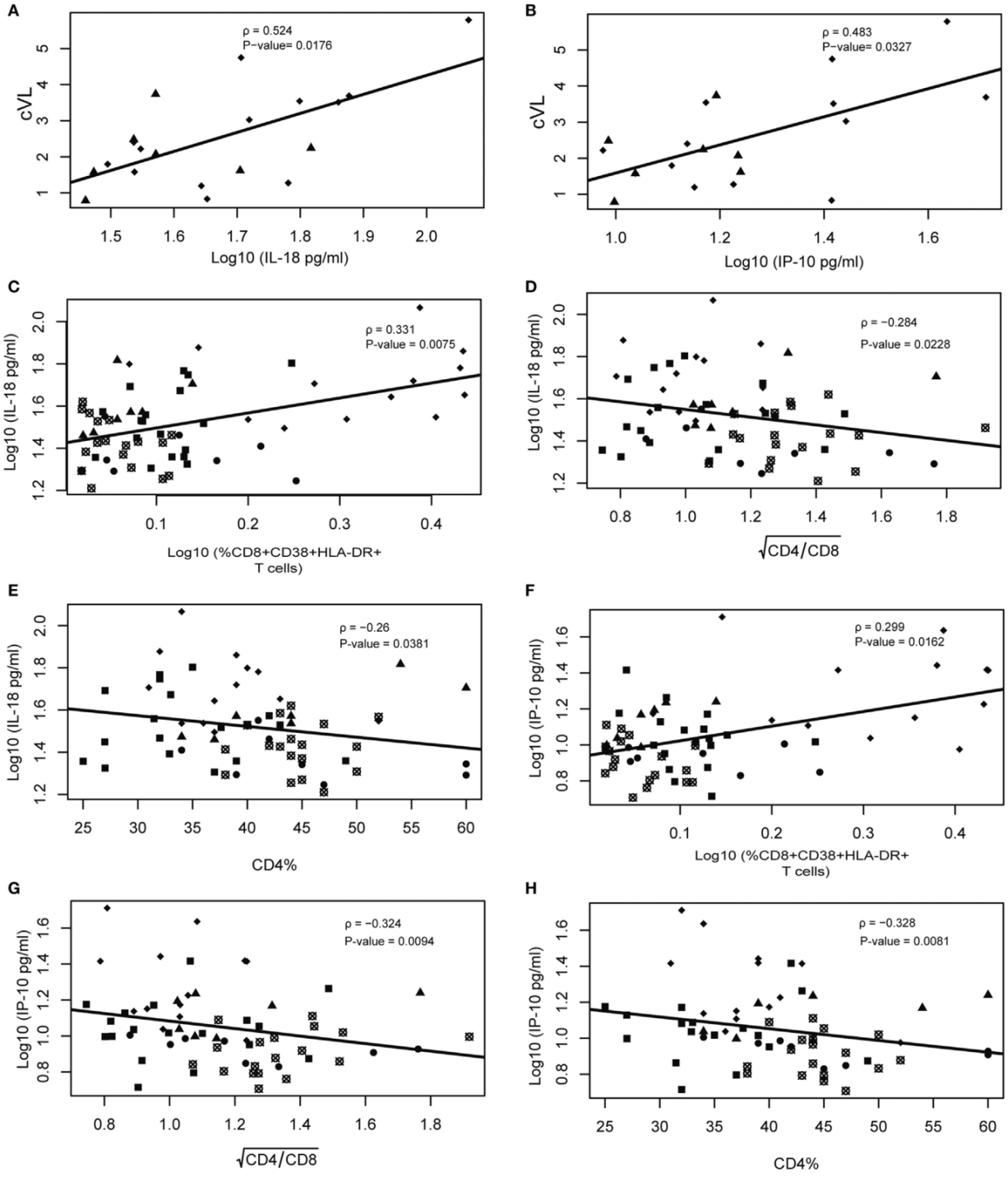

FIGURE 4 | IL-18 and IP-10 correlate with cumulative viral load, CD4/CD8 ratio, and CD4%. Viral load correlations were performed only using ebbing elite controller (EEC) and viremic controller (VC) groups. Spearman rank correlation of IL-18 vs cumulative viral load (cVL) **(A)** and IP-10 vs cVL/year **(B)**. Correlation between IL-18 vs %CD8$^+$CD38$^+$HLA$^+$DR$^+$ **(C)**, $\sqrt{\text{CD4 / CD8}}$ **(D)**, and %CD4 **(E)**; and between IP-10 vs %CD8$^+$CD38$^+$HLA$^+$DR$^+$ **(F)**, $\sqrt{\text{CD4 / CD8}}$ **(G)**, and %CD4 **(H)**. Spearman's rank correlations coefficient analysis was performed among these variables with type I error adjustment for multiple comparisons following the Holm–Bonferroni method. Crossed open black circles, HIV-neg; solid black squares, combined antiretroviral therapy; solid circles, persistent elite controller; solid black triangles, EEC; and solid black lozenges, VC.

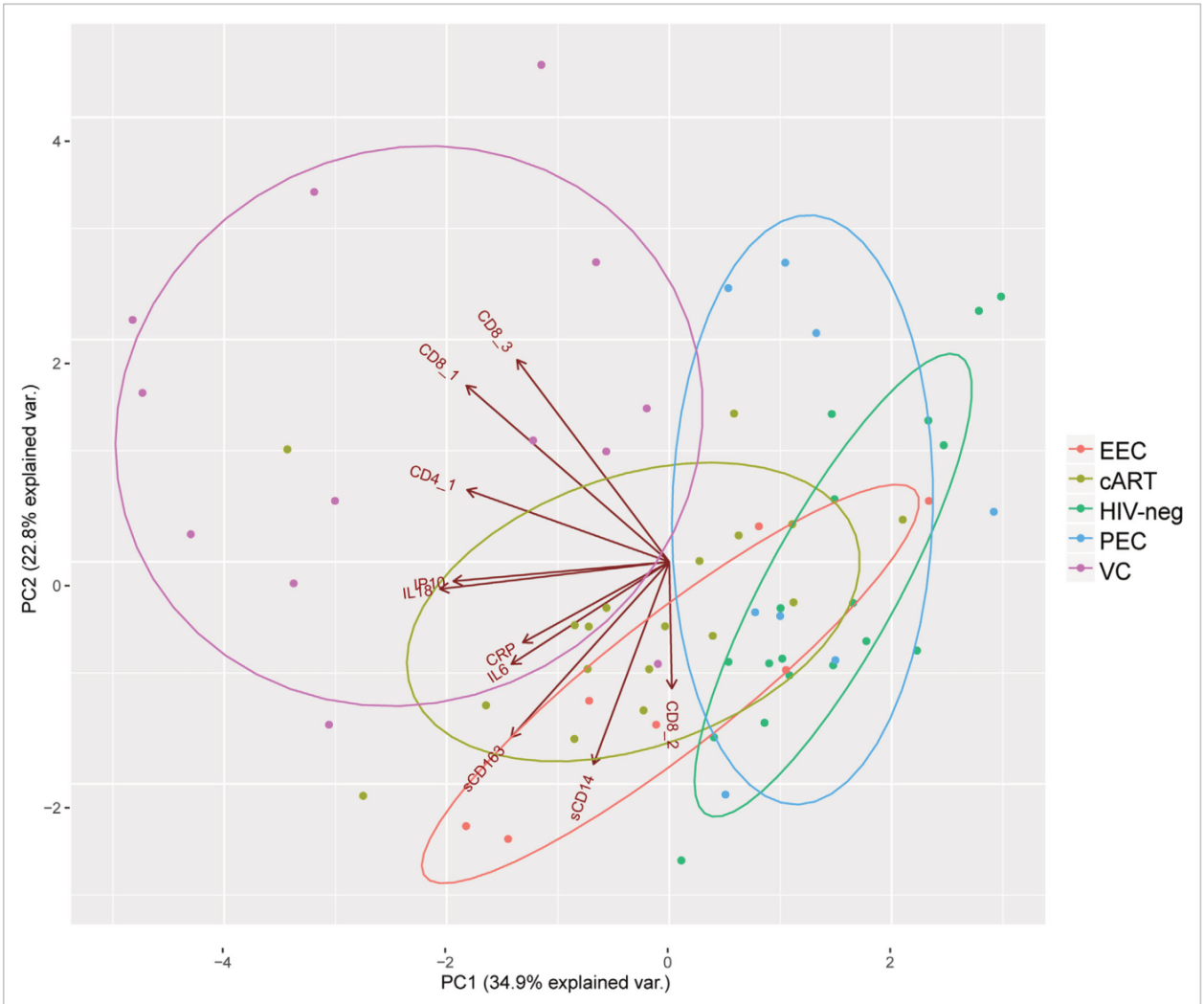

FIGURE 5 | Principal component analysis highlights elite controllers with an immune profile similar to HIV-1 negative individuals: CD8_1, $CD8^+CD38^+HLA\text{-}DR^+$; CD8_2, $CD8^+HLA\text{-}DR^+$; CD8_3, $CD8^+CD38^+$; CD4_1, $CD4^+CD38^+HLA\text{-}DR^+$. Brown directed vectors represent the contribution of each variable for the formation of the two-principal components. Colored ellipses represent the 68% of the normal concentration for each group.

presented an inflammation and activation profile similar to that observed among HIV-1 negative individuals, in agreement with previous studies using comparable stringent criteria for EC definition (10–12). In sharp contrast, we found that a persistent low-level viremia (80–2,000 copies/mL) is sufficient to drive elevated chronic levels of immune activation and inflammation in long-term VCs.

Plasmatic levels of IP-10 and IL-18 correlated with cVL control and $CD8^+$ T cell activation in our HICs cohort. There is increasing evidence of the positive relationship between IP-10 and HIV-1 VL (31–33), and previous studies have demonstrated a higher level of IP-10 in some ECs than in HIV-negative individuals (25, 34). Thus, the normal level of IP-10 in our ECs could reflect an extremely low level of residual viral replication, consistent with the very low frequency of HIV-1 Gag-specific responses in the IFN-γ ELISPOT assay previously described for some of these ECs (11). HIV-1-infected individuals present higher levels of IL-18 than do HIV-uninfected individuals (35–37). To the best of our knowledge, only two previous studies analyzed the IL-18 levels in HICs (38, 39) but did not compare EC with HIV-1 uninfected individuals.

Our analyses also support that inflammation and T cell activation reflects the past cumulative viral replication better than VL at a single point as we did not observe a direct linking between the presence of VL blips and higher levels of inflammation and/or T cell activation in EC. For instance, individual EEC36 was analyzed during a blip (1,086 copies/mL), but displayed a low cVL (3.17 copies/mL/year) and one of the lowest levels of inflammation (IP-10 = 89.39 pg/mL and IL-18 = 278.44 pg/mL) and T cell activation (0.47%). On the other hand, individual EEC18 was

analyzed at a point with undetectable VL, but displayed a relative high cVL (8.47 copies/mL/year) and a high levels of inflammation (IP-10 = 161.73 pg/mL and IL-18 = 362.62 pg/mL) and T cell activation (2.15%) among EC.

Although the mean levels of inflammatory and activation markers in our EC cohort were similar to those observed in the HIV-uninfected group, the combined analysis of the inflammatory markers IP-10 and IL-18 and of activation marker $CD8^+CD38^+HLA\text{-}DR^+$ T cells suggest the existence of two subgroups of ECs. The subgroup EC^{low} displayed inflammatory and activation markers indistinguishable from HIV-uninfected subjects and probably represent the best model of natural functional cure. The subgroup EC^{high}, by contrast, displayed inflammatory and/or activation markers that were above the normal range. Interestingly, the EC^{low} and EC^{high} subgroups did not match the PEC and EEC subgroups, suggesting that some EC are able to maintain normal levels of inflammatory and activation markers despite occasional blips, while some ECs display high activation despite persistent undetectable viremia. Further studies using longitudinal analyses and a higher number of individuals will certainly contribute to a better definition of these groups.

The pathogenic effect of the high levels of $CD8^+CD38^+HLA\text{-}DR^+$ T cells observed in three PECs, however, should be interpreted with caution. In sharp contrast to the activation profile typically observed in HIV-1 infection, the three PEC subjects presenting the highest expression of HLA-DR in $CD8^+$ T cells also showed very low expression of CD38. Notably, HIV-1 specific $CD8^+$ T cells expressing only HLA-DR present a better functional profile (higher frequency of IFN-γ^+IL-2^+-producing cells, higher proliferative and cytotoxic capacities) than those expressing both HLA-DR and CD38 (16, 40, 41). Although we only evaluated CD38 and HLA-DR expression in the bulk $CD8^+$ T cells, these observations suggest that the high levels of $CD8^+HLA\text{-}DR^+$ T cells detected in some PECs may reflect a more functional anti-HIV $CD8^+$ T cell condition, instead of a harmful non-specific immune activation. However, more analyses are necessary to test this hypothesis, as compare CD107a, IFN-γ, and IL-2 expression, with a HIV-1 specific stimulus, between $CD8^+HLA\text{-}DR^+$ and $CD8^+HLA\text{-}DR^-$ T cells in these patients.

Some studies have reported $CD4^+$ T cell depletion in some HICs (3–5, 42, 43), although the frequency of this phenomenon greatly varies among the different cohorts evaluated, mainly due to the criteria of HIC definition and the number of subjects evaluated. Leon et al. (42), using the criteria of VL <50 copies/mL (ECs) and <2,000 copies/mL (VCs) for at least 1 year, identified 475 HICs and showed a significant $CD4^+$ T cell decline in 48% of them. By contrast, a study including 217 HICs from the ANRS C021 CODEX (43) with VL <400 copies/mL and at least 5 years of HIV-1 diagnosis found that only 5% experienced immunological progression. We detected no evidence of $CD4^+$ T cell depletion in any of the long-term HIC groups evaluated in our study. This suggests that $CD4^+$ T cell depletion may be a rare phenomenon in HICs maintaining viral suppression for more than 5 years, even for VCs and EC^{high} subgroups.

Beyond the $CD4^+$ T cell decrease, one of the characteristics of HIV-1 infection is $CD8^+$ T cell increase, resulting in a lower CD4/CD8 ratio and CD4% over time (44). The CD4/CD8 ratio predicts the time to AIDS in HIV-1-infected untreated individuals (26), and a low CD4/CD8 ratio (0.51–0.80) is associated with an increased risk of losing virological control in HIC individuals (45). The CD4% is also one of the best predictors of AIDS-related events, even after therapy start (34), and it was highlighted that ECs with CD4% >40% had normal levels of T cell activation and reduced expression of exhaustion markers compared with ECs presenting CD4% <40% (10). All ECs included in our study displayed a median CD4/CD8 ratio >1 and a median CD4% >40%, except for one PEC individual. Half of the VC subjects, by contrast, displayed a CD4/CD8 ratio <1 (47%) and CD4% <40% (86%). Thus, although we detected no evidence of $CD4^+$ T cell depletion in the HIC groups evaluated in our study, persistent low-level viremia in VC appears to be associated with $CD8^+$ T cell increase and consequently lower CD4/CD8 ratio and CD4% over time.

Our study has some limitations. First, the small number of HICs evaluated may have imposed a limitation on our capacity to detect significant differences between groups, although that limitation was compensated by the more stringent criteria of HIC definition and long-term follow-up. Second, an ultrasensitive VL assay could permit a better characterization of the accumulative residual viremia in our ECs and its relationship with markers of inflammation and immune activation. Finally, we classified the group as EC^{low} and EC^{high} based in only one point, a longitudinal study could help to minimize transient fluctuations in the levels of inflammation and T cell activation within each individual and to identify better plasmatic biomarkers of functional cure in ECs.

Taken together, our results confirm that the use of more stringent criteria for EC classification, combined plasmatic levels of IL-18 and IP-10, and a frequency of $CD8^+CD38^+HLA\text{-}DR^+$ T cells could help to identify individuals with true preserved immune integrity, which is essential in the search for correlates of a functional cure in HIV-1 infection. The lack of $CD4^+$ T cell depletion in all long-term ECs analyzed here raises the question about the benefits of cART for this population, especially for those EC subjects with normal immune activation and inflammation profiles. This study reinforced the notion that initiation of cART in ECs should be evaluated individually and supports the importance of EC follow-up with elevated plasmatic levels of IL-18, IP-10, and activated $CD8^+$ T cells to identify those ECs at most risk for disease progression.

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

FC, GB, and MM conceptualized and designed the study. FC, GB, MM, and MG contributed to the experimental design and provided intellectual input; FC, DC, and SA performed sample processing, clinical data, and intellectual input. FC and HP performed the experiments. ST performed the genetic analyses of HICs. BH, BG,

and VV included patients and provided clinical data. FC, HP, GB, and MR-A analyzed data and wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at https://www.frontiersin.org/articles/10.3389/fimmu.2018.01576/full#supplementary-material.

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Reduction of inflammation and T cell activation after 6 months of cART initiation during acute, but not in early chronic HIV-1 infection

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Abstract

Objectives: To investigate the impact of early combined antiretroviral therapy (cART) on inflammation biomarkers and immune activation during acute and early chronic HIV-1 infection.

Methods: We included 12 acute (AHI), 11 early chronic (EcHI), and 18 late chronic HIV-1-infected (LcHI) individuals who were treated with cART and 18 HIV-1-uninfected (HIV-neg) individuals. Plasmatic levels of inflammation biomarkers, $CD8^+CD38^+HLA\text{-}DR^+$ T cell frequencies, CD4 T cell counts, CD4/CD8 ratio, total HIV-1 DNA and plasmatic viral load were evaluated. Mann–Whitney test, Pearson and Spearman correlation, and linear regression models were used for statistical analyses.

Results: IP-10, IL-18, and sCD163 were significantly elevated at pre-ART in the AHI and EcHI groups, showing a significant reduction after 6 months of cART in the AHI group, achieving similar levels to the HIV-neg group. For the EcHI group, the IP-10 and sCD163 levels were also significantly reduced on M6-ART; however, IP-10 levels remained higher than in the HIV-neg group, and no significant reduction of IL-18 levels was observed. The $CD8^+$ T cell activation levels were elevated in the AHI and EcHI groups at pre-ART and showed a significant reduction on M6-ART, but they were similar to levels seen for HIV-neg only after 12 months of cART. At pre-ART, IP-10 levels but not IL-18 levels were positively correlated with HIV-1 viral load in the AHI group.

Conclusions: Early initiation of cART in HIV infection can reduce systemic inflammation, but the earlier normalization of the inflammation markers was only observed when cART was initiated in the acute phase of infection. A slower dynamic of reduction was observed for $CD8^+$ T cell activation.

Keywords: HIV-1 acute infection, Inflammation, cART, Immune activation, IP-10, IL-18

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Introduction

High systemic immune activation has a pivotal role in HIV-1 pathogenesis [1]. Studies have associated the elevated immune activation with $CD4^+$ T cell depletion and progression to AIDS [2–7]. During primary HIV-1 infection (PHI), viral loads can reach values higher than one million HIV-1 RNA copies/mL, and a significant decrease in $CD4^+$ T cell counts occurs [8, 9]. A high production of pro-inflammatory cytokines, called a "cytokine storm", arises in response to virus replication [10]. A controlled initial pro-inflammatory immune response may be beneficial, as observed in natural SIV hosts, which displayed significant increases in plasma cytokines and interferon-stimulated gene (ISG) expression, normalized four weeks after infection [11]. However, in the context of HIV-1 infection, the pro-inflammatory response remains elevated after the end of the acute phase, and most individuals without cART develop progressive immune dysregulation, culminating in AIDS [8].

The cART has had an enormous impact on mortality and morbidity in HIV-1 infection [12–14]. Studies in the cART era have demonstrated a shift in the causes of death and morbidities in HIV-1-infected individuals, with a proportional increase of non-AIDS-related events, such as cardiovascular, liver, and renal diseases, when compared with AIDS-related events [15–20]. Also, a higher prevalence of age-related noninfectious comorbidities was observed in HIV-1-infected individuals than in the general population [21]. Alongside this, a strong association between inflammation and AIDS and non-AIDS-events became evident [22–24]. Several biomarkers of inflammation have been associated with disease progression and mortality in HIV-1 infection [25]. Among these markers, IL-6 and CRP were associated with an increased risk of AIDS [23] or death [22, 24]. IL-6 levels, at time of seroconversion, also predicted HIV-1 disease progression [26]. The markers of monocyte activation sCD14 and sCD163 are associated with a higher risk of death [27–29]. And, during the PHI, CRP levels were significantly higher in HIV-1 infected individuals than in HIV-1 uninfected individuals [5]. Other biomarkers elevated during PHI were IP-10 and IL-18, with an association between IP-10 and a faster disease progression [30, 31]. Continued IL-18 and sCD14 elevation were also associated with clinical cART failure [32].

Although cART reduces immune activation and inflammation [5, 22], these remain higher in HIV-1-infected individuals than in HIV-1-uninfected individuals, even after long-term viral suppression [33]. Studies have shown the benefits of initiation of cART during the acute or early infection [2–4, 6, 7]. The early therapy decreases immune system damage and the establishment of a large viral reservoir [6, 7]. HIV-1 infected patients who started cART during PHI presented an earlier immune reconstitution ($CD4^+$ T cell count > 500 cells/mm^3, CD4% > 30%, and CD4/CD8 ratio > 1) than cART in chronic patients [34]. Moreover, early cART decreases T cell activation [3, 7, 35] and markers of inflammation [3, 5]. Moreover, early ART is associated with a lower risk of development of non-AIDS morbidities [36, 37], and the study with the ANRS VISCONTI cohort demonstrated the possibility of a functional cure in individuals starting ART in primary infection [38].

In the present study, we compared the impact of cART on the levels of immunological markers between primary HIV-1 infected Brazilian individuals, who initiated cART, during the acute (Fiebig I-V) and early chronic phases (Fiebig VI). We evaluated $CD4^+$ T cell counts, CD4/CD8 ratio, $CD4^+$ and $CD8^+$ T cell activation before (Pre-ART) and six (M6 ART) and 12 months (M12 ART) after cART initiation. We also evaluated plasmatic markers of inflammation and monocyte/macrophage activation at Pre-ART and M6 ART and total HIV-1 DNA at M6 and M12. We hypothesized that earlier cART would normalize levels of biomarkers associated with disease progression and death in HIV-1 infection.

Materials and methods

Study population and ethical statement

Study participants were recruited at the Instituto Nacional de Infectologia Evandro Chagas (INI), Rio de Janeiro, Brazil. The inclusion criteria include those who were over 18 years of age and with documented seroconversion within the previous 6 months. Recruitment occurred among those who sought INI or community-based out-of-health care units for HIV testing or care with HIV diagnostic tests suggesting acute HIV infection. Documentation of seroconversion could be the following: (a) a negative result for a third-generation HIV rapid test, followed by reactive HIV Ag/Ab combination assay, or a detectable HIV RNA on pooled HIV RNA testing subsequently confirmed with an individual HIV RNA test; (b) a reactive HIV serology and a documented HIV negative serology within the prior 6 months or a reactive western blot lacking p31 (pol) reactivity. The exclusion criteria were the following: lacking plasma or peripheral blood mononuclear cells (PBMC) on the baseline or M6 ART visits until March, 15th 2016 or less than 10 million cells on the PBMC samples. Of the fifty patients initially included, two did not confirm HIV infection, one abandoned the treatment, one withdrew from the study early, and 23 were excluded, as shown in Fig. 1; thus, the remaining 23 patients were included. The patients were categorized into Fiebig stages, as described in Fiebig et al. [39]. Briefly, Fiebig I was characterized by the presence of HIV-1 RNA in plasma samples (Abbott RealTime

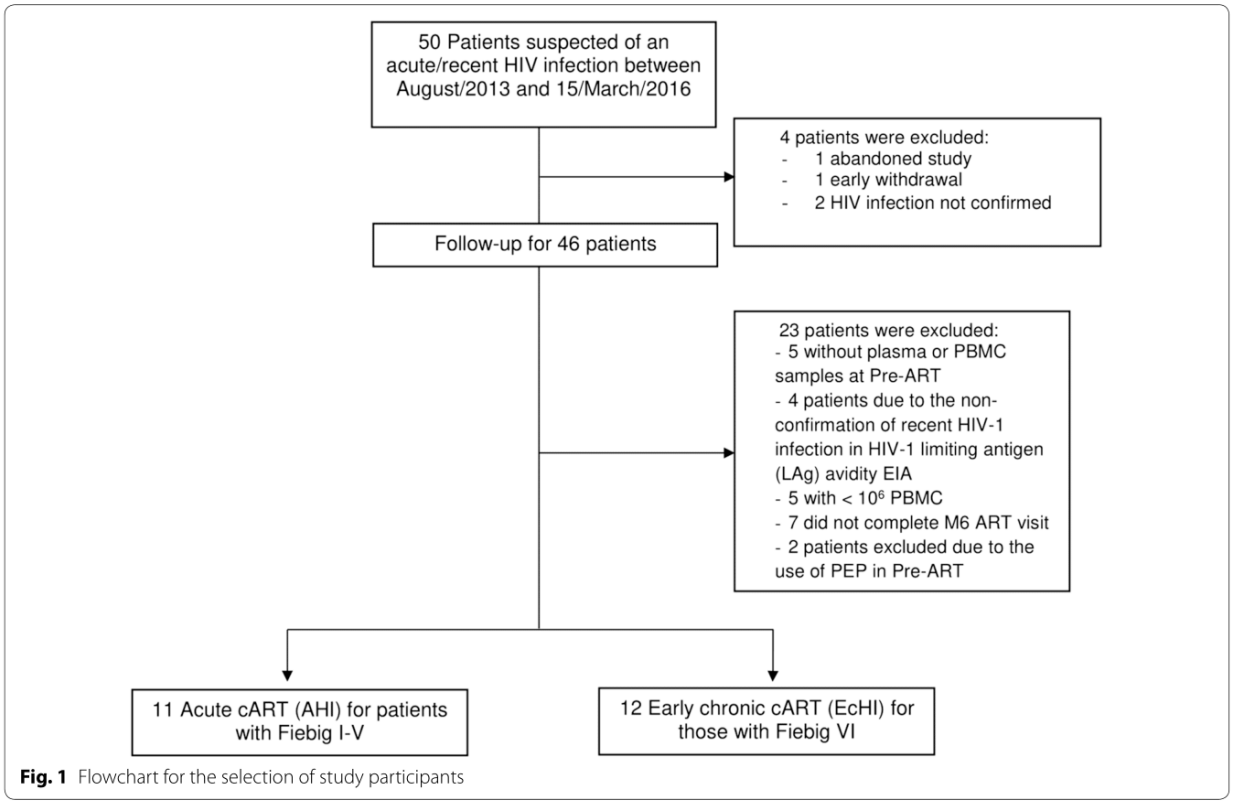

Fig. 1 Flowchart for the selection of study participants

HIV-1) together with a fourth generation ELISA negative (Enzyme Linked Fluorescent Assay BioMérieux HIV DUO Ultra Assay); Fiebig II by a HIV-1 RNA detectable in plasma and the fourth generation ELISA with antigen positive and antibody negative; Fiebig III by a HIV-1 RNA detectable in plasma and a reactive HIV-1 antibody assay (by a 3rd generation assay with detection of IgG and/or IgM anti-HIV-1 or an antibody detection in the 4th generation BioMérieux ELISA HIV DUO Ultra Assay) but a Western Blot (Western Blot Cambridge Biotech HIV-1 negative (defined by the absence of HIV-1 specifc bands); Fiebig IV reactivity profile is identical to that present in the stage III, but with undetermined pattern in Western Blot (presence HIV-1 specific bands, but do not meet the criteria for the interpretation of reactive Western Blot, which is defined by the presence of two of the following three bands: p24, gp41 or gp120/160); Fiebig V reactivity profile is identical to that verified in stage IV, but with a reactive Western Blot result (defined by the presence of two of the following three bands:p24, gp41 or gp120/160); Fiebig VI by reactivity profile is identical to that observed in the stage V, but with the complete WB reactivity pattern, including the p31 band. After, they were divided into two groups: (a) starting cART in acute HIV-1 infection (AHI) for patients with Fiebig I-V (n = 11) and (b) starting cART in early chronic HIV-1 infection (EcHI) for those with Fiebig VI (n = 12). We also included late chronic treated HIV-1 infected patients (n = 18), with more than 5 years of HIV-1 viral load suppression by cART (LcHI), and HIV-1 uninfected individuals (HIV-neg, n = 18). All patients gave informed consent, and the INI Ethical Review Board approved this study.

Sample collection and preparation

Blood samples were collected before cART initiation (Pre-ART), after six (M6 ART) and 12 months (M12 ART) of cART in EDTA and sodium heparin containing tubes. Peripheral blood mononuclear cells (PBMCs) were isolated from whole blood by Histopaque-1077 (Sigma-Aldrich, USA) density gradient centrifugation and kept in liquid nitrogen until use. Plasma aliquots were stored in a −80 °C freezer until use.

HIV-1 limiting antigen (LAg) avidity EIA

To confirm the recent HIV-1 infection status, EDTA-plasma specimens of all patients were subjected to a quantitative limiting antigen (LAg) avidity enzyme immunoassay (Sedia™ HIV-1 LAg-Avidity EIA, Sedia Biosciences Corporation, USA).

$CD4^+$ and $CD8^+$ T cell count and plasma viral load determination

Absolute $CD4^+$ and $CD8^+$ T cell counts and %CD4 were obtained using the MultiTest TruCount-kit and the MultiSet software on a FACSCalibur flow cytometer (BD Biosciences, USA). Plasma HIV-1 viral loads were measured using the Abbott RealTime HIV-1 assay (Abbott Laboratories, Germany). For two AHI patients, data of $CD4^+$ T cell counts were not available for the Pre-ART visit.

Enzyme-Linked Immunosorbent Assay (ELISA)

The plasmatic concentrations of IP-10/CXCL10, IL-18, IL-6, sCD14, sCD163, and CRP were determined in EDTA-plasma samples by ELISA according to the manufacturer's protocol at Pre-ART and M6 ART (R&D Systems, USA). For statistical analyses, points above the detection limit were replaced by the highest value in the assay. To evaluate in more detail the IP-10 and IL-18 levels in the AHI group, we also analyzed samples collected after 1, 2, and 3 months of cART initiation.

Flow cytometry

Cryopreserved PBMCs from preART, M6 ART, and M12 ART were thawed and rested overnight. Then, the PBMCs were stained with anti-CD3 APC-H7, anti-CD4 PECF594, anti-CD8 APC, anti-CD38 PerCPCy5.5 and anti-HLA-DR PE to determine the percentage of activated $CD4^+$ and $CD8^+$ T cells by flow cytometry (all antibodies were purchased from BD Biosciences). The Fixable Viability Stain 450 (FVS 450-BD Biosciences, USA) was used to exclude nonviable cells. Samples were acquired using a BD FACSAria™ IIu flow cytometer (BD Biosciences, USA), and analyses were performed with FlowJo software v.10.0.7 (Tree Star, USA). For three EcHI and one AHI patients, PBMCs from Pre-ART presented viability below the levels accepted for testing.

Quantification of HIV-1 total DNA in PBMCs

Total cellular DNA was extracted from 1×10^7 cryopreserved PBMCs from Pre-ART and M12 ART visits using the QIAamp DNA Mini Kit (Qiagen, Germany). The Generic HIV® DNA Cell Kit (Biocentric, France) was used to quantify the cell-associated HIV-1 DNA, following the manufacturer's recommendations. The lower limit of detection was 40 HIV-1 DNA copies/10^6 cells.

HIV-1 subtyping

All HIV-1 *pol* (PR/RT) sequences were submitted to REGA HIV-1 Subtyping Tool. And them pure HIV-1 subtypes and circulating recombinant forms identified were confirm by Maximum-likelihood (ML) phylogenetic trees that were reconstructed with the PhyML 3.0 program [40] using the most appropriate nucleotide substitution model selected using program jModeltest v. 3.7 [41] and the approximate likelihood-ratio test (aLRT) was used to estimate the confidence of the branching on the tree. For unique recombinant forms we performed the bootscan analysis implemented in Simplot v3.5.1 software with the following parameters: 300nt window, 20nt increments, NJ method under Kimura's two-parameter correction with 100 bootstrap replicates [42].

Statistics

The Kruskal–Wallis test and Fisher's exact test were used for the comparison of continuous variables and categorical variables, respectively, between groups. $CD4^+$ T cell absolute counts and CD4/CD8 ratio, inflammatory and activation markers were analyzed using the Mann–Whitney test to compare variables between two groups and Wilcoxon matched pairs test to compare variables between Pre-ART, M6 ART and M12 ART intragroup. Correlations were performed using the Pearson test or Spearman test according the normality of the data. Linear regression models were used to evaluate factors (age and group) associated with cytokine levels at pre-ART and M6 ART. Statistical analyses were performed using GraphPad v6.0 (Prism Software, USA) and R software.

Results

Characteristics of the study population

Fifty-nine individuals were included in this study, divided into four groups: AHI, EcHI, LcHI, and HIV-neg (Table 1). The groups with PHI (AHI and EcHI) comprised the youngest individuals (median age 30.6 [interquartile range (IQR): 27.7, 39.5] for AHI, 28.3 [IQR 26.7, 29.9] for EcHI), and the LcHI group comprised the oldest individuals (median age 44.2 [IQR 40, 49.8]). We also found a different sex distribution among the groups; the HIV-neg group had 50% women, and the EcHI and AHI groups were composed exclusively men, all men who have sex with men (MSM). The HIV-1 viral load of the AHI group was significantly higher than that of the EcHI group at Pre-ART (median of 5.9 log [IQR 4.8, 6.5] in AHI, 4 log [IQR 3.7, 4.9] in EcHI and 1.6 log [IQR 1.6–1.6] in LcHI). After 6 months of cART, most individuals with PHI had undetectable viral load, except two EcHI (VL = 94 and 96 copies/mL) and one AHI (VL = 280 copies/mL). At 12 months of cART, all PHI subjects had an undetectable viral load, except one that has suppressed on M6 and presented a blip of 460 copies/mL at M12. The $CD4^+$ T cell counts were higher in the HIV-neg group (median 831 cell/mm^3 [IQR 757.2, 1129.5]) and the LcHI group (median 853 cell/mm^3 [IQR 762, 983]) and lower in the EcHI group (median 566 cell/mm^3 [IQR 389, 666.5]) and the AHI group (median 634 cell/mm^3 [IQR 484.2, 885.5]). To EcHI and AHI individuals the HIV-1

Table 1 Demographic and clinical characteristics of study participants

	HIV-neg (n = 18)	LcHI (n = 18)	EcHI (n = 11)	AHI (n = 12)	Total (n = 59)	*P* value
Age						0.001
Median (IQR)	37.1 (31.6; 49.3)	44.2 (40;49.8)	28.3 (26.7;29.9)	30.6 (27.7;39.5)	34.5 (27.9;46.5)	
Gender (%)						< 0.001
Female	9 (50)	7 (38.9)	0 (0)	0 (0)	16 (27.1)	
Male	9 (50)	11 (61.1)	11 (100)	12 (100)	43 (72.9)	
HIV-1 viral load						< 0.001
Log_{10}, median (IQR)	NA	1.6 (1.6;1.6)	4 (3.7;4.9)	5.9 (4.8;6.5)	3.5 (1.6;4.9)	
CD4 + T cell count						0.001
Cells/mm^3, median (IQR)	831 (757.2;1129.5)	853 (762;983)	566 (389;666.5)	634 (484.2;885.5)	784 (648;909)	

Analyses with age, HIV-1 viral load and $CD4^+$ T cell count were performed using the Kruskal–Wallis

To EcHI and AHI we analyzed the pre-ART visit and for LcHI a pos-cART visit (median of 5 years after cART initiation)

Analyses with gender were performed using Fisher's exact test

LcHI late chronic HIV-1 infection; *EcHI* early chronic HIV-1 infection; *AHI* acute HIV-1 infection; *NA* not applicable; *IQR* interquartile range

subtypying was performed and the subtype B was the prevalent (Additional file 1: Table S1).

The avidity enzyme immunoassay results confirmed all AHI and EcHI individuals included in the study as recently HIV-1 infected, considering the window of 130 days established for this test.

cART initiation during acute infection can normalize $CD4^+$ T cells

We compared the $CD4^+$ T cell absolute counts and the CD4/CD8 ratio between individuals who started cART in Fiebig I-V (AHI) and Fiebig VI stages (EcHI) with HIV-neg individuals (Fig. 2). The AHI group had $CD4^+$ T cell counts (median = 838 cells/μL; IQR = 610–971) similar to the HIV-neg group (median = 831 cells/μL, IQR = 742–1227) 6 months after cART initiation, while the EcHI group, even after 12 months of cART (CD4 median = 670 cells/μL, IQR = 582–926), did not normalize $CD4^+$ T cell counts ($P = 0.0139$) compared with the HIV-neg group (Fig. 2a). However, when we analyzed the CD4/CD8 ratio (Fig. 2b), both groups with recent HIV-1 infection presented lower CD4/CD8 ratios than the

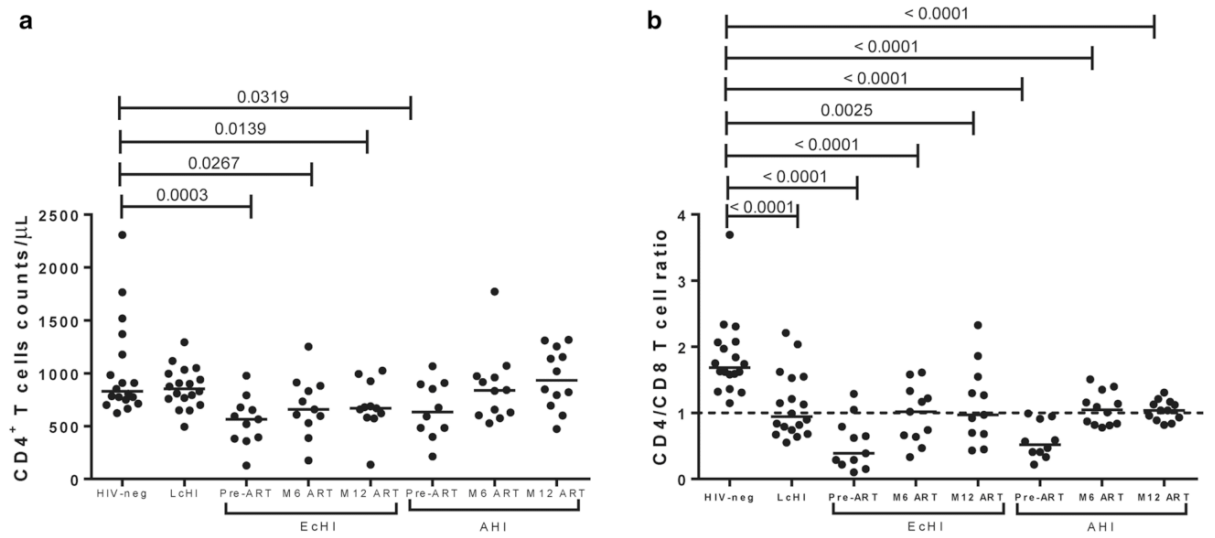

Fig. 2 $CD4^+$ T cell count and CD4/CD8 ratio on pre-ART and after six months. $CD4^+$ T cell counts (**a**) and CD4/CD8 (**b**) ratios were evaluated using the MultiTest TruCount kit. *P* values were calculated using the Mann–Whitney test. All values < 0.05 were considered statistically significant. HIV-neg, HIV-1 uninfected individuals; LcHI, late chronic treated HIV-1 infection (median of 5 years after cART initiation); EcHI, early chronic HIV-1 infection; AHI, acute HIV-1 infection; M6 ART, six months after ART start; M12 ART, 12 months after ART start

HIV-neg group after 6 and 12 months of cART. Moreover, we included a group of individuals who started cART in the late chronic phase (LcHI) and, despite a long time in cART with viral suppression and $CD4^+$ T cell counts similar to the HIV-neg group, this group also presented a lower CD4/CD8 ratio than HIV-neg.

IP-10 and IL-18 levels reduced after 6 months of cART in acute individuals

We evaluated six plasmatic markers of inflammation (IP-10/CXCL10, IL-18, IL-6, sCD14, sCD163, and CRP) before cART and 6 months after cART initiation (Fig. 3 and Additional file 1: Figure S1). These markers were chosen based in the following criteria: having a pro-inflammatory action, elevated levels in acute or chronic HIV-1 infection or being associated to AIDS progression or mortality [22, 25, 26, 28, 30, 31, 43]. IP-10 and IL-18 ($P<0.0001$ for EcHI and AHI) and sCD163 ($P=0.0049$ for EcHI and $P=0.0019$ for AHI) were higher at pre-ART in both groups of PHI individuals than in the HIV-1 uninfected group. After 6 months of cART, IP-10 levels were significantly reduced in EcHI ($P=0.0351$) and AHI ($P=0.0049$) groups when compared with the pre-ART levels. However, only the AHI group reached similar levels to those presented by the HIV-neg group. Moreover, IP-10 was higher in the LcHI group ($P<0.0001$) than the HIV-neg group (Fig. 3a). In the AHI group, cART was also able to reduce significantly ($P=0.0005$) the IL-18 levels 6 months after cART initiation compared with pre-ART levels and reached similar levels to those observed in HIV-1 negative individuals (Fig. 3b). Despite the significant reduction of sCD163 levels after 6 months of cART in the EcHI group ($P=0.0186$), but not in AHI,

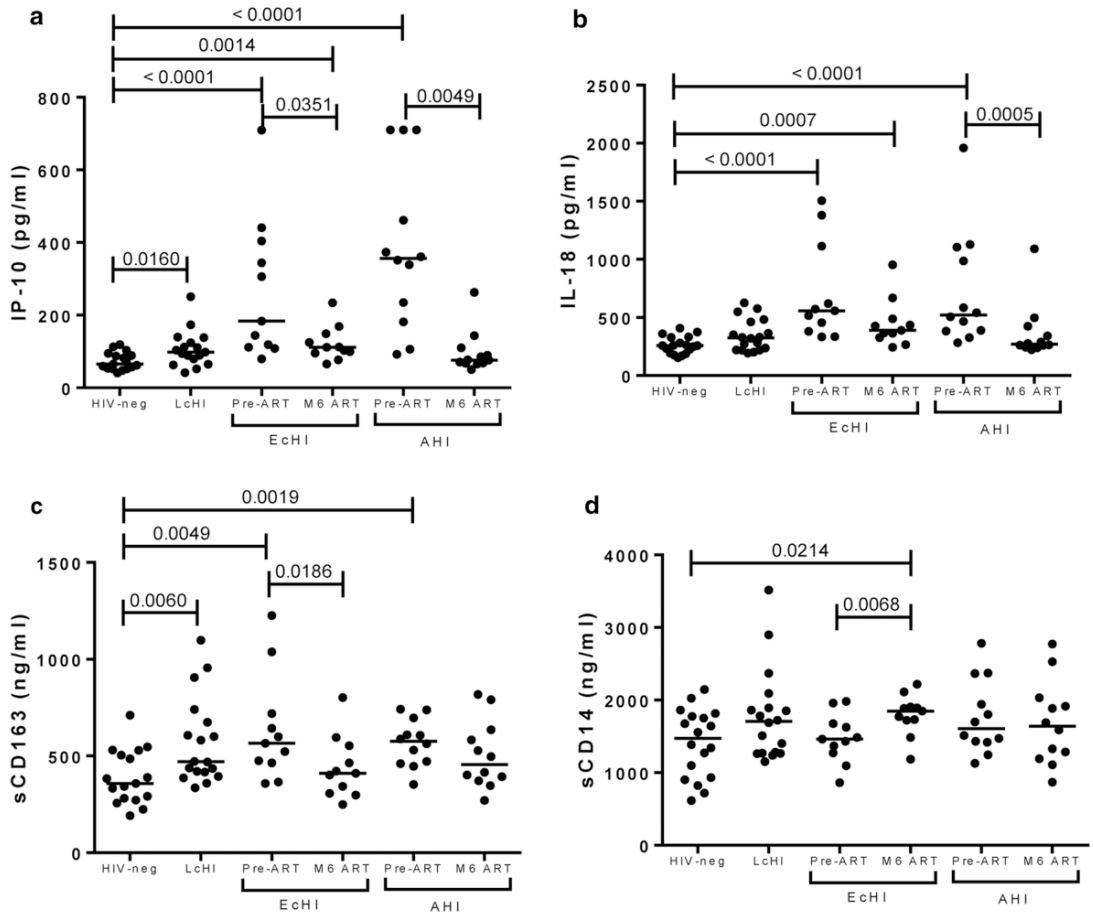

Fig. 3 Plasmatic markers of inflammation on pre-ART and after 6 months. IP-10 (**a**), IL-18 (**b**), sCD163 (**c**), and sCD14 (**d**) were assessed by ELISA. *P* values were calculated using the Mann–Whitney test. All values < 0.05 were considered statistically significant. HIV-neg, HIV-1 uninfected individuals; LcHI, late chronic treated HIV-1 infection (median of 5 years after cART initiation); EcHI, early chronic HIV-1 infection; AHI, acute HIV-1 infection; M6 ART, 6 months after ART start; and M12 ART, 12 months after ART start

compared to the pre-ART levels, both groups presented levels similar to the HIV-1-uninfected group at this visit. This marker was higher in the LcHI group than in the HIV-neg group ($P=0.0060$) (Fig. 3c). We observed higher sCD14 levels in the EcHI group after 6 months of cART than in the HIV-neg group ($P=0.0214$) and the pre-ART point of the group ($P=0.0068$) (Fig. 3d). No significant differences were observed in CRP and IL-6 among the studied groups (Additional file 1: Figure S1). We also evaluated IP-10 and IL-18 levels after 1, 2 and 3 months of cART initiation in the AHI group and observed that after 2 months, IP-10 and IL-18 reached levels that were not different from HIV-neg individuals (Additional file 1: Figure S2).

In analyses adjusted by age and group, at the pre-ART visit, the levels of IP-10 ($P<0.0001$ for EcHI and AHI), IL-18 ($P=0.0005$ for EcHI and $P<0.0001$ for AHI) and sCD163 ($P=0.0001$ for EcHI and $P=0.0056$ for AHI) remained higher than those in the HIV-1-neg group. After adjustment, we found a slightly higher level of IL-6 in the AHI group than the HIV-1-neg group at the Pre-ART visit ($P=0.0418$).

CD8$^+$ and CD4$^+$ T cell activation reach levels that are comparable with HIV-1 uninfected individuals after 12 months of cART initiation during primary infection

We observed a progressive decrease in CD8$^+$ T cell activation after cART initiation in both groups of individuals recently infected with HIV-1 (Fig. 4a). A significant decline in the frequency of activated CD8$^+$ T cells was observed comparing pre-ART and M6 ART visits in both groups. However, CD8$^+$ T cell activation was similar to the HIV-neg group only after 12 months of cART. The CD4$^+$ T cell activation, as observed to CD8$^+$ T cells, only reached similar values to the observed in HIV-1-uninfected individuals after 12 months of cART. In the AHI group, we did not observe a decrease at M6, but after 12 months after cART the levels of CD4$^+$ T cell activation were similar to the observed in HIV-neg group. In the analyses adjusted by age and group at the pre-ART visit, the frequency of activated CD8$^+$ T cells remained higher than the HIV-neg group for both the EcHI ($P=0.0264$) and AHI ($P<0.0001$) groups, data not shown.

IP-10 correlates with HIV-1 viral load at the pre-ART visit

Among the PHI individuals (AHI and EcHI groups), viral loads showed a strong positive correlation with IP-10 at pre-ART visit ($P=0.0006$ and $r=0.6610$) (Fig. 5). In the adjusted analysis, this correlation remained significant ($P=0.0019$), and we found a significant association between sCD14 and viral load ($P=0.0012$) after age adjustment (Additional file 1: Table S2). No correlation was found between IL-18 or sCD163 and viral load among the PHI individuals. We also evaluated the total HIV-1 DNA in PBMCs of AHI individuals at the pre-ART visit and found no correlation with T cell activation or markers of inflammation.

CD4/CD8 ratio correlates with inflammatory and activation markers

CD4/CD8 ratio have been pointed as a more accurate marker of immune dysfunction than absolute CD4$^+$ T cell count [44]. Negative correlations between CD4/CD8 ratio and IP-10 ($P=0.0112$ and $r=-0.5416$), IL-18

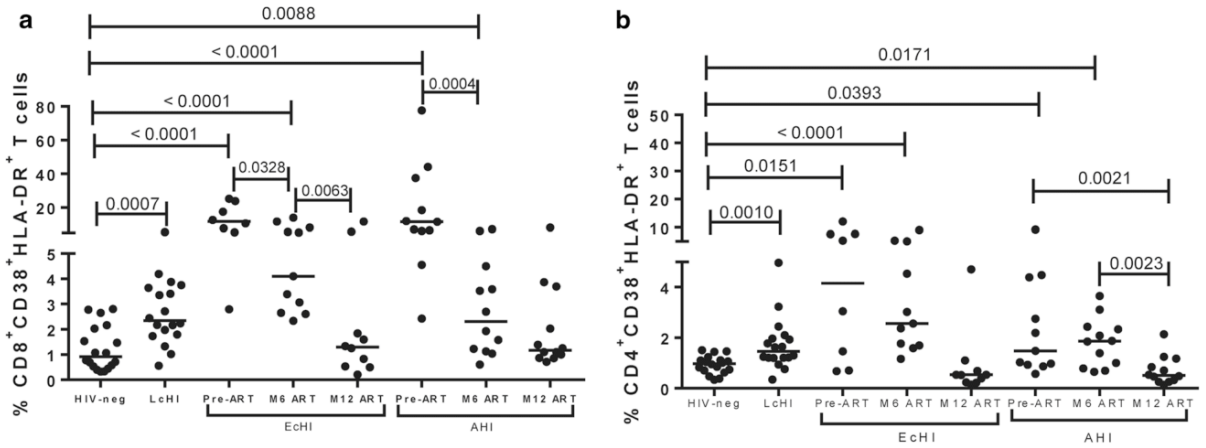

Fig. 4 T cell activation on pre-ART and 6 months after. CD4$^+$ (**a**) and CD8$^+$ (**b**) T cell activation were measured by flow cytometry. *P* values were calculated using the Mann–Whitney test. All values < 0.05 were considered statistically significant. HIV-neg, HIV-1 uninfected individuals; LcHI, late treated chronic HIV-1 infection (median of 5 years after cART initiation); EcHI, early chronic HIV-1 infection; AHI, acute HIV-1 infection; M6 ART, 6 months after ART start; and M12 ART, 12 months after ART start

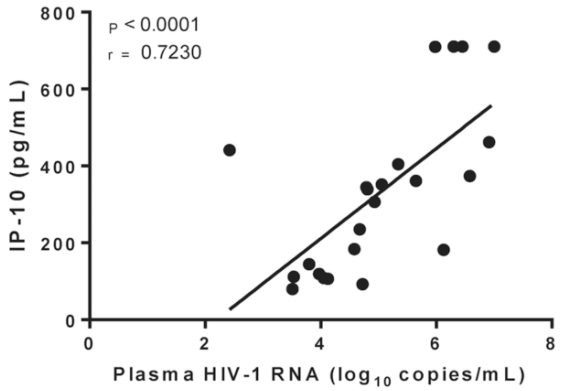

Fig. 5 Correlation between IP-10 and HIV-1 plasmatic viral load. P and r values were calculated using Pearson correlation test. All values < 0.05 were considered as statistically significant

($P=0.0068$ and $r=-0.5713$) and $CD8^+$ T cell activation ($P=0.0124$ and $r=-0.6000$) were also observed (Additional file 1: Fig S3). No correlation with sCD14, sCD163, IL-6 or CRP with CD4/CD8 ratio was observed.

Discussion

This is the first study evaluating inflammation and T cell activation in Brazilian recent HIV-1 infected individuals. In this longitudinal study, we found that cART initiation in the HIV-1 acute infection had a higher impact on inflammation reduction and $CD4^+$ T cell recovery than cART initiation in early chronic phase. IP-10, IL-18, sCD163, and $CD8^+$ T cell activation were elevated in early infected individuals from a Rio de Janeiro, Brazil when compared with the HIV-neg group. However, among the inflammatory markers, only IP-10 presented a positive correlation with the HIV-1 viral load.

There is clear evidence of the benefits of the early initiation of cART [2–7], and the WHO currently recommends the immediate cART initiation in individuals with a positive HIV-1 diagnosis, which is a recommendation followed by Brazil since 2013 [45]. The primary goals of this strategy are the following: to preserve the immune system, to avoid the early development of AIDS and non-AIDS related diseases and to reduce HIV-1 transmission. Although the $CD4^+$ T cell count is no longer a criteria to define the moment of cART initiation, it is still a valuable marker of immune reconstitution in individuals starting cART and is also routinely used in clinical follow-up in settings were viral load monitoring is poor. Here, we observed an earlier normalization of $CD4^+$ T cell counts in the group that started cART during the Fiebigs I-V acute phase (AHI). After 12 months of cART, the group that started cART only in the early chronic phase remained with lower $CD4^+$ T cell counts than the HIV-neg group. Studies have explored the CD4/CD8 ratio as a marker of persistent immune activation and inflammation, even after long-term suppressive cART [46–48]. A large study conducted with more than 400 MSM showed that 56% inverted the CD4/CD8 ratio around 3 months after seroconversion [49]. In contrast with the normalization of $CD4^+$ T cell counts in the AHI group after 6 months of cART, we observed a lower CD4/CD8 ratio in both the AHI and EcHI groups than in the HIV-neg group even at 12 months of cART. A previous study demonstrated that an altered CD4/CD8 ratio remains after 2 years of ART in individuals who started ART in acute infection, even during the Fiebig I stage [4]. In our study, the inverted CD4/CD8 ratio is mainly explained by the high $CD8^+$ T cell counts (data not shown). Once cART suppressed viral loads after 6 months of treatment, it is probably that other factors, possibly the persistent inflammation and immune activation, than only the viral loads, affect the $CD8^+$ T cell compartment. Indeed, we found negative correlations between the CD4/CD8 ratio and IP-10, IL-18, and $CD8^+$ T cell activation. Microbial translocation also correlated with $CD8^+$ T cell counts [50], and in our study, we found no higher levels of sCD14, a monocyte activation marker also used as a microbial translocation marker [51], in both AHI and EcHI groups when compared to the HIV-neg group at the pre-ART visit. An unexpected increase in sCD14 at M6 was observed in EcHI group, which could be explained by the later cART initiation in this group compared with AHI, allowing a more extensive damage in the gut mucosa. In contrast, we did not observe a higher level of this marker in LcHI group, composed by individuals with more than 5 years in cART, compared to HIV-neg group, this could indicate that a longer time of cART is necessary to decrease sCD14 to similar levels to the observed in HIV-neg individuals. A previous study [5] demonstrated that sCD14 levels remained higher in individuals treated during acute HIV infection than in HIV-uninfected individuals after 2 years, even though this group, after 12 months of treatment, presented lower sCD14 levels compared to individuals who started cART in the chronic phase.

Early cART initiation reduces inflammation and immune activation [3, 4, 6, 7]. IP-10 was one of the markers of inflammation elevated in a pre-ART visit in AHI and EcHI individuals, significantly reducing after 6 months of cART in both groups, although EcHI individuals did not achieve normal values. This marker was associated with an increased risk of HIV-1 acquisition, faster disease progression, and high plasmatic viral loads [31, 52]. IP-10 binding CXCR3 and $CD4^+CXCR3^+$ T cells are the main targets of HIV-1 infection. Thus, it is likely

that a high level of IP-10 contributes to the elevated viral replication by attracting target cells that subsequently induces further IP-10 production [53]. After 2 months of treatment the levels of IP-10 in AHI group reached similar levels to the observed in HIV-1 neg-group. At this time, most individuals presented undetectable or very low (< 100 copies/mL) viral load, except for one individual (1311 copies/mL), who presented pre-ART viral load > 10 million copies/mL. We also found a high level of IL-18 at the pre-ART visit in this group. After 2 months of cART, these levels in the AHI group were similar to that observed in the HIV-neg group, following the same dynamics observed for IP-10. IL-18 is produced in response to inflammasome activation, as reviewed by Dinarello et al. 2013 [54]. The inflammasome is activated in response to several stimuli, including LPS, and HIV-1 can activate inflammasome [55, 56]. The comparison of our results of IP-10 and IL18 levels with other studies is limited by variations in assay and characteristics of study population, as age and coinfections. However, in a general way, we founded studies which showed higher [3, 6, 30, 57], lower [43], and similar [31] values of IP-10, and a similar level of IL-18 when compared with a French study [30].

Studies have demonstrated a decrease in T cell activation, mainly $CD8^+$ T cell activation, after cART initiation during the acute phase of HIV-1 infection [3, 35]. Here, we observed an earlier decrease in markers of inflammation than in T cell activation. However, we detected an earlier normalization of $CD8^+$ T cell activation after cART initiation than that reported by two previous studies, which demonstrated higher levels of this marker, even after 12 [7] or 24 months [35], than those observed in HIV-1-uninfected individuals. In both studies, the median of $CD8^+$ T cell activation was higher than that observed here, which could explain the longer time required for $CD8^+$ T cell activation normalization. Moreover, as observed to markers of inflammation, other factors, as coinfections, may also explain the differences.

The HIV-1 subtype B was the prevalent in our study groups of recent infected individuals, followed by B/C and B/F recombinants. When we compared HIV-1 subtype B versus non-B individuals, we founded no differences in inflammation or T cell activation markers. One individual from EcHI group was infected by HIV-1 subtype D, which have been associated with a faster disease progression [58]. However, this individual presented viral load and $CD4^+$ T cell count comparable with group median, and the same was observed to markers of inflammation and T cell activation.

Our study has some limitations that should be highlighted. The first limitation is the small size of our cohort. Indeed, the identification of individuals in the acute or early phase of HIV-1 infection is a major challenge. Second, our groups of recently infected individuals were composed exclusively by MSM, different from the LcHI and HIV-neg groups. Moreover, we only quantified the total HIV-1 DNA levels, with no differentiation between 2-LTR and integrated DNA and no functional assay was performed to test the size of the competent reservoir. A longer follow-up certainly will help to achieve a better understanding of the dynamics of immune activation, inflammation, and immune reconstitution after the early cART initiation.

Alltogether, our results reinforce that early cART initiation during HIV infection can reduce systemic inflammation, but the earlier normalization of the markers related to this phenomenon was only observed when cART is initiated in the acute phase. We suggest that IP-10 was the best marker to evaluate inflammation on recent HIV-1 infection, once it was elevated at pre-ART visit and showed a positive correlation with plasmatic viral load. $CD8^+$ T cell activation presented a different reduction dynamic when compared to IP-10 and IL-18, and it only reached normal levels one year after cART initiation.

Additional file

Additional file 1: Figure S1. Plasmatic markers of inflammation. CRP (**a**) and IL-6 (**b**) were measured by ELISA. *P* values were calculated using the Mann–Whitney test. All values < 0.05 were considered statistically significant. HIV-neg, HIV-1 uninfected individuals; LcHI, late chronic HIV-1 infection; EcHI, early chronic HIV-1 infection; AHI, acute HIV-1 infection; M6 ART, 6 months after cART start after cART start. **Figure S2.** Decreasing dynamics of inflammatory markers among acute treated HIV infected individuals. IP-10 (**a**) and IL-18 (**b**) were measured by ELISA at different time points after cART start. *P* value were calculated using the Mann–Whitney test. All values < 0.05 were considered statistically significant. HIV-neg, HIV-1 uninfected individuals. **Figure S3.** Correlations between markers of inflammation activation with CD4/CD8 ratio at pre-ART visit. IP-10 and CD4/CD8 ratio (**a**), IL-18 and HIV-1 CD4/CD8 ratio (**b**), CD8 activation and CD4/CD8 ratio (**c**). *P* and *r* values were calculated using the Spearman test. All values < 0.05 were considered statistically significant. **Table S1.** HIV-1 subtypes. **Table S2.** Age adjusted analyzes of the association between sCD14 levels and HIV-1 viral load at pre-ART visit.

Authors' contributions

MLG and FHC conceptualized and designed the study; FHC, MGM, and MLG contributed to the experimental design and provided intellectual input. FHC and DGC performed the sample processing, provided the clinical data, and provided intellectual input. FHC, HHSP, ED, and SLMT performed the experiments. BG, SWC, VG, EGJ, MMA and ACGF included patients and provided the clinical data. HHSP, FHC, ACGF, and LEC analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

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

Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Consent for publication
Not applicable.

Ethics approval and consent to participate
All participants gave informed consent, and the INI Ethical Review Board approved this study.

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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

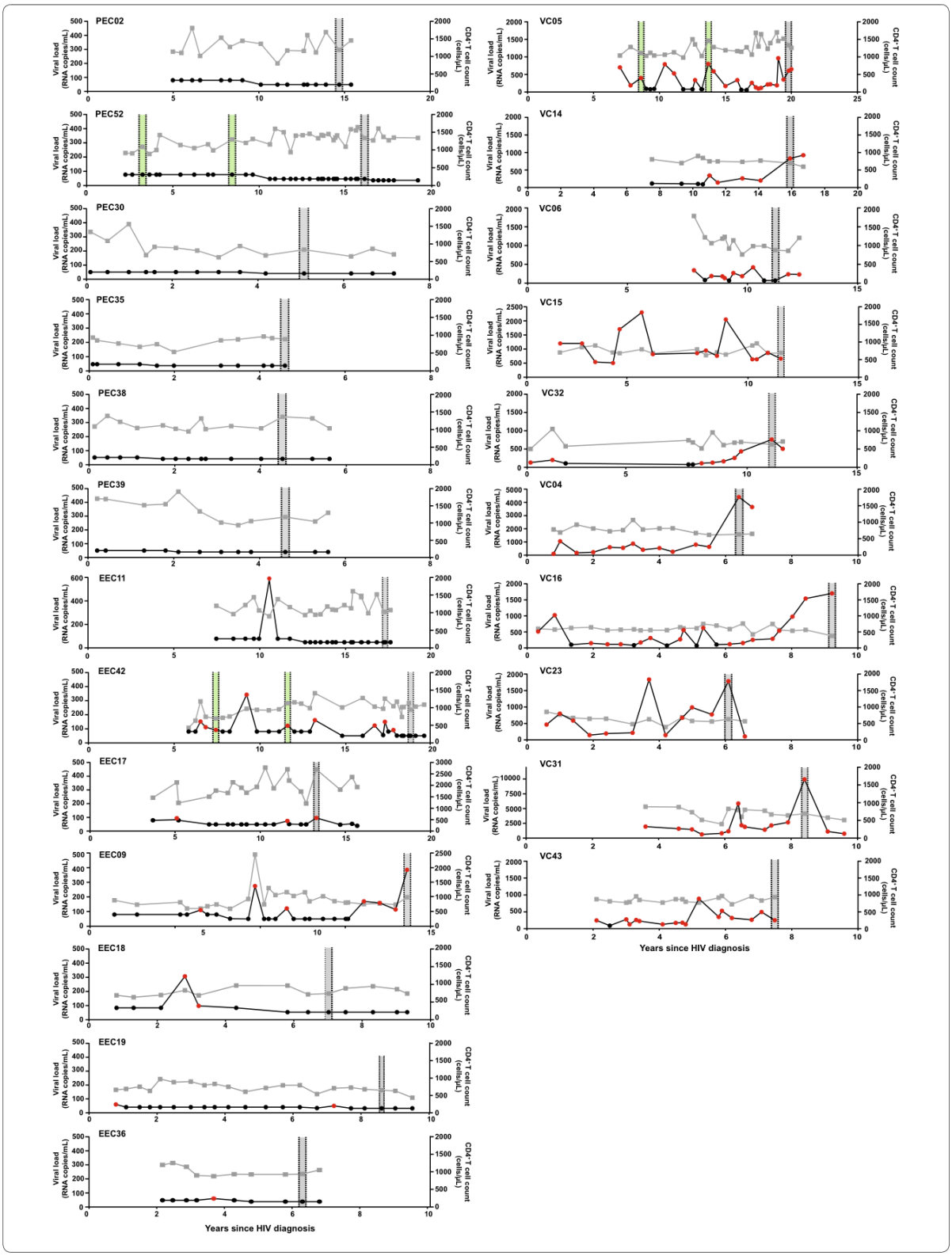

PEC02
PEC52
PEC30
PEC35
PEC38
PEC39
EEC11
EEC42
EEC17
EEC09
EEC18
EEC19
EEC36
VC05
VC14
VC06
VC15
VC32
VC04
VC16
VC23
VC31
VC43
Viral load (RNA copies/mL)
CD4+T cell count (cells/μL)
Years since HIV diagnosis

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

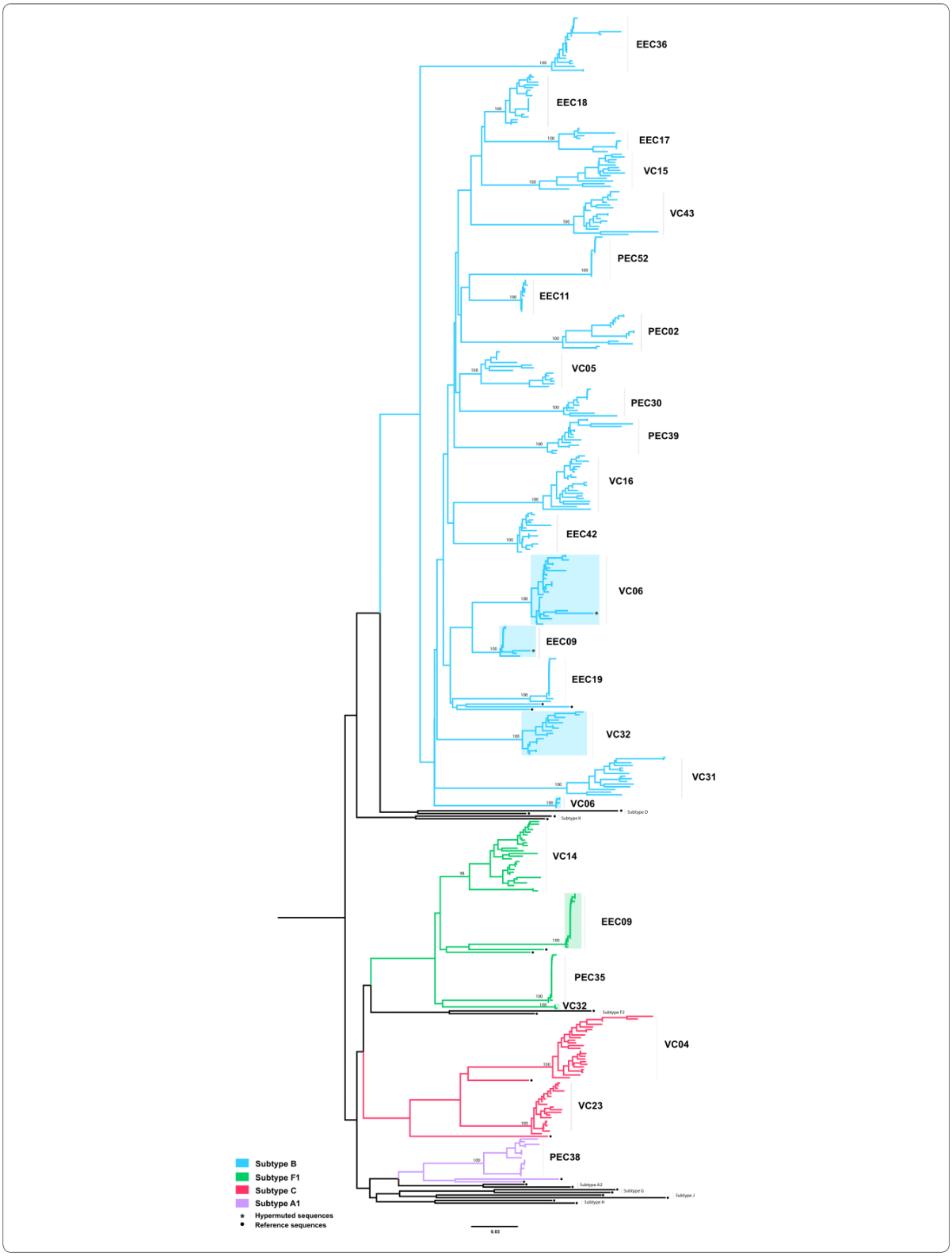
dual infection. The subject VC06 was double infected by two HIV-1 subtype B variants, while individual EEC09 and VC32 were double infected by HIV-1 subtypes B and F1 variants. A second sample from these three individuals was analyzed confirming the previous result (data not shown). Overall, subtype B (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
PEC38
Subtype B
Subtype F1
Subtype C
Subtype A1
Hypermuted sequences
Reference sequences
0.03

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

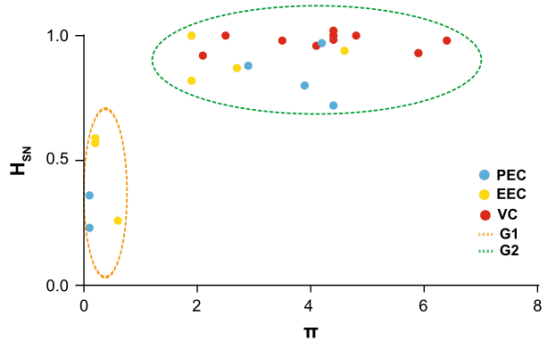


Fig. 4 Identification of divergent patterns of intra-host viral diversity among EC subjects. The normalized Shannon entropy (H_{SN}, *y* axis) of each subject's proviral *env* quasispecies was plotted against the corresponding mean nucleotide diversity (π, *x* axis). The colors of the *circles* represent 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)

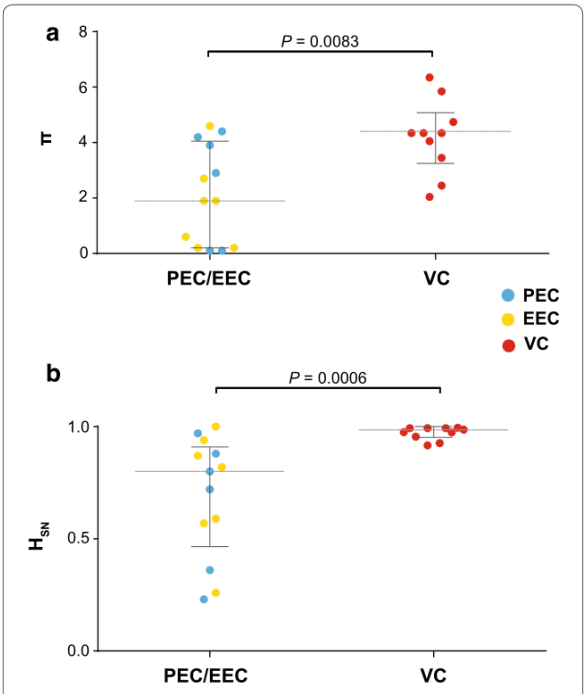


Fig. 3 Mean nucleotide diversity (π, **a**) and normalized Shannon entropy (H_{SN}, **b**) of proviral *env* quasispecies from EC and VC. The colors of the *circles* represent the different levels of systemic viral suppression in HIV-1 controllers as indicated in the legend. *Dotted* and continuous *gray 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

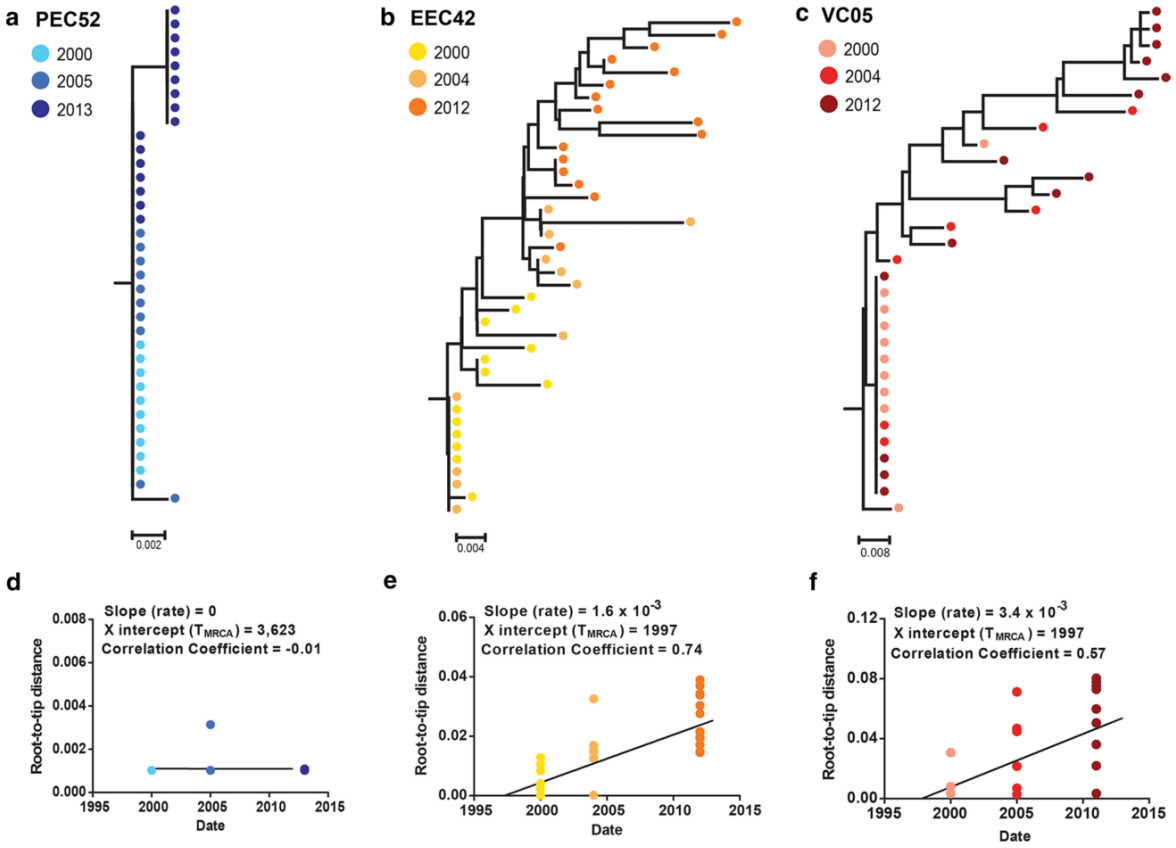


Fig. 5 Longitudinal analysis of HIV-1 proviral *env* sequences obtained from subjects PEC52 (**a**, **d**), EEC42 (**b**, **e**) and VC05 (**c**, **f**) between 2000–2013, 2000–2012 and 2000–2011, respectively. **a**–**c** ML phylogenetic trees for each individual are shown, in which horizontal branch 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 ($\pi = 4\text{-}8\%$) 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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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.

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