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Identification of Th17-Polarized CD4+ T-Cells as Key Players in HIV-1 Pathogenesis and Novel Targets for Cure/Remission Interventions

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Identification des celules T CD4+ à profil de polarisation Th17 comme des acteurs clés dans la pathogénèse de l'infection à VIH-1 et des cibles pour des nouvelles interventions thérapeutiques vers l'éradication ou la cure

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Résumé

La découverte du virus de l'immunodéficience humaine de type 1 (VIH-1) en 1983 a été suivie d'avancées majeures dans la compréhension de la pathogenèse de la maladie et la caractérisation moléculaire du cycle de réplication virale. Ces connaissances ont permis la conception et l'implantation de thérapies antirétrovirales (TAR) capables de contrôler efficacement la réplication virale à des niveaux plasmatiques indétectables, réduisant ainsi fortement le risque de transmission et transformant l'épidémie mortelle en une maladie chronique gérable. Malgré ces progrès, l'infection par le VIH-1 demeure un problème de santé important au niveau international. Le principal obstacle à la guérison du VIH-1 comprend la persistance de réservoirs viraux dans les cellules immunitaires à longue durée de vie, nécessitant ainsi un traitement à vie chez les personnes vivant avec le VIH (PVVIH).

Au cours de l'infection par le VIH, une déplétion progressive des lymphocytes T CD4+ est observée. Une fraction de ces cellules retrouvée majoritairement aux muqueuses sous l'appellation Th17 représente la première cible de l'infection par le VIH, ce qui mène à leur déplétion dans le tractus gastro-intestinal. Les cellules Th17 sont essentielles au maintien de l'homéostasie intestinale; par conséquent, l'infection par le VIH provoque des altérations majeures de l'immunité intestinale. Malgré des stratégies thérapeutiques efficaces capables de supprimer la réplication du VIH, les cellules Th17 ne sont pas reconstituées dans l'intestin, et ce, même lors d'une initiation précoce de la TAR. La perte des cellules Th17 dans l'intestin des PVVIH entraîne une translocation microbienne et une inflammation systémique et, de ce fait, peut contribuer à des comorbidités non liées au SIDA chez des individus virologiquement supprimés. Malgré leur appauvrissement, il a été démontré que des sous-ensembles spécifiques de cellules Th17 à longue durée de vie supportent un réservoir viral chez les PVVIH sous TAR. En effet, nous avons montré qu'une sous-population de cellules CCR6+ Th17 infiltrant le côlon est un réservoir de VIH enrichi chez les PVVIH sous TAR. Cette permissivité à l'infection et cette contribution à la persistance virale appuient l'hypothèse selon laquelle les cellules Th17 jouent un rôle fondamental dans l'immunopathologie du VIH.

Afin de mieux comprendre l'interrelation entre ces cellules immunitaires et le VIH, dans la première partie de cette thèse, je me suis intéressé à déterminer l'effet de l'IL-17A, la cytokine clé de ce compartiment cellulaire, sur la capacité des cellules épithéliales intestinales à favoriser l'infection et la réactivation du réservoir viral. Nous avons observé que l'IL-17A agit en synergie avec le TNF pour favoriser la production de CCL20, une chimiokine dont le récepteur, CCR6, est fortement exprimé par les cellules Th17. Cette activité synergique promeut également la trans-infection par le VIH des cellules T CD4 + et l'expansion virale dans les cellules de PVVIH sous TAR. Par ailleurs, l'IL-17A médie une signature moléculaire pro-inflammatoire et provirale caractérisée par une diminution de l'expression des facteurs de restriction du VIH induits par l'interféron de type I. Ces résultats soutiennent que, malgré le rôle bénéfique de l'IL-17A sur l'homéostasie muqueuse, cette cytokine peut contribuer à la dissémination et à la persistance du VIH.

Puisque le développement et la différenciation des cellules Th17 dépendent fondamentalement de l'expression du facteur de transcription RORC2, dans la deuxième partie de cette thèse, nous évaluons l'Implication de RORC2 comme facteur de dépendance de la réplication du VIH et de la réactivation virale. Nous avons constaté que RORC2 joue un rôle majeur lors de l'infection par le VIH et que son inhibition pharmacologique à l'aide de petites molécules qui se lient au domaine de liaison du ligand RORC2 réduit la réplication du VIH dans la lignée cellulaire Jukart et dans les cellules primaires T CD4 infectées in vitro. De plus, l'interférence génétique de l'expression de RORC2 prévient la réplication du VIH, tandis que sa surexpression a l'effet opposé. Dans les cellules de PVVIH traitées, il a été constaté que les lymphocytes T CD4 exprimant RORC2 étaient surreprésentés dans le réservoir viral par rapport aux lymphocytes T CD4 RORC2-. Tout comme dans les cellules infectées in vitro, l'inhibition pharmacologique de RORC2 dans les lymphocytes T CD4 de ces participants bloque l'excroissance du VIH. Ces résultats suggèrent que ce régulateur transcriptionnel central aux cellules Th17 représente une cible à ce compartiment cellulaire pour le traitement du VIH.

Enfin, la déplétion des cellules Th17 au niveau de la muqueuse intestinale est un moteur crucial de la translocation microbienne et de l'activation immunitaire chronique. Cette activation

immunitaire est d'ailleurs associé à la survenue de comorbidités non liées au SIDA. Ainsi, dans la troisième partie de la thèse, nous avons cherché à identifier une signature immunologique liée à l'athérosclérose subclinique chez les PVVIH sous TAR. Nous avons constaté que les PVVIH atteintes d'athérosclérose subclinique présentaient une augmentation des taux plasmatiques de fibrinogène, une réduction de la fréquence des Th17 et des rapports Th17/Treg, ainsi qu'une augmentation de la fréquence des monocytes non classiques à phénotype CCR9low HLADRhigh.

En conclusion, les travaux de cette thèse ont permis d'approfondir les connaissances sur l'importance des cellules Th17 au cours de l'évolution de l'infection par le VIH, notamment dans l'établissement de comorbidités non liées au SIDA, telles que les maladies cardiovasculaires. Ces nouveaux résultats se montrent essentiels dans la considération des cellules Th17 comme une nouvelle cible potentielle d'interventions thérapeutiques pour la rémission et la guérison du VIH.

Mots-clés : VIH-1, cellules T CD4+ à profil de polarisation Th17, IL-17A, RORC2, cellules epithéliales intestinales, immunologie muqueuse, maladie cardiovasculaire.

Abstract

The discovery of human immunodeficiency virus type 1 (HIV-1) in 1983 was followed by major advances in understanding disease pathogenesis and the elucidation of the viral replication cycle at the molecular level. This knowledge allowed the design and implementation of antiretroviral therapy (ART) that efficiently controls viral replication at undetectable plasma levels, thus robustly reducing the risk of transmission and transforming the deadly epidemic into a manageable chronic disease. Despite these advances, HIV-1 infection remains a significant health issue at the international level. The major barrier to HIV-1 cure is the persistence of viral reservoirs in long-lived immune cells, therefore requiring life-long treatment in people living with HIV (PLWH).

During HIV infection, there is a progressive depletion of CD4+ T-cells. A subset of mucosal CD4+ T-cells denominated Th17 cells are the first targets of HIV infection and are depleted in the gastrointestinal tract. Th17 cells are critical for maintaining intestinal homeostasis; therefore, HIV infection causes major disruptions of intestinal immunity. Despite effective ART regimens able to suppress HIV replication, Th17 cells are not replenished in the intestine, not even when ART is initiated during the early phases of acute infection. The depletion of Th17 cells in the gut of PLWH leads to microbial translocation and systemic inflammation and, therefore, may contribute to non-AIDS co-morbidities in fully virological suppressed subjects. Despite their depletion, specific subsets of long-lived Th17 cells were demonstrated to carry viral reservoirs in ART-treated PLWH. Previous studies by our group and others have shown that CD4+ T-cells expressing the Th17 marker CCR6 are enriched in HIV reservoirs in the blood and the colon of ART-treated PLWH. This evidence supports the hypothesis that Th17 features play a pivotal role in HIV immunopathology and viral reservoir persistence during ART.

Considering the importance of Th17 cells in HIV infection, in the first part of this thesis, I was interested in determining the effect of IL-17A, the Th17 hallmark cytokine, on intestinal epithelial cells (IEC) ability to promote HIV trans infection and viral reservoir reactivation in CD4+ T-cells. We observed that IL-17A acts in synergy with TNF to promote the production of CCL20, a Th17-

attractant chemokine, and promote HIV *trans*-infection of CD4+ T cells and HIV outgrowth from cells of ART-treated PLWH. IL-17A-mediated a pro-inflammatory and pro-viral molecular signature. The pro-viral molecular signature was characterized by a decreased expression of type I interferon-induced HIV restriction factors. These results demonstrate that despite the beneficial role of IL-17A on mucosal homeostasis, it also contributes to HIV dissemination and viral reservoir reactivation.

Since Th17 development and differentiation depend on the expression of the master transcriptional regulator RORC2, in the second part of this thesis, we evaluate RORC2 as a positive regulator of HIV replication and viral reactivation. We found that RORC2 is a critical host dependency factor during HIV infection. The pharmacological inhibition of RORC2 using small molecules that bind to the RORC2 ligand-binding domain (LBD) reduced HIV replication in Jurkat cells and primary CD4+T cells *in vitro*. Additionally, the genetic interference with RORC2 expression inhibited HIV replication, while RORC2 overexpression boosted it. In people living with HIV receiving ART, RORC2+ were enriched in HIV reservoirs compared to RORC2- CD4+ T-cells. The pharmacological inhibition of RORC2 blocked HIV outgrowth in CD4+ T-cells from ART-treated PLWH. These results suggest that the Th17 master transcriptional regulator RORC2 represents a novel putative Th17-specific target for HIV therapy.

Finally, Th17 cell depletion at the intestinal mucosal level is a crucial driver of microbial translocation and chronic immune activation. The latest was associated with the occurrence of non-AIDS comorbidities. Thus, in the third part of the thesis, we sought to identify an immunological signature associated with subclinical atherosclerosis in PLWH receiving ART. We found that PLWH with subclinical atherosclerosis had increased plasma fibrinogen levels, reduced Th17 frequency and Th17/Treg ratios, and increased frequencies of $CCR9^{low}HLADR^{high}$ nonclassical monocytes.

In conclusion, the work of this thesis extends the knowledge of the relevance of Th17 cells during the course of HIV infection, including the establishment of non-AIDS comorbidities such as cardiovascular disease. This knowledge is vital when considering Th17 cells as a novel potential target of therapeutic interventions for HIV remission and cure.

Keywords: HIV-1, Th17 CD4+ T-cells, RORC2, IL-17A, intestinal epithelial cells, mucosal immunology, cardiovascular disease.

Appendix

Figures List

Abbreviations

A

ADCC: Antibody-Dependent Cellular Cytotoxicity
AIDS: Acquired Immune Deficiency Syndrome
AhR: Aryl hydrocarbon Receptor
APC: Antigen Presenting Cell
Apo: Apolipoprotein
APOBEC3: Apolipoprotein B mRNA Editing Catalytic Polypeptide Subunit 3
ART: Antiretroviral Therapy
ARV: Antiretroviral
ASXL1: Additional sex combs like-1

B

BATF: Basic leucine zipper ATF-like Transcription Factor
BST-2: Bone Marrow Stromal Cell Antigen 2

C

CA: Capsid, p24
CAD: Coronary Artery Disease
CCD: Catalytic Core Domain
CCR: CC Chemokine Receptor
CD: Cluster of Differentiation
cDNA: Complementary DNA
CLEC10A: C-Type Lectin Domain Containing 10A
CMV: Cytomegalovirus
CTD: C-Terminal Domain
CTL: Cytotoxic T Lymphocyte
CLTA-4: Cytotoxic T Lymphocyte Antigen-4
CVD: Cardiovascular Disease
CXCL: CXC chemokine Ligand
CXCR: CXC chemokine Receptor

D

DAMP: damage-associated molecular patterns
DC: Dendritic Cell
DNMT3A: DNA Methyltransferase 3 Alpha
DNA: Deoxyribonucleic Acid
dsDNA: Double Strand DNA

E

EAE: Experimental Autoimmune Encephalomyelitis
EC: Elite Controller
eNOS: endothelial NO Synthase
ESCRT: Endosomal Sorting Complex Required for Transport

F

FOXp3: Forkhead box P3 (needs to be introduced at line 1347)

G

Gag: Group-specific Antigen
GalCer: Galactosylceramide
GALT: Gut-Associated Lymphoid Tissue
GFP: Green Fluorescent Protein
GM-CSF: Granulocyte-Macrophage Colony-Stimulating Factor
gp: Glycoprotein

H

HDF: HIV Dependency Factor
HIES: hyper-IgE Syndrome
HIV: Human Immunodeficiency Virus
HLA: Human Leukocyte Antigen
HLA-DR: Human Leukocyte Antigen – DR isotype
HTLV: Human T cell lymphoadenopathy virus

I

ICAM: Intercellular Adhesion Molecule
IDO-1: Indoleamine-2,3-dioxygenase
IEC: intestinal epithelial cell
IFN: Interferon
Ig: Immunoglobulin
IL: Interleukin
IMT: Intima-Media Thickness
IN: Integrase, p32
iNOS: Inducible Nitric Oxide Synthase
IRF: Interferon Regulatory Factor
Itgax: Integrin subunit alpha X

J

JAK2: Janus Kinase 2

K

KLF2: Kruppel-like factor 2

L

LAG-3: Lymphocyte Activation Gene 3
LAV: Lymphoadenopathy-associated virus
LBD: Ligand-Binding Domain
LDL: lysophosphatidylcholine
LDL-C: Low-density lipoprotein cholesterol
LDL-R: lysophosphatidylcholine receptor
LEDGF: Lens Epithelium-Derived Growth Factor
LFA-1: Lymphocyte function-associated antigen 1
LI: Link protein, p6
LPS: Lipopolysaccharide
LTR: Long Terminal Repeat

M

MA: Matrix, p17
MAF: Musculoaponeurotic Fibrosarcoma
MCP-1: Monocyte Chemoattractant Protein-1
MDDC: monocyte-derived Dendritic cell
MDM: Monocyte-Derived Macrophages
MDR1: Multidrug Resistance 1 protein
MHC: Major Histocompatibility Complex
MNDA: Myeloid Cell Nuclear Differentiation Antigen
mTOR: Mammalian Target of Rapamycin
MXD1: MAX Dimerization Protein 1

N

NAC: Nucleic Acid Chaperone
NC: Nucleocapsid, p7
Nef: Negative Factor (not well abbreviated at line 344)
NES: Nuclear Export Signal
NF-kb:Nuclear factor kappa B
NLRP3: NOD-, LRR- and pyrin domain-containing protein 3
NNRTI:Nonnucleoside Reverse Transcriptase Inhibitor
NOS: Nitric Oxide Synthase
NPC: Nuclear Pore Complex
NTD: N-Terminal Domain

O

ORF: Open reading frame

OxLDL: Oxidized LDL
OXPHOS: Oxidative Phosphorylation

P

PAMP: pathogen-associated molecular patterns
PD-1: Programmed Death 1
pDC: Plasmacytoid Dendritic Cell
PIC: Pre-Integration Complex
PLWH: People Living with HIV
PPT: Polypurine tract
PR: Protease, p12
PSGL-1: P-selectin glycoprotein ligand
P-TEFb: Positive Transcription Elongation Factor b
Ptt: Pan troglodytes troglodytes

Q

R

RALDH: Retinaldehyde dehydrogenase
RARA: Retinoic acid receptor alpha
RER: Rough Endoplasmic Reticulum
Rev: Regulator of Expression of Virion Protein
RM: Rhesus Macaque
RNA: Ribonucleic Acid
ROS: Reactive oxygen species
RORC: Rar-related Orphan Receptor C
RRE: Rev Response Element
RT: Reverse Transcriptase, p51
RTC: Reverse transcription complexe

S

SEB: Staphylococcal Enterotoxin B
SFB: Segmented Filamentous Bacteria
Siglec10: Sialic acid-binding Ig-like lectin 10
SIV: Simian Immunodeficiency Virus
Slan: 6-sulfo LacNac
SM: Sooty Mangabeys
SP: Spacer peptides
ssRNA: Single Strain RNA
STAT: Signal Transducer and Activator of Transcription

T

TAR: Trans-activation response
Tat: Trans-activator of Transcripton
TB: Tuberculosis
T-bet: T-Box Expressed in T cells
TBX21:T-box transcription factor 21, T-bet
TCR: T Cell Receptor
TET2: Tet Methylcytosine Dioxygenase 2
TFBS: Transcription Factor Binding Site
Tfh: T follicular helper
TGF: Transforming Growth Factor
Th: T helper
TIM-3: T-cell immunoglobulin and mucin domain 3
TLR: Toll-like Receptor
TNF: Tumor Necrosis Factor
Treg: Regulatory T cells
TREM: Triggering Receptor Expressed On Myeloid Cells
TRM: Tissue-resident macrophages
$tRNA^{Lys3}$: Transfer RNA Lys3

U

UN: United Nations
USA: United State of America

V

VCAM-1: Vascular cell adhesion protein 1
Vif: Viral Infectivity Factor
VNN2: Vanin2
Vpr: Viral Protein R
Vpu: Viral Protein U
Vpx: Viral protein X
VSMC: Vascular smooth muscle cells

W

WHO: World Health Organization

X

Y

YS: Yolk Sac

Z

ZO-1: Zonula Occludens-1

« How much better is it to get wisdom than gold! And to get understanding is rather to be chosen than silver »

Proverbs 16:16

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Chapter 1: Introduction

1. Human Immunodeficiency Virus

1.1. HIV Discovery

Cases of a newly acquired immunodeficiency disease syndrome (AIDS) were first reported in 1981 and were characterized by malignancies such as Kaposi's sarcoma and lymphomas, in addition to opportunistic infections with *Pneumocystis cariini*, *Cryptococcus neoformans* and *Candida Albicans* to name but a few(1-3). Although AIDS was first reported in The United States of America (USA), it was rapidly identified in Europe, as well as in Central and East Africa. The loss of cellular immunity and altered distribution of T-cells subsets suggested that the tropism of this pathogen was a T-cell subset(4).

The AIDS-associated pathogen was first identified in 1983 when Drs. Françoise Barré-Sinnoussi and Luc Montagnier at the Pasteur Institute isolated a new virus from the lymph nodes of a patient with pre-AIDS manifestations (i.e., lymphadenitis)(5). Similar to the previously identified human T cell lymphadenopathy virus type I and II (HTLV-I/HTLV-II)(6, 7), the new virus was a retrovirus with a reverse transcriptase (RT) activity dependent on Mg(5). This virus was initially named lymphadenopathy-associated virus (LAV)(8) and was demonstrated to replicate in Cluster of differentiation (CD) 4+ T-cells and use CD4 as the main entry receptor(9). The sequencing of the new virus indicated an outstanding genetic variability(10), explained by the incapacity of the RT to repair errors during the process of reverse transcription(11). In parallel, Dr. Robert Gallo from USA, the discoverer of HTLV-I and II(7), also isolated and associated the presence of this new retrovirus with pre-AIDS and AIDS conditions and proposed the name of HTLV-III(12-15). Finally, this retrovirus was named Human Immunodeficiency Virus (HIV)(16) and nowadays is referred to as HIV-1. Another less pathogenic retrovirus with different antigen characteristics was isolated in 1986 from 2 patients from West Africa(17) and was called HIV-2.

1.2. Epidemiology and current situation

Since the beginning of the HIV pandemic, it has been estimated that 84.2 million people have been infected, of whom 40.1 million have died due to HIV-related causes. As of the end of 2021, there were around 38.4 million people living with HIV (PLWH) and 650,000 reported deaths due to the virus. This highlights that despite great progress in treatment and medical care, HIV is still an important health concern today(18).

A few years ago, the United Nations (UN) set a strong and ambitious objective in the fight against HIV. By 2020, the plan was to achieve an HIV care continuum of 90-90-90, meaning that 90% of PLWH will be diagnosed, of these people, 90% will receive and adhere to ART leading to a viral suppression in 90% of them(19). Despite great strives made by the international scientific community and healthcare professionals in partnership with PLWH, the consortium fell short in fulfilling the 2014 emblematic goal. In 2020, among the 37,7 million PLWH(20), only 84% knew their status and 87% of them were under ARV with 90% being virally suppressed(21). Since the goals of 90% PLWH diagnosed and 90% of diagnosed PLWH under ARV were not achieved, there is a gap of 2,7 million people that is not with an undetectable viral load(22). In consequence, the 95-95-95 targets of 2030 will require more efforts and a stronger investment from the international community to address the remaining challenges and gaps in care for the eradication of HIV.

Among the main issues in the HIV pandemic is the inequity in the access to care between the countries. In 2016, the WHO recommended that everybody living with HIV, regardless of his or her CD4 count should receive ART and a total of 187 countries adopted this recommendation by 2021. This large adherence that should, in theory, covers 99% of PLWH(23) gives yet results far from reality, as shown by the 90-90-90 data of 2020. This highlighted the difficulties in providing a sustainable cascade of care in many regions of the world. In fact, although present worldwide, the burden of this disease is strongly biased toward African countries in its distribution. Indeed, the eastern-sourthern and west-central regions of Africa account for more than two thirds of the estimated reported cases(23) with 60% of the new infections being in sub-Saharan Africa(20).

The disparities in HIV care do not only exist between the different regions of the world, but also among individuals from the same community. Indeed, inequities in human rights and gender considerations remain a considerable obstacle in the establishment of a proper prevention system and medical care. This is reflected mostly in high risk populations that include sex workers, people injecting drugs, prisoners, transgender individuals, and men having sex with men. Indeed, in 2020, sex workers had 26 times more risk of becoming infected than the general population(20). This risk was 35 times greater than in the rest of the community for injectable drug users(20). New efforts should be invested in education to inform the high risk groups on strategies to prevent the transmission/acquisition of HIV and other infectious diseases.

In Canada, the latest HIV epidemic data provided by the federal government stated based on predictions that 62 050 people were living with HIV in 2018, of which 87% were diagnosed(24). A large proportion of these individuals, 85% to be exact, were on ART leading to an undetectable viral load in 94% of them. Even though no observation has been reported since then, Canada was on the good track to reach the 90-90-90 objectives at that time. The biggest challenges toward the eradication of the HIV threat on public health in Canada are common to many other developed countries. Among the most vulnerable populations in Canada are the Indigenous people. Representing only 4.9% of the Canadian population, they accounted for 14% of the new cases in 2018, an increase of 1.7% since 2016 (24).

Even though important challenges persist in the eradication of HIV as a global health threat, the progresses made are substantial with a decrease of 47% in AIDS-related deaths(22). Despite inadequate access in many parts of the world, we have also witnessed a significant improvement in innovative therapeutic approaches with the commercialization of simplified, more efficient, and less toxic antiretrovirals(25), including in prophylaxis.

The progress achieved in the field is the result of thorough investigations on the origin, diversity, replication cycle, and immunopathology of HIV, which are described in the following sections.

1.3. HIV Origin

The discovery of similar retroviruses named simian immunodeficiency viruses (SIV), causing pathogenic and non-pathogenic(26) infections in non-human primates open the path for a new understanding of HIV-1 and HIV-2 origins(27). It is now proposed that HIV originated from multiple cross-species transmissions of the SIV of African primates to humans. Each independent cross-species transmission gave origin to the different HIV types (HIV-1 and HIV-2) and lineages. HIV-1 is separated into groups M (main), N (non-M, non-O), O (outlier), and P, which were originated from an independent cross-species transmission event. Group M and N originated from SIV-infected chimpanzee species *Pan troglodytes troglodytes* (*Ptt*). Group O closest SIV relative was found in gorillas (Gorilla gorilla; SIVgor), but the original host are likely chimpanzees. HIV-2 is separated into 8 groups based on independent cross-species transmissions and is originated from SIV in sooty magabeys (*Cercocebus atys*)(27).

1.4. HIV Diversity and Classification

Two major types of HIV are currently identified, HIV-1 and HIV-2, and despite their genetic similarities, replication cycle and transmission routes, they are distinguished from each other by their genetic sequence but also by their epidemiology and their immunopathology. Of note, unlike HIV-1, very few updated epidemiological data on HIV-2 are currently available since it is not specifically or well enough tracked around the world. It is, however less prevalent than HIV-1 and is mostly distributed in West Africa, although it has been reported in various regions of the world(11, 28, 29).

In terms of genome structure, both HIV-1 and HIV-2 originate from a zoonotic transmission, but rather than stemming from the chimpanzee strain of SIV as is the case for HIV-1, HIV-2 would

come from the SIV infecting the sooty mangabeys. HIV-1 and HIV-2 share around 60% of homology in the *gag* and *pol* genes and between 30 to 40% in the LTR and the rest of the genique sequence(30). It is worth mentioning that this genetic disparity leads to protein structural and functional differences that affect multiple aspects of its infectivity. For example, the HIV-2 RT is resistant to nonnucleoside reverse transcriptase inhibitors (NNRTI) used against HIV-1(31). Its capsid is also more sensitive to Trim5α than HIV-1(32) and presents distinct characteristics in terms of stability and polymerization properties(33). Furthermore, the genome sequence of HIV-1 and HV-2 gives rise to exclusive proteins, as demonstrated by the absence of Viral protein U (Vpu) and Viral protein X (Vpx) in HIV-2 and HIV-1, respectively(34). By differentially expressing Vpu and Vpx, HIV-1 and HIV-2 exhibit distinct abilities to counteract the restriction factors BST-2 and SAMHD1, respectively.

The multiple genetic divergences between HIV-1 and HIV-2 have various consequences in their immunopathology(34, 35). Indeed, HIV-2 is less pathogenic compared to HIV-1, leading to AIDS less rapidly and resulting in a reduced mortality rate(11, 29, 36, 37). Moreover, HIV-2 infectivity is significantly inferior to that of HIV-1, as reflected by its low incidence and prevalence worldwide. This observation can be mostly explained by a lower viral load found in people living with HIV-2, which could result in an incomplete viral fitness in humans or an effective natural immunity against this virus(38). It was indeed observed that HIV-2 had a slower heterosexual spread(39) and a diminished genital shedding(40). It was also reported, in a cohort of individuals living either with HIV-1 or HIV-2 in Senegal, that both groups present similar HIV integration levels but that the latter group showed a reduced mRNA level, suggesting that there is a difference in the replicative capacity at a post-integration stage between the two viruses(41). Host factors are also involved in the control of HIV-2 replication. It was indeed previously demonstrated that there was a significant correlation between the HIV-specific cytotoxic T-lymphocytes (CTL) responses and the control of HIV-2 replication in infected individuals(42), and that this immune defense strategy could persist better with HIV-2 due to a lack of human leukocyte antigen (HLA) associated viral polymorphism that would efficiently elude de selective pressure of CTL(43).

Despite HIV-2 reduced infectivity, adopting a proper therapeutic strategy is essential since infected individuals have a disease evolution similar to HIV-1, with the development of opportunistic infections and progression toward AIDS and death in absence of treatment(38). Establishing an efficient therapeutic strategy against HIV-2 requires, in the first place, to differentiate it from HIV-1, as treatments differ from one another, and to recognize dual infections in some cases. As mentioned before, it was reported that NNRTI are inefficient against the RT of HIV-2, and this appears to be caused by modifications in the interacting regions of NNRTI with the RT of HIV-2(31). Although some clinical trials were and are done with people living with HIV-2, large knowledge gaps remain in what are the optimal standard of care and therapies for the management of these infections and most of the current data are extrapolated from HIV-1 guidelines(29, 44). Moreover, still today, specific tools for diagnosis, viral load quantification and resistance assessment are not widely available and even less in low-income countries, which creates clinical obstacles in the management of this infection(29, 38, 45).

In addition to the existence of HIV-2, the high infidelity of HIV-1 RT, has given rise to multiple groups and clades of HIV-1, some of which are now well recognized and spread throughout the world. In fact, HIV-1 is divided into four groups, as follows: M (major), N (non-M, non-O)), O (outlier) and P, which all originated from a distinct cross-species transmission case(46): The M lineage was the first to be discovered and is the most prevalent across the world(46, 47). Within the M groups, nine subtypes are recognized (A-D, F-H, J and K) which are furthermore divided into sub-subtypes(48), supporting the great evolutionary diversity of this virus. In North America, the subtype B is predominant among PLWH. This is also the case in Europe and in Australia, whereas the African continent is characterized by a strong genetic heterogeneity(46), with the predominance of clade C in South Africa(46). Interestingly, more recently, a new variant in the clade B was identified in the Netherlands(49). It is considered that this new clade has been circulating in that area since the 90s and is described as more virulent, with a viral load 3.5 to 5.5 times higher compared to other HIV-1 strains and a twice as fast loss of CD4+ T-cells in the absence of treatment(49). This new discovery reminds us of the importance of solidifying our diagnostic and surveillance infrastructures to rapidly implement the antiretroviral treatment (ART) in recently infected individuals especially in high-risk communities.

As for the lineage O, it mostly affects the population of West-Central Africa(27, 47). Regarding the group N, it is found very rarely, but would come, similarly to the M lineage, from a cross-species transmission with the chimpanzee *Ptt*(47). Finally, the group P is the most recent one to have been identified. Its prevalence is significantly thin, with only two cases reported by 2018(50, 51). It has been reported that it could be less deleterious due to less adapted viral properties in human hosts(52).

1.5. HIV-1 Genome and Structure

HIV-1 is part of the Lentivirus genus and is classified in the Retroviridae family under the Orthoretrovirinae subfamily. The mature virion is around 100 nm in diameter and consists of a two single-stranded RNA (ssRNA) genome of 9kb enclosed, along with its RT, its integrase (IN) and some other proteins, in a conical capsid made of p24 proteins. The capsid is surrounded by a lipid bilayer envelope that exhibits multiple copies of a unique viral protein named Env or glycoprotein (gp) 160. The latter is in fact a trimer of heterodimers composed of the gp120 (surface) and the gp40 (transmembrane). Linked together via non-covalent bonds, this trimer is responsible for initiating the entry process in the host cells by interacting with CD4 (Figure 1)(53, 54).

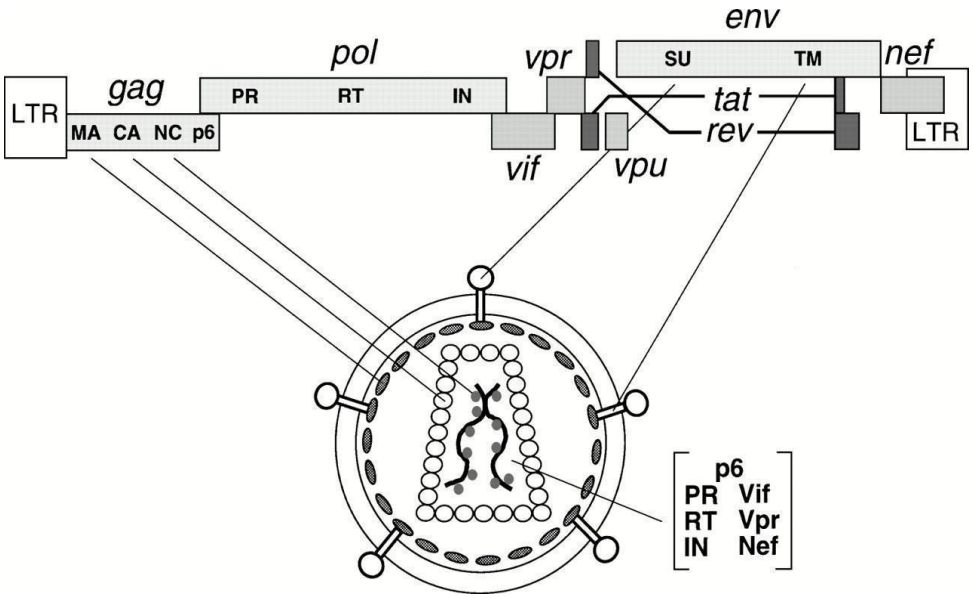

Figure 1 : Genome and Structure of HIV

The genome of HIV is structured as two single-stranded RNA coding for nine genes delimited by two long terminal repeat sequences. These genes give rise to 15 viral proteins that fulfill different structural, non-structural and enzymatic functions. In terms of structure, the viral particle relies on the Matrix (MA), the Capsid (CA), the Nucleocapsid (NC), the Link protein (p6) and the surface protein Env (gp120 and gp41). The non-structural protein consists of the accessory proteins Vif, Vpu, Nef and Vpr and the regulatory proteins Tat and Rev. Among the enzyme proteins are the Protease (PR), the Reverse Transcriptase (RT), and the Integrase (IN). Used with permission of Annual Reviews, Inc., from Frankel AD, Young JA. HIV-1: fifteen proteins and an RNA. Annu Rev Biochem. 1998;67:1-25. doi: 10.1146/annurev.biochem.67.1.1. Permission conveyed through Copyright Clearance Center, Inc licence 1279629-1

The genome of HIV-1 consists of nine open reading frames (ORF) surrounded by two long terminal repeats (LTR) sequences containing three functional elements named U3 (containing the basal, the enhancer and the modulatory promoter elements), R (encoding the trans-activation response [TAR]) and U5. These regions are enriched in different transcription factor binding sites (TFBS) that allow the interaction with either viral (Tat, Trans-Activator of Transcription) or cellular host factors such as NF-κb(53-55). Variation in these TFBS among the diversity of HIV subtypes affects the viral replication rate and its fitness in response to the host cellular environment and status(55).

Although the 3' and the 5' LTR have the same sequences, their role for HIV regulation is different(56). The 5' LTR is a key element in regulating the initiation of the transcription of the integrated provirus by the host machinery(56, 57). As for the 3'LTR, although its promoter activity is minimal compared to the 5' LTR, Tat can still induce transcription(57). In fact, the 3'LTR is mostly involved in the polyadenylation of the viral mRNA(58, 59).

Among the nine genes of the HIV genome, three are considered to play a fundamental role in the constitution of the viral particle by coding for nine of the 15 viral proteins. These three genes, *env*, *gag*, and *pol*, are transcribed as polyproteins named Env, Gag, and Gag-Pol, respectively, and require a series of proteolytic processes to generate the functional proteins, all of which are classified as structural components (Figure 1). More precisely, the proteolytic maturation of Env generates two proteins, gp120 and gp41, which as discussed earlier, constitute the only surface

protein of the viral particle. Gag maturation provides the Matrix (MA, p17), the Capsid (CA, p24), Nucleocapsid (NC, p7) and the Link protein (LI, p6), along with two spacer peptides (SP1 and SP2). Finally, the proteolysis of the fused Gag-Pol(60), results in the Protease (PR, p12), the RT (p51), and the integrase (IN, p32). In addition to these, the viral genome generates non-structural proteins that have either a regulatory (Tat and Rev [Regulator of Expression of Virion Protein]), or an auxiliary (Vif- viral infectivity factor, Vpu and Nef- negative regulatory factor) function(54, 61, 62).

1.5.1. Enzymes and Structural Proteins

Enzymes

Protease

The newly released viral particle is yet immature and requires the proteolytic processing of the polyproteins Gag and Gag-Pol, which is ensured by the protease, itself embedded in the Gag-Pol precursor. The protease requires first an auto-processing step of its precursor to initiate the maturation of the virions(54, 63-65). Its role is thus essential to produce replication-competent viruses. In 1988, Kohl *et al.* showed that in the absence of a functional PR, virions were losing their infectivity, showing the relevance of this enzyme as a therapeutic target(66). Considered as an aspartic protease, it contains this typical Asp-Thr-Gly catalytic triads under a homodimer structure that was initially reported in 1989(67-69). Still today, the exact molecular mechanism regarding its rather complex proteolytic activities in the virion maturation is still not fully understood and questions remain to be answered, especially relative to the timing and the kinetic of this sequence of events(64, 65, 70).

Reverse Transcriptase

The second enzyme of the HIV repertoire is the RT, a heterodimer protein consisting of the p66 and p51 subunits that differ in their structure while sharing the same sequence(54, 71-73). Its role is essential to convert the viral single-stranded RNA genome into a linear double-stranded DNA (complementary DNA, cDNA) format. To complete this retrotranscription, the RT has two different enzymatic functions, DNA polymerase and RNase H, that are both assumed by the p66

subunit, as the p51 plays a more structural role(71). It is through the use of the host transfer RNA Lys3 ($tRNA^{Lys3}$) that the RT can assemble the required nucleotides to produce the cDNA strand. This $tRNA^{Lys3}$ acts, in fact, as a primer for the enzyme and binds the primer binding site located in the 5' extremity of the viral ssRNA(71). As for the RNase H function, it allows the degradation of any viral RNA that would form a duplex with the newly produced DNA strand. It is still unclear, however, if both roles of the RT are necessarily accomplished by the same enzyme and in a coordinated manner during retrotranscription(73). Furthermore, the exact location of the initiation of this reverse transcription is still controversial.

Integrase

Finally, the last viral enzyme is the Integrase, which, as proposed by its name, is in charge of the integration of the reverse transcribed viral dsDNA into the host genome. To mediate the integration, the IN adopts a tetramer conformation on the viral cDNA, which constitutes the intasome (two dimers on each end of the targeted dsDNA strand)(74). Each subunit has three functional domains: the N-terminal domain (NTD), the central catalytic core domain (CCD) and the C-terminal domain (CTD). The first domain is involved in the oligomerization of the IN monomers as well as in the maintenance of the protein structure. As for the CCD, it has a highly conserved motif and binds to the viral cDNA and the host genome to allow the catalytic functions of the protein. On its side, the CTD also binds to the DNA in a nonspecific way and is responsible of stabilizing the assembly of two IN dimers at the extremity of the reverse transcribed viral genome(74-77). By the activity of these different domains, we can identify two main enzymatic functions which are the 3' processing (endonucleotide cleavage) and the DNA strand transfer. Indeed, once the intasome is formed, the IN cuts 2 nucleotides at the 3' end of both DNA strands, which creates a 3'-hydroxil extremity that acts as the nucleophilic agent in the following transfer/covalent binding reaction into the host genome(74, 75, 77, 78). In addition to this integration activity, it was discovered, in 2016, that the IN has also a role in the morphogenesis of the virus, by binding to preferred regions of the viral genome and ensuring its location within the capsid. Abrogation of this function leads to an immature virion thus unable to complete its replication cycle(79). Further details on the involvement of the IN in the virus maturation were since reported(80).

Viral structure protein

Gag polyprotein is the fundamental precursor of the structural proteins by regrouping the MA, the CA, the NC, and the LI/p6 (Figure 1). It represents around 50% of the mass of the virions(81). This polyprotein is fundamental in the packaging of the viral unspliced RNA(82) and its mature form is only found after the budding of the virion(81, 83).

Matrix

The matrix performs two different sets of functions that depend on its conformation and on the viral replication steps. Indeed, under its precursor form (within the Gag polyprotein), the MA is, along with other factors, responsible for bringing the polyprotein to the host membrane where the budding will happen to ensure its packaging in the future virion(81, 83). It is also thought that the uncleaved MA is also involved in guiding the Env at the budding site by interacting with the gp41 tail, although this is still controversial. Once proteolytically processed, the mature MA is oligomerized and is found between the capsid and the lipid bilayer envelope (Figure 1). It interacts directly with the inner part of this lipidic membrane and protects the core of the virus(84). After the entry of the virus to a newly infected cell, the matrix plays a role in the recruitment of the cell host factors for the nuclear import of the pre-integration complex (PIC). The pre-integration complex is composed of the double-stranded(dsDNA) viral genomic DNA coated with viral (IN and Vpr), and host cellular proteins (Barrier to autointegration factor- BAF and Lens epithelium-derived growth factor- LEDGF/p75)(83-85).

Capsid

The capsid is an essential protein of the viral structural core that protects the viral genome from host cell factors that could negatively impact its infectivity. Indeed, through its polymerization into pentamers and hexamers, it forms a conical layer, composed of around 1,500 Ca monomers, directly around the viral genome complex(86, 87). In addition to the protection and stability that the capsid provides, it is also involved in the trafficking of the viral complex to the nucleus, as it

is part of both the reverse transcription complex (RTC) and the PIC. Indeed, the capsid shields the virus of any host restriction factors and thus provides a safe environment for the reverse transcription and the incorporation of the viral genome into the nucleus at a transcriptionally active site(86, 87). The uncoating happens progressively and is completed in the nucleus(86) and the timing of this event has a fundamental impact on the competence of the virus to infect, although questions remain about the exact kinetic of this phenomenon(87).

Nucleocapsid

As a domain of the Gag precursor, the nucleocapsid orchestrates the packaging of the viral genome into the capsid by binding the RNA with its zinc fingers. It is also important for the Gag-Gag interaction during the viral assembly(81, 88, 89). Under its mature form, the NC binds and covers the viral genome which protects the integrity of the two RNA strands. Acting as a nucleic acid chaperone (NAC), the nucleocapsid condenses the latter to provide them with better stability and protects the viral genome in the capsid. This NAC activity is both seen on the RNA and the dsDNA form of the viral genome, which highlight its involvement at many stages of the viral replication, including during the proviral DNA synthesis and integration(88, 90, 91).

Surface protein gp120 and gp41

The entry of the viral particle into a cell is mediated by trimers of the surface gp120 and the transmembrane gp41 non-covalently linked together(78). As mentioned earlier, these two glycoproteins originated from the cleavage of the polyprotein Env which, unlike the Gag and Gag-Pol polyproteins, is performed by the host furin-like protease at the Golgi rather than the viral Protease(92, 93). The gp120 subunit is known to contain 5 conserved (C1-C5) and 5 variable regions (V1-V5)(92, 93). Regarding the transmembrane protein gp41, it has three domains: the extracellular domain that carries the fusion peptide, the transmembrane domain, and the cytoplasmic tail(94).

Each of the two Env subunits plays a distinct role in the viral entry. The surface gp120 is responsible for the interaction with the host receptor CD4. The viral spike is characterized by substantial conformational flexibility, this first contact induces a structural reorganization that allows the exposure of the binding site to link the co-receptor CCR5 and/or CXCR4(95). This sequence of events leads to further changes in the gp41, whose role is to mediate the fusion. More precisely, the refolding of gp41 translocates its N terminal fusion peptide and allows its insertion within the host cell membrane which results in fusion(96-99).

The Env protein is the only antigen presented at the surface of the viral particle and it is also expressed at the surface of an infected cell right before budding. Thus, the virus is required to develop various mechanisms to avoid its recognition by the immune system. One of the main escape techniques relies on the hyper-glycosylation of the gp120 that presents between 18 and 33 glycosylation sites with a median of 25(100, 101). Since there is a high variability in the structure and the composition of the glycan shield surrounding Env and that the architecture of this shield is dynamic(100), the generation of an efficient neutralizing antibody response is rarely achieved in PLWH(102, 103) and requires extensive somatic mutations to shape the antibody repertoire(104). Another immune escape feature of the Env is the inaccessibility of its highly conserved regions due to its conformation. Indeed, the exposure of these functional regions leading to the required virus-host interactions for the entry is only seen through conformational changes that take place after the first binding of gp120 to the CD4. This prevents any blockage of the virus by the immune response (i.e., antibodies)(102, 105, 106).

1.5.2. Non-structural proteins

Accessory (Vif, Vpu, Nef & Vpr)

Among the proteins encoded by the HIV-1 genome, Vif, Vpu, Nef and Vpr are classified as accessory proteins since they do not fulfill a so-called essential or direct function in the infection. Indeed, this set of proteins is often not required for producing replication competent viruses in various cell lines or in highly permissive cells. However, they are key in multiple aspects of the

pathogenesis when it comes to natural infections by inhibiting the host restriction factors, by modulating the cell environment, by allowing immune escape and by strengthening the viral infectivity(102, 105, 106). Still today, new discoveries are reported related to the role of these auxiliary proteins, and these lead to a better understanding of the host pathways and its interactome.

The viral protein Vif has an extended range of interactions with the APOBEC3 enzymes and PPP2R5 phospho-regulators. It is noteworthy that APOBEC3G was the first HIV-1 restriction factor identified(107) and its discovery open the path for multiple laboratories worldwide to study the mechanism of action(108). The APOBEC3 family consists of deoxycytidine deaminases, which transform a cytidine into uridine (C-U). In the context of HIV, in the absence of Vif, the APOBEC3 is incorporated in the viral particle and at the time of reverse transcription, it introduces U instead of C into the negative ssDNA. In consequence, when the second strand of DNA (positive) is synthesized, there is integration of G-to-A hypermutations and the resulting dsDNA is defective. Vif counteracts this activity by promoting the polyubiquitination of APOBEC3 and inducing its proteasomal degradation(109-111). Furthermore, by degrading the PPP2R5 phospho-regulators, Vif alters the cell cycle by provoking an arrest in the G2/M phase(112).

As for Vif, Vpu interacts with a host restriction factor, BST-2/tetherin in this case, and induces its ubiquitination that leads to its degradation. In the absence of Vpu, BST-2/tetherin prevents the release of newly assembled viral particles at the cell surface(110, 113-115). It was also shown that BST-2/tetherin plays an innate immune sensor function against viruses by inducing a pro-inflammatory response that goes through the NF-kB pathway(116). In addition to the degradation of BST-2/tetherin, Vpu can suppress this immune activation independently of BST-2/tetherin by stabilizing the NF-kB inhibitor IκB and by promoting the retention of NF-kB in the cytoplasm, thus preventing the expression of antiviral genes(117). Furthermore, Vpu was recognized as being able to retain newly synthesized CD4 at the endoplasmic reticulum and to induce its proteasomal degradation(118). This prevents the interactions of CD4 with the Env proteins at the surface of newly produced viruses(110).

To date, the mechanism of action of Vpr is still not well understood. It is known to alter the DNA damage mechanism of the cells without provoking real damage to the genome but rather by inducing the degradation of a wide range of cell targets. It was also reported to cause an arrest in phase G2/M of the cell cycle, which could be a consequence of the proteasomal degradation(112). The cell cycle arrest provoked by Vpr could also result from its interaction with the SLX4 complex, which, through a series of events, would cause a stress response and lead to a cell cycle arrest(119, 120). Despite the unresolved questions remaining, the intervention of Vpr during infection enhances the HIV gene expression and inhibits the induction of the IFN type I response(112).

The last accessory protein of HIV-1 is Nef(121, 122). Like Vpu, Nef can modulate the expression of CD4 at the surface of the infected cells (123). However, it does not retain the expression of newly synthesized CD4 molecules but rather down-regulates them from the surface of the cells. Indeed, Nef induces its endocytosis through the clathrin/AP-2 pathway and induces its lysosomal degradation(124, 125). However, the interactions of Nef with the host proteins are not limited to CD4. Indeed, Nef was reported to bind over 70 proteins and its capacity to use the cellular trafficking machinery gives it a wide range of functions that contribute to the pathogenesis of HIV(124, 126). Another example of these Nef-interacting proteins that are down-regulated by Nef are the ones from the major histocompatibility complex (MHC) class I molecules, which then prevent the infected cells to be recognized and eliminated by cytotoxic T-cells(110, 126, 127).

Regulatory (Tat & Rev)

In addition to its enzymes and other structural proteins, the HIV genome codes for two regulatory proteins named Tat and Rev. More precisely, Tat is an activator of the transcription of the viral genome and ensures the proper elongation of the mRNA by the RNA polymerase II. It accomplishes this function by binding the TAR domain of the LTR and recruiting the cellular

complex whose core is called positive transcription elongation factor b (P-TEFb) and includes the factors CDK9 and Cyclin T1(78, 128, 129).

The transcription of the viral genome generated multiple mRNA that presented various splicing patterns. Unlike the host mRNA that is degraded in the nucleus if not spliced, the virus needs to export these partially spliced and unspliced transcripts as they are. That allows an adequate expression of the viral proteins, but also the packaging of the viral genome in its proper form. Bypassing the cell export requirement depends on Rev(130). The latter coordinates this transition by binding to the Rev response element (RRE) in the *env* domain where it polymerizes to enhance its affinity to the RRE. Since Rev contains a nuclear export signal (NES), this Rev-mRNA complex is then able to interact with the cellular nuclear export factor CRM1 and the export machinery to reach the cytoplasm(78, 131, 132).

1.6. Replication Cycle

The HIV replication cycle can be divided in two phases where the first one, the early stage, regroups the steps from the entry up to the integration and the second one, the late stage, starts from the integration and finishes with the release of newly produced virions (Figure 2). In addition to its own proteins, the replication cycle of the HIV requires the engagement of a wide range of cellular proteins to establish a competent infection(133).

1.6.1. Early Stage

Viral Entry

The replication cycle of HIV begins with the interaction of the HIV-1 Env protein with the molecule CD4 at the surface of target cells. As previously presented, the binding of Env to the CD4 induced conformational changes that allow the exposure of the binding site for the interaction with the co-receptor CXCR4 or CCR5 and then, of the gp41 N terminal fusion peptide that leads to the fusion of the viral membrane with the one from the targeted cells(11, 98, 134).

Although CD4 T-cells are the principal targets, myeloid cells have also been reported to manifest a permissiveness potential, although to a limited extent compared to CD4 T-cells(135-137). In fact, monocytes and macrophages can also be the host of the viral reservoir, but the magnitude of their contribution is still controversial nowadays(138, 139).

Uncoating and reverse transcription

Once the fusion is completed, the capsid is released in the cytoplasm, where it begins to uncoat. During the transition into the nucleus, multiple events take place, although the exact kinetic and location of these steps are still not fully elucidated. For example, the uncoating of the viral core was previously reported to occur completely in the cytoplasm. However, recent publications showed that the capsid persists until the nuclear import and disassemble near the integration site(140-142). The importance of a well regulated uncoating process was supported by the discovery of the restriction factor TRIM5alpha. TRIM5alpha binds to the capsid and promotes an accelerated uncoating abrogating reverse transcription(143, 144). As the capsid plays a central role in the reverse transcription and the nuclear import processes, it is of great relevance to better understand the uncoating mechanisms and the capsid interactome(145).

Despite the persistent knowledge gaps remaining, it is well established that prior to its integration, the viral RNA genome first requires to be reverse transcribed to a dsDNA version by the RT. As mentioned earlier, the reverse transcription necessitates various proteins, including some host factors, to form the reverse transcription complex that later transits into the PIC(73, 146). Among the host factors, the $tRNA^{Lys3}$ is critical as it intervenes as a primer by binding the primer binding site and allowing the RT to generate the minus DNA strand at the 5'end of the HIV ssRNA(71, 73). Through its RNase H activity, the RT degrades the RNA coupled with the DNA strand, which allows its transfer to the R region of the 3' end of the viral RNA genome. The reverse transcription can then continue in parallel to the RNA degradation. Only a small RNA sequence,

called polypurine tract (ppt) resists this degradation and acts as the primer for the transcription of the plus strand to finally generate a dsDNA(73, 146).

Trafficking and Integration

Prior to its integration, the HIV genome must migrate into the nucleus. To accomplish this step, the newly synthesized viral DNA is coupled with several factors to form the PIC whose molecular weight is significant. In consequence, rather than being a passive diffusion through the cytoplasm and the nuclear pore complex (NPC), this step is in fact energy-dependent(76, 147), and mobilizes over 15 host cellular proteins and nucleic acids elements(133). Although demanding, the PIC and this active nuclear import process give the virus the chance to access the chromatin of non-dividing cells. This constitutes an important characteristic of the lentivirus genus to which HIV belongs(148). In that aspect, the capsid was also shown to be of first importance in the ability of the HIV to infect non-dividing cells(149, 150). As discussed earlier, the capsid is known to be a key factor in the recruitment of those cell host factors for the nuclear import of the PIC (83, 84).

Once the virus is in the nucleus under its dsDNA conformation, it can finally be integrated into the host chromosome. This requires, again, multiple viral and cell factors, but most predominantly, the intervention of the viral IN. The characterization of the integration profile of HIV has been of great interest to the scientific community. It was reported that this integration is performed in preferential regions of the genome rather than randomly. Indeed, the viral genome favored genes as its integration sites, but more precisely, genes that are transcriptionally active(151). This preference is first partially explained by the fact that in this state, the chromatin is more accessible. In addition to this aspect, it was demonstrated that the position of the cellular genes relative to the nuclear periphery/pore impacts the integration rate and that the peripheral genes often bear open chromatic marks and are often paired with the NPC which has been shown to be involved in the infection(152). It was also demonstrated that the host factor LEDGF/p75, a transcriptional activator, plays a significant role in this process by binding both the IN in the PIC and the host genome, and thus, guiding the integration location of the HIV DNA(153-155). The inhibition of LEDGF/p75 greatly modifies the integration map and affects the viral

infectivity(156). Considering the wide range of protein interaction that the PIC can perform with the host factors, it is most likely that this also plays a central role in furthermore modulating the integration process(157).

Aside from the topographic characteristic of the integration site, the integration preferences of the viral genome are also influenced by some genomic sequence motifs. Indeed, the HIV integrates preferentially near Alu sequences, which are predominantly present in genes, but this bias is independent of the transcriptional activity and the nature (gene or intergene) of the region(158). Despite the identification of preferential integration sites and architectural traits, it remains unclear how this affects the immunopathology of the infection in PLWH and the viral latency and replication pattern. More work needs to be done to better understand the importance of this selective integration on the progression scheme of the disease, but also in the persistence of the virus as it represents a major challenge for its eradication.

1.6.2. Late Stage

After integration, the virus can either stay latent for an extended period, or it can pursue its replication cycle. In the latter case, the virus genome will first be transcribed by the host cell machinery. Then, the viral transcript will be exported to be translated or brought to the assembly site to be inserted in the viral particle. Finally, the virus will bud for the cell and complete its maturation process.

Transcription and translation

The first transcripts to be synthesized during viral replication are the ones of Tat and Rev, which are the only ones to be fully spliced and, thus, the only ones following the normal cellular course of events regarding RNA splicing for nuclear export(131, 159). As discussed in a previous section, Tat and Rev are essential in regulating and orchestrating the viral gene expression at a transcriptional and post-transcriptional stage, respectively. Indeed, Tat ensures the efficient elongation of the viral mRNA by the RNA polymerase II(78, 128, 129) and Rev allows the nuclear

export of the unspliced or partially spliced viral transcripts containing RRE, among which is the viral RNA genome itself(78, 131, 132).

As Tat improves the efficiency of viral transcription, longer viral transcripts with a partial splice pattern emerge and can be exported by Rev. In total, the splicing patterns of the HIV mRNA lead to over 40 forms of transcripts in an infected cell. Like Tat and Rev, Nef mRNA is completely spliced whereas Env, Vif, Vpr and Vpu are partially spliced by the cell spliceosome(131). Finally, at a later stage, the full-length viral RNA is produced which leads to the Gag-Pol polyprotein, but also to the viral genome to be encapsidated(131, 159).

Whether they are fully or alternatively spliced by the cell spliceosome, the viral mRNA molecules then need to be translated by the host machinery. As mentioned earlier, the genes env, gag and pol are transcribed as the respective polyproteins Env, Gag and Gag-Pol and will further be processed by proteolysis into individual functional units(60). The generation of Gag and Pol polyproteins from the full-length viral RNA arises from a ribosomal frameshift that takes place 5% of the time resulting in 20 times more Gag than Gag-Pol polyproteins(131).

Trafficking and Assembly

Following transcription, the virus particles get assembled near the budding area. This represents a complex and crucial step as all the different viral components need to be gathered in a specific location to produce complete infectious virions. This step is mostly coordinated by the Gag polyprotein as its four domains interact with many of the viral materia(160). More precisely, the nucleocapsid domain is able to bind and protect the viral genomic RNA with its zinc fingers and orchestrates the Gag-Gag interactions during the viral packaging(81, 88, 89). The matrix domain ensures the connection between Gag and the cellular membrane(160, 161).

Although Gag plays a dominant role in viral assembly and is able to interact with Env through its matrix subunit, its interplay with the envelope glycoproteins for their recruitment at the assembly site is still not well defined(94, 160). In fact, the path of Env toward the formation of the viral particle is slightly different from the rest of the components. Indeed, while Gag is synthesized in the cytosol, Env is translated and glycosylated at the rough endoplasmic reticulum (RER). Afterward, it is transferred to the Golgi where it is cleaved, as mentioned in a previous section, by the host furin-like protease(92-94). The mature subunits, gp120 and gp41, form multimers and migrate to the cell membrane via the secretory network. At the plasma membrane, Env is expressed at a very low level, being rapidly endocytosed due partially to the endocytosis motif on the gp41 cytoplasmic tail(162). This is thought to inhibit recognition of this viral antigen by the immune system(94). However, similar to Gag multimers, Env is associated with lipid raft microdomains at the plasma membrane(163). This shared characteristic is also the subject of one of the models for Env incorporation by which this cellular structure would serve as an assembly site where both Env and Gag are targeted(94, 160). Of note, these microdomains constituted of specific lipids such as sphingomyelins, cholesterol, and phosphoinositides play an important role in the production of new viral particles and their infectivity considering that the they are enriched in the envelope of mature virions and that this lipidome differs from the usual lipid distribution of the plasma membrane(164, 165).

Budding and Maturation

Once the packaging of the virion is completed, the latter buds from the cells and its maturation pursue to produce infectious particles. As for the assembly, Gag plays a central role in the budding of the virus, but this process also relies on one main cellular machinery called endosomal sorting complex required for transport (ESCRT) that involves around 20 different proteins, although only a portion is directly involved with HIV release(160). Indeed, the p6 subunits of Gag comprise two motifs, also called late domains, that allow the recruitment of members of this cellular complexe that induces the scission of the cellular plasma membrane to release the budding viral particle. The first late domain, PTAF, interacts with TSG101 that is in the ESCRT-I complex, and the second motif, YPXL, binds ALIX(166). To a lesser extent, another late domain

found in the CA subunit, PPXY, helps the budding by interacting the E3 ubiquitin-protein ligase NEDD4(160, 167, 168).

As mentioned previously, the IFN induced protein BST-2 restricts HIV release(114). By inhibiting viral release, BST-2 increases the susceptibility of cells to antibody-dependent cell-mediated cytotoxicity(169). Conversely, it was also shown that BST-2 could also play an important role on the propagation of HIV-1. BST-2 restricts cell free virus transmission but not cell-to-cell transmission. Indeed, the basal expression of BST-2 is required to tether virions on the surface and facilitate HIV spreading(170).

The maturation of the viral particle consists of the cleavage of the Gag and Gag-Pol polyproteins into their subunits by the viral protease. The processing of the polyproteins varies in terms of speed, but the rate of cleavage is, to some extent, structured in a sequence of events that trigger one another and lead to a reshaping of the viral particle into the well-known mature form characterized by the conical core comprising the viral genome and its associated proteins as described in the previous section. At the end, this restructuring is essential for the infectivity of the newly produced viral particle(160, 167, 168).

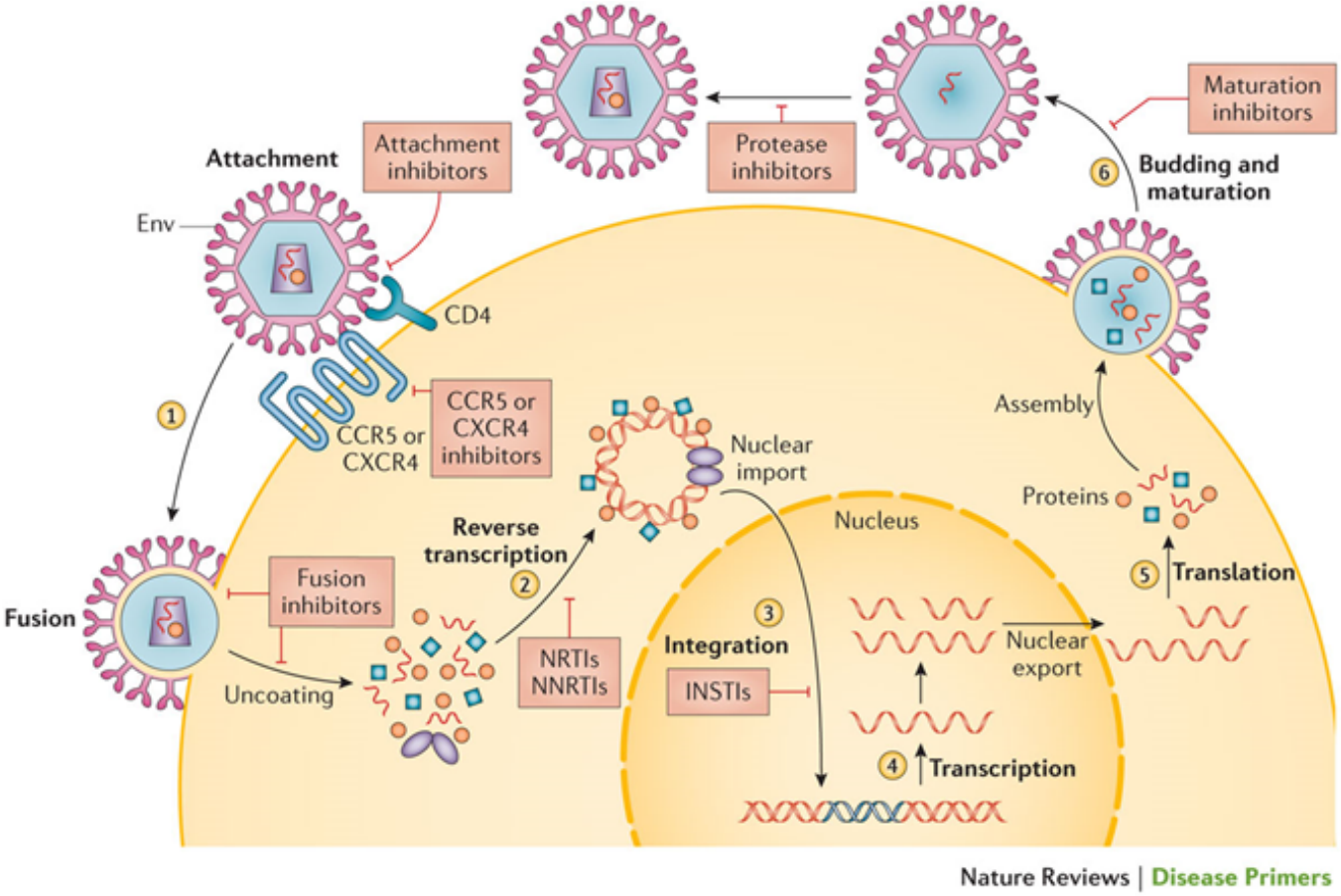

Figure 2 : HIV-1 replication cycle

The genome of the HIV is structured as two single-stranded RNA coding for nine genes that are delimited by two long terminal repeat sequences. These genes give rise to 15 viral proteins that fulfill different structural, non-structural and enzymatic functions. In terms of structure, the viral particle relies on the Matrix (MA), the Capsid (CA), the Nucleocapsid (NC), the Link protein (p6) and the surface protein Env (gp120 and gp41). The non-structural proteins consist of the accessory proteins Vif, Vpu, Nef and Vpr and the regulatory proteins Tat and Rev. Among the enzyme proteins are the Protease (PR), the Reverse Transcriptase (RT) and the Integrase (IN). Used with permission of Annual Reviews, Inc., from Frankel AD, Young JA. HIV-1: fifteen proteins and an RNA. Annu Rev Biochem. 1998;67:1-25. doi: 10.1146/annurev.biochem.67.1.1. Permission conveyed through Copyright Clearance Center, Inc license 1279629-1

1.7. Immunopathology

Typically, in the absence of therapy, the HIV infection follows a well-defined course of events that is divided into three major phases following the eclipse period: the acute stage, the chronic one, and finally, AIDS (Figure 3), which despite our progress in the HIV care continuum, was related to 680 000 deaths in 2020(20). Although this sequence of events is well characterized, the rate at which the disease progresses through those stages remains substantially among PLWH. These variations in HIV control are attributable to environmental factors, the host genetic characteristics, and the virus genotype itself(171-174).

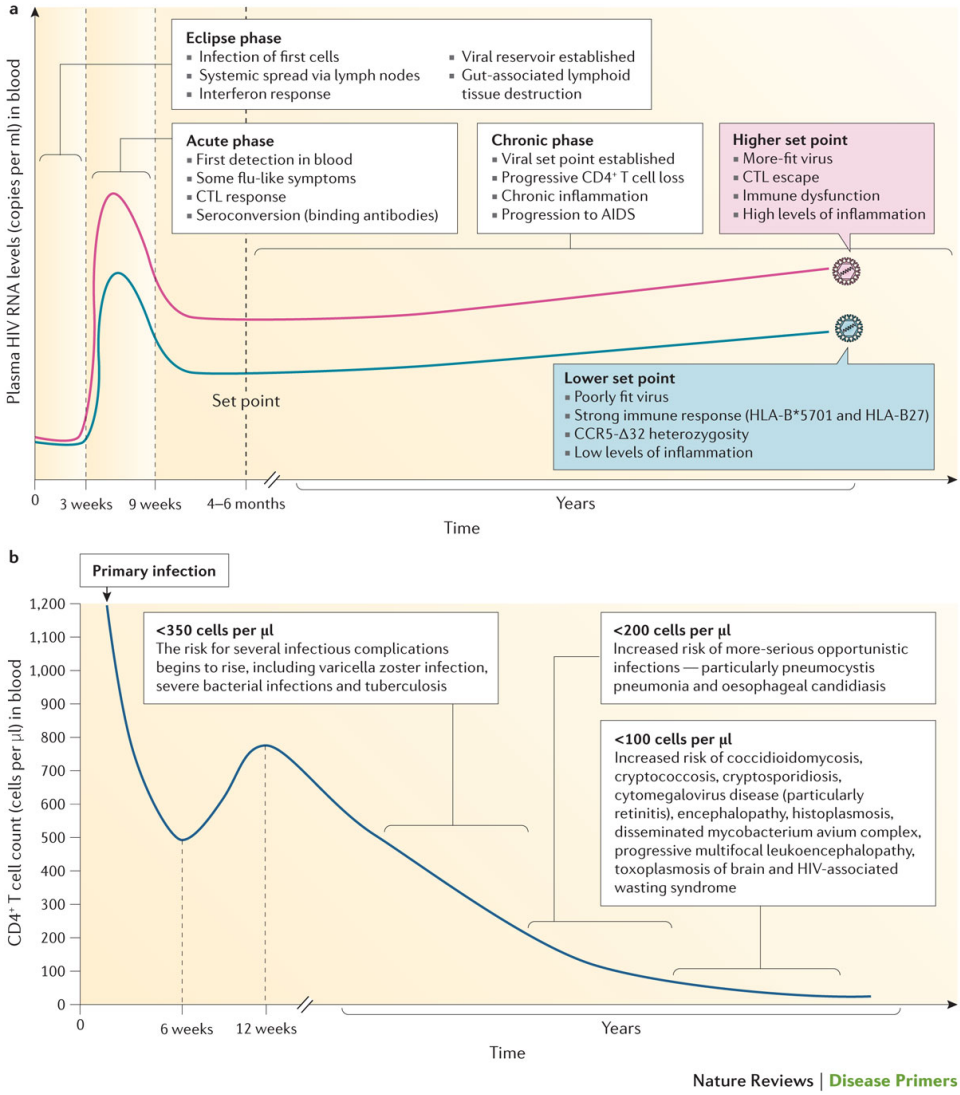

Figure 3: HIV Immunopathological kinetic

After transmission, the first stage of HIV infection is referred to as the eclipse. This phase is followed by the acute and chronic stages before reaching the AID stage in absence of ART. Each step is defined by clinical parameters, including the CD4 T cells counts, the development of an immune response directed against the viral products, and the sensitivity of the host to opportunistic infection. Reprinted by permission from Springer Nature, license 5410421060780, Deeks, S., Overbaugh, J., Phillips, A. et al. HIV infection. Nat Rev Dis Primers 1, 15035 (2015). https://doi.org/10.1038/nrdp.2015.35

1.7.1. HIV Transmission

HIV transmission takes place through the exchange of specific body fluids such as blood, semen, vaginal secretions, and breast milk from a PLWH in the absence of effective ART(175). Thus, acquisition of this viral infection can occur during sexual intercourses, injections with shared needles, percutaneous injuries with infected material, blood transfusions, pregnancy, delivery, and breastfeeding. Among those routes of infection, contaminated blood transfusions represent the riskiest act of transmission per exposure. However, the most common way of acquiring HIV is through the exposure of mucosal sites, such as the genital tract and the rectum, to the virus(176-178). More precisely, out of different unprotected sexual practices, receptive anal intercourse has been reported as the riskiest act for contracting HIV per exposure(179).

Despite the route of exposure weighting significantly on the risk of HIV transmission, the viral load of PLWH also plays a fundamental role in this event(180-184). Indeed, it was demonstrated, initially with the HPTN 052 and then with the PARTNER and Opposites Attract studies, that the risk of transmission during sexual intercourse is inexistent when the sex partner living with HIV is under ART and has a suppressed viral load under 200 HIV-RNA copies/ml(180, 181, 185). This observation gave birth to the concept Undetectable equals Untransmissible, or U=U, which reflects well the importance of early ART initiation(186).

1.7.2. Eclipse

During the first few days following infection, the viral RNA is not yet detectable in the blood, and this period has thus been named the eclipse. Although undetectable, the virus spreads

throughout the mucosa within the first hours of the eclipse phase and then starts disseminating in the draining lymphatic system(11, 177, 178).

Based on estimations from longitudinal cohorts and self-reports of exposure events, it was previously established that this stage lasts between 7 to 21 days. Still, a great heterogeneity exists between studies(11, 177, 178). This heterogeneity stems, above all, from the fact that for a large proportion of individuals, the initial phase of an HIV infection does not always coincide with the appearance of specific symptoms, if any, pointing to an HIV diagnosis(177). However, a recent study by the RV217 team could refine this period to approximately seven days using a molecular approach in their cohort instead(187).

1.7.3. Acute phase

After the eclipse, the infection transits to the second stage, called the acute phase, which starts when the viral RNA, also referred to as the viral load, is first detected in the plasma(177). For PLWH reporting symptoms, it is often during this period that the latter will be noticeable, resembling the ones of a Flu(188). These symptoms include fever, sore throat, swollen lymph nodes, and mouth(189).

The acute period is characterized by the large-scale replication and dissemination of the virus in the gut-associated lymphoid tissue (GALT) and peripheral lymphoid tissues. This soon leads to the detection of other viral components than the viral RNA in the blood, such as the Gag p24 protein. The sequential appearance of these products is even used to characterize the progression of the early infection better. Indeed, it defines what is called the Fiebig stages, which range from stage I, where only viral RNA is detected, to phase VI, where the p31 antigen is detected by western blot(176-178, 190).

The systemic dissemination of HIV during the acute phase has multiple impacts on the immune status of the PLWH. Among the most well-known is lymphopenia driven by the depletion of the CD4+ T cell compartment, initially identified as Leu-3+ T-cells(191). While easily observed in the periphery, this depletion occurs mainly at a mucosal level, more specifically in the gastrointestinal tract, where enrichment of CD4+ T-cells is observed(192-197). Indeed, within the GALT, nearly 20% of the CD4+ T-cells become infected in the early phase of the infection(193). Among the most affected CD4+ T-cells are the T helper (Th) 17 compartment(198-200) as will be further described later (see section 2.2). The integrity and functionality of this compartment remain partially disturbed despite ART initiation(196, 201).

Several mechanisms are associated with viral-induced lymphopenia. Indeed, HIV-1 can induce cytolysis of infected cells, apoptosis through caspase-3 signaling, and pyroptosis through the caspase-1 pathway(192). The infected cells can also be targeted by the host immunity via a cytotoxic T lymphocyte (CTL) response and also through antibody-dependent cellular cytotoxicity (ADCC)(192). Additionally, HIV can indirectly cause cell death, as evidenced by the low ratio of infected cells vs. the extent of cell loss and by the depletion of uninfected CD4 T-cells in PLWH(192, 193, 202, 203). More precisely, some viral proteins released by infected cells, in addition to the inflammation caused by the infection, will impact the responsiveness and proliferation capacities of the surrounding cells, but most importantly, it can lead to bystander apoptosis which is thought to be a major contributor to CD4 decline during HIV infection(192, 202).

In addition to lymphopenia, the systemic dissemination of HIV during the acute phase causes a significant surge in the production of inflammatory cytokines, mainly from the innate immune compartment(193). This rise of inflammation, also known as a cytokine/chemokine storm, concurs with the rapid elevation of the viremia and occurs mainly at the mucosal sites rather than in systemic lymphoid tissues(204). Although a great variability exists in the literature regarding the kinetic of this cytokine/chemokine storm due not only to the tools used but the time points considered and the biological compartment studied, the cytokine/chemokine storm in HIV-1

infection is characterized as being composed of sequential waves of transiently exacerbated factors and sustainably elevated ones which strongly impact the progression of the disease in PLWH(205, 206). In fact, it is thought that even though it primes further immune responses against the virus, this cytokine/chemokine storm also contributes to the spread of the virus by providing more activated targets for the virus to infect in absence of a proper or strong enough antiviral response that emerges later on(193, 204, 207). In the gut, a cytokine profile similar to the one of chronic inflammatory disease is observed, and this contributes most probably to the massive CD4 T cell depletion at this site, by recruiting activated T-cells permissive to HIV infection and by offering an environment rich in Fas and Fas ligand, important inducers of apoptosis(204). In fact, an association exists between immune activation and depletion of CD4 T-cells independently of the viremia(208). High levels of TRAIL were also reported in PLWH at an early stage of the infection, suggestive of an environment favorable to cell death(209, 210).

Among the important components of this inflammatory surge are interleukin (IL) 15, IL-1α/β, Tumor Necrosis Factor (TNF) α, Interferon (IFN) y, IL-22, IFN-α, and IP-10(177, 193, 206, 211, 212). The immunoregulatory cytokine IL-10 is also present in this cascade(212, 213) , but is slightly delayed compared to the others(212) and may contribute to the persistence of the reservoir later on(214).

Despite this cellular and activation imbalance observed in PLWH, the immune system eventually succeeds in gaining partial control of the viral replication and the viral load finally reaches its peak after approximately 30 days with a value of around 10^6 to 10^7 copies per ml before decreasing of 100-fold(215). This concurs with a modest transient increase in the CD4 counts and the appearance of the first signs of seroconversion(176, 215). The viral RNA level eventually reaches a plateau, reflecting the immune system's certain control of the virus. This plateau, also called the viral set point, is defined as a relatively stable level of viral replication that is maintained over an extended period of time(193, 215) and is achieved by a delicate balance between the immune pressure and the virus ability to escape these host defenses. The value of this set point is often

reflective of the speed to which the disease will progress(216-220), and it is determined by host(171, 174, 221, 222) and viral(172, 223-226) factors(227).

Important contributors to this control and to the acute immune response are the production of antiviral cytokines by the innate cells, the ADCC(228), but most of all, the CD8 T cell compartment. Indeed, HIV-specific CTL are fundamental in the decline of viremia and its control(193, 229-232). Those CTL cells are first seen right before the peak of viral replication, and by mediating the killing of productively infected cells, they contribute to the fall of viral replication in the acute phase, and they select escape mutations of the virus(233, 234). Initially, this response recognizes mainly the consensus sequence of Nef(235), but is later on directed toward more conserved viral proteins such as Gag p24 and Pol, which allows greater control of the replication rate(193). In fact, some of these selective mutations lead to a lesser replication capacity or fitness of the virus, as seen in PLWH who naturally control the virus(229, 233, 234). However, as the name says, these escape mutations confer to the virus the ability to not be quickly recognized by the CTL response that requires the generation of new CTL able to target its emergent epitopes(233, 234). Consequently, the virus can persist, and a perpetual battle occurs between the immune system and the virus throughout the chronic phase(177, 193, 236) .

CD8+ T-cells also exert noncytotoxic antiviral responses that contribute to viral suppression. This suppression is independent of CD8+T cells cytotoxicity and cell killing(237, 238). The noncytotoxic antiviral activity is attributed to a soluble factor and does not require cell to cell contact. It does not require major histocompatibility complex and it is not the result of the block of entry, reverse transcription or integration but most probably the block of transcription(237).

1.7.4. Chronic phase

Seroconversion and the reach of the set point are indicators of the transition from the acute phase of the infection to the chronic one. As mentioned earlier, the depletion of the CD4+ T cell compartment caused by HIV infection is not restricted to the acute phase, but it represents a

continuum throughout the subsequent steps of the disease. In the chronic phase, in addition to the physical loss of CD4+ T-cells, disturbances in both the functionality and proliferation arise for the CD4 as well as the CD8 compartment, leading to the progression of the disease toward AIDS.

In general, after infection, the immune system is able to clear the infection, which results in the elimination of the viral antigens and the resolution of the inflammation. However, in the presence of HIV-1, this clearance is not observed, and this results in a persistent inflammatory milieu that causes progressive functional impairment of both CD4 and CD8 T-cells(236, 239), impacting their survival(240). This is well reflected by the upregulation of several immune checkpoint inhibitors, including programmed death 1 (PD-1)(241-243) cytotoxic T lymphocyte antigen-4 (CTLA-4)(244, 245), lymphocyte activation gene 3 (LAG3)(246), and T-cell immunoglobulin and mucin domain-3 (TIM-3)(236, 239, 247-249) .

This exhaustion not only attenuates their responsiveness toward HIV-1 antigens but inhibits their proliferative and self-renewal capacities(250-253). Furthermore, HIV-1 causes dysfunction of the lymphoid tissues, through the induction of fibrosis. This results in a limited output of naive T-cells, impacting, even more, the CD4+ T cell counts and the ability of the T compartment to be restored under ART(254-258).

Of note, in addition to the virus itself and the cytokine storm, microbial products translocating from the intestinal lumen into the periphery are another source of stimulus responsible for the persistent immune activation during the chronic phase of the infection(259, 260). Indeed, during the progression of HIV-1, an increased level of plasma lipopolysaccharides (LPS) is found and is associated with indicators of immune activation(260).

From a more clinical point of view, HIV-1 infection has a major impact on the general health of PLWH and has been associated or it thought to be linked to various diseases such as liver cirrhosis, end-stage renal disease, digestive complications and cardiovascular events(215, 261, 262). For

example, in the clinical trial NCT00113126, HIV RNA replication has been associated with higher levels of markers for cardiovascular risk(263). The higher risk of development of these pathologies can be attributable to HIV-induced inflammation and to the tissue damage that the infection causes, although the mechanisms behind the development of these co-morbidities are probably multifactorial (ex. lifestyle and co-infections)(262). In addition to these co-morbidities, untreated HIV infection is considered as a model of accelerated ageing because it induces an immune state similar to the one seen in an elderly population in terms of immunosenescence and dysfunction(261).

In general, in the absence of therapeutic interventions, a person will die from HIV or HIV-related complications in less than 10 years. However, certain groups of individuals demonstrate a completely different kinetic of infection where they either progress faster or slower toward AIDS and death or do not show any aggravation of their condition(264-266). In any case, the last stage of HIV infection is AIDS.

PLWH that is able to control viremia to undetectable levels in the plasma without ART and do not progress to AIDS are called elite controllers (ECs)(266, 267). EC also preserve CD4+ T-cells counts over many years in the absence of ART. The immunological mechanisms associated with EC have been the focus of intense research(266, 267). As an example, EC have an overrepresentation of HLA variants that are denominated as protective. Among those variants, we have HLA-B57 or B27 restricted CTL responses that target HIV gag(268). CD8+ T- cells of EC compared to chronic progressors have also shown superior properties related to polyfunctionality, stemness potential and trafficking to lymphoid tissues(267, 269-271).

Individuals that are homozygous for a 32-nucleotide deletion (delta 32) within CCR5 receptor gene remain uninfected despite constant exposure to HIV-1. The same protection was not observed in heterozygotes for CCR5 receptor gene after exposure to HIV-1 but these individuals showed a slower loss of CD4+ T cells after acquiring HIV-1. This slower decay of CD4+ T cells was

not correlated with a reduction of time to develop AIDS. Therefore, these results showed a limited protective role of delta 32 CCR5 heterozygous genotype against disease progression(272).

1.7.5. AIDS

In the absence of a proper antiretroviral regimen, the HIV infection progresses, and the CD4 T cell depletion continues until what is called AIDS. At this last stage of the infection, defined by a CD4 T cell count below 200 CD4+ T-cells per µl of blood, the PLWH are immunocompromised, and as a consequence, they are significantly susceptible to opportunistic infections and more prone to develop cancer(215).

Among the most frequent opportunistic pathogens, *Mycobacterium tuberculosis* is of the greatest importance as it is the predominant cause of death among PLWH(273). In fact, the risk of developing active Tuberculosis (TB) is amplified by more than 20 times in PLWH with advanced infection. This risk is also exacerbated in the early stage of HIV(273). In 2020, the WHO estimated an incidence of 787 000 HIV/TB coinfections(274). Other opportunistic infections include Candidiasis, Coccidioidomycosis, Cryptococcosis, Cytomegalovirus (CMV), Pneumocystis pneumonia and Toxoplasmosis(275).

As mentioned earlier, if not treated, people with AIDS will eventually succumb to the disease or of complications related to opportunistic infections.

2. HIV Infection and Persistence at Mucosal Barriers

As mentioned earlier, HIV preferentially targets CD4+ T-cells where it actively replicates. The molecular characteristics and function of CD4+ T-cells define their ability to allow HIV replication and harbor the HIV reservoir(276). More specifically, Th17 cells were reported to be highly

permissive to the infection. As they play an important role in maintaining mucosal immunity, their impairment has a great impact on HIV pathogenesis.

2.1. Overview of Th17 cells

CD4+ T-cells play a major role in regulating the immune response through the secretion of cytokines and activation of cells of the innate immune system, B-lymphocytes, cytotoxic T-cells, and non-immune cells such as epithelial or endothelial cells(277).

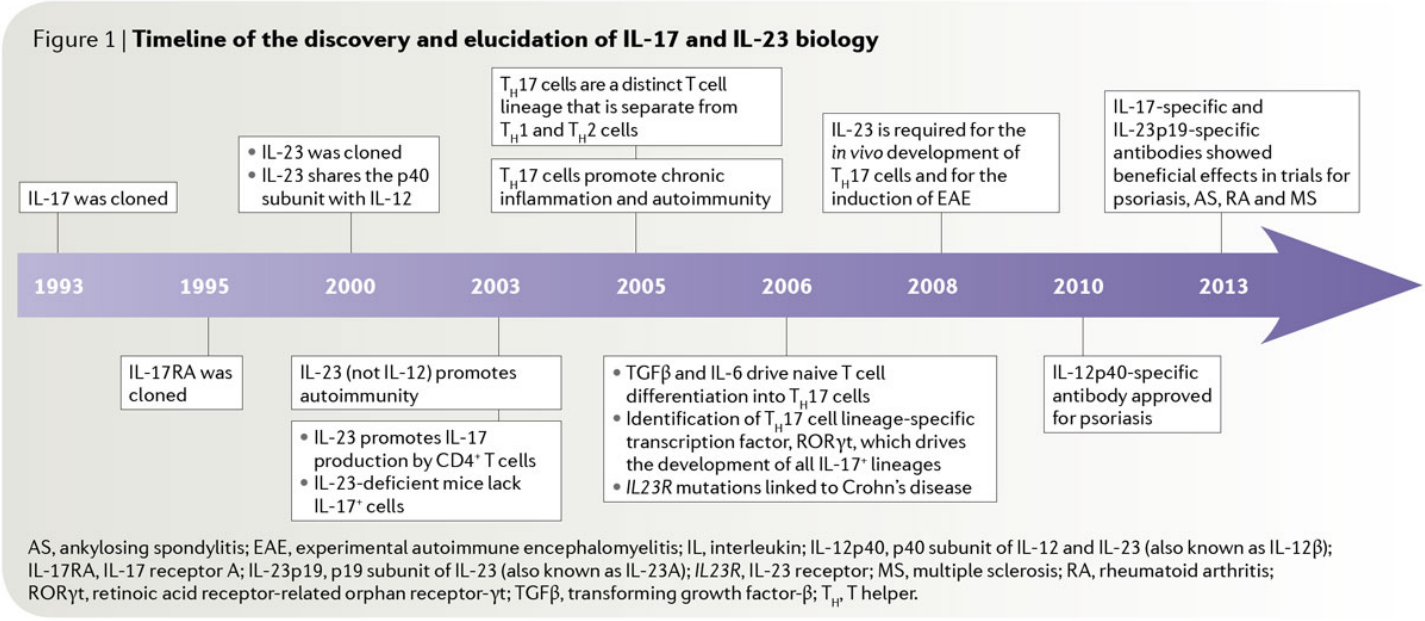

Figure 4: Overview of the discovery of Th17 cells

Timeline of the crucial discoveries defining Th17 cell lineage, its immune functions, and its implication in pathological settings. Reprinted by permission from Springer Nature, License Number 5411000814478, Gaffen, S., Jain, R., Garg, A. et al. The IL-23–IL-17 immune axis: from mechanisms to therapeutic testing. Nat Rev Immunol 14, 585–600 (2014). https://doi.org/10.1038/nri3707

Mature T-lymphocytes develop from committed lymphoid progenitors in the thymus. The T-cell receptor (TCR) is generated during their maturation process, and the CD4 receptor is expressed(278). Mature CD4+ T-cells that emigrated from the thymus and have not been activated are denominated naïve CD4+ T-cells. The activation of naïve CD4+ T-cells occurs when they encounter an antigenic stimulus resulting from the interaction between the TCR and CD4

receptor with the antigen-MHC II complex of professional antigen presenting cell (APCs). CD4+ T-cells responses are highly antigen-specific, this specificity is determined by the TCR. After activation, naïve CD4+ T-cells proliferate and differentiate into effector or memory CD4+ T-cells(279-281), which can be classified based on their function, homing, and expression of surface markers, cytokines, and transcription factors in 4 major subsets, called Th1, Th2, Th17, and T follicular helper(Tfh)(282-284).

The Th1/Th2 subsets paradigm was established when Mossman & Coffman identified Th1 and Th2 cells in the late 1980s(284-286). Th1 cells were carachterized by the production of IFN-γ and Th2 by the production of IL-4, IL-5, and IL-13. Th1 and Th2 cells play different effector functions driven by their cytokine production. Briefly, Th1 cells activate macrophages and enhance antigen presentation, facilitating phagocyted microbes' killing. Thus, Th1 cells give protection against intracellular pathogens. In 2000, T-bet was identified as the transcription factor that regulates Th1 function(287).

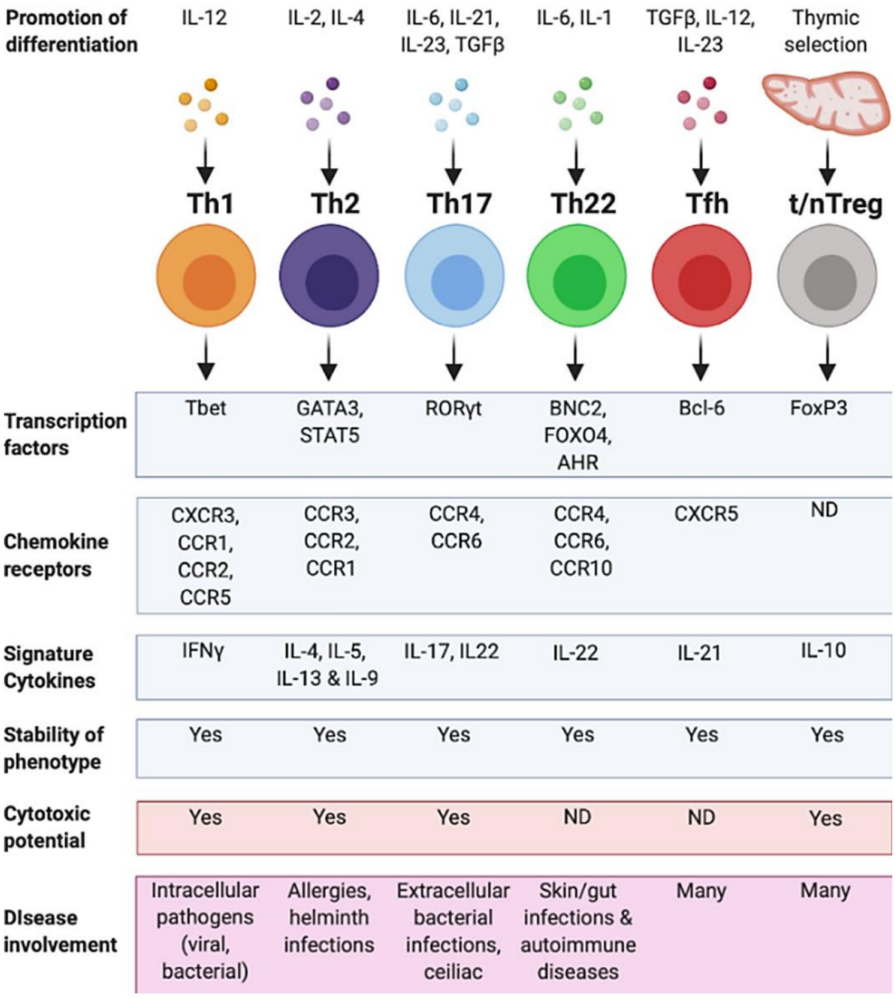

Figure 5 : Human CD4+ T cell subsets

Overview of the different Th subsets and their key phenotypic, regulatory and functional characteristics. Chatzileontiadou, D.S.M.; Sloane, H.; Nguyen, A.T.; Gras, S.; Grant, E.J. The Many Faces of CD4+ T Cells: Immunological and Structural Characteristics. Int. J. Mol. Sci. 2021, 22, 73. https://doi.org/10.3390/ijms22010073. This is an open access article distributed under the Creative Commons Attribution License which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

In contrast, Th2 cells promote the expansion of B cells and immunoglobulin (Ig) E production and give protection against extracellular pathogens such as helminths. Th2 cells execute its antihelmintic effect by activating eosinophils and mast cells(288). In 1997, GATA-3 was identified as the transcription factor that regulates Th2 cells(289).

The study of the involvement of Th1 cells in autoimmunity opened the door for the discovery of Th17 cells. At first, it was reported that mice lacking the expression of Th1 master transcription factor T-bet were resistant to experimental autoimmune encephalomyelitis (EAE)(290). In contrast, mice that did not express IFN-γ or the receptor of IFN-γ as well as mice that were deficient for factors necessary for Th1 development such as IL-12-p35, IL-12 receptor, and IL-18 were not protected from EAE(291). These findings supported the hypothesis that another T-cell subset was required to establish EAE. The evidence that IL-23 was a driver of EAE enlightened the path toward identifying Th17 cells(292). Indeed, IL-23 knock-out mice did not have affected Th1 responses, had reduced expression of IL-17, and were resistant to EAE(292). Furthermore, IL-23 induced the expansion of T-cells that produce IL-17 which showed different transcriptomic characteristics compared to IL-12-differentiated Th1 cells(293). In 2005, Harrington et al. and Park et al. showed the existence of a Th CD4+ T-cell lineage different from Th1 and Th2 cells that produced IL-17. These cells were then called Th17 cells(294, 295).

Th17 cells can also be differentiated from Th1 and Th2 cells by the expression of surface markers. In 2007 different groups reported that human memory CD4+ T-cells producing IL-17 (Th17 cells) express the chemokines receptors CCR6 and CCR4, whereas CD4+ T-cells producing IFN-γ (Th1 cells) express CXCR3 and do not express CCR6. CD4+ T-cells producing IL-4 (Th2 cells) expressed CCR4 and did not express CCR6. Another group of CD4+ T-cells producing IL17 and IFN-γ (Th1Th17) express CCR6 and CXCR3(296, 297).

In terms of function, Th17 cells have been associated with both mucosa protective and inflammatory processes. In the first case, Th17 cells were denominated “non-pathogenic or homeostatic Th17” and have been reported to be enriched in tissue barriers and give protection against pathogenic bacteria and fungi(298, 299). In the context of a pro-inflammation program, Th17 cells are known as pathogenic or Th1/Th17 cells, and are implicated in autoimmune diseases such as EAE in mice and psoriasis, psoriasis arthritis, ankylosing spondylitis, multiple sclerosis and inflammatory bowel disease in humans(300).

2.1.1. Cytokines involved in Th17 differentiation

IL-23 alone could not drive Th17 differentiation, although it maintains Th17 lineage specification, suggesting that other factors were required for its polarization(301). It was identified that Th17 differentiation is induced by TGF-β and IL-6(302, 303). The ablation of TGF-βr or TGF-β gave mice resistance to develop EAE and also weak Th17 responses(304, 305). On the other hand, mice that overexpressed TGF-β developed more severe EAE and had an increased frequency of Th17 cells(306). In addition, the block of IL-6 prevented the development of Th17 cells *in vitro*. IL-21, a family of the IL-2 cytokine family, is induced by IL-6 during Th17 differentiation and acts as an autocrine cytokine that promotes the Th17 program(303, 307, 308). IL-1 is another cytokine that strengthens the Th17 program at the early and late stages of differentiation. IL-6 induces IL-1 receptor (IL-1R) after Th17 induction. Mice that lack IL1-R showed fewer symptoms in the EAE model(309).

The microenvironment and cytokines in which Th17 cells differentiate contribute to whether the cells will have a pathogenic profile. As an example, after adoptive transfer, Th17 cells that were *in vitro* differentiated in the presence of IL-6, IL-1β and IL-23 can induce more severe EAE, whereas Th17 cells that were differentiated with IL-6, IL-1β and TGF-β were poorly pathogenic and did not induce EAE(310).

2.1.2. Transcription factors and Th17 cells

In 2006, the group of Dan Littman identified the rar-related orphan receptor C (RORC) as a master regulator of Th17 cell differentiation(311). They demonstrated that RORγT is induced by TGF-β and IL-6. CD4+ T-cells that did not express RORγt were incapable of differentiating in Th17 cells and lacked expression of IL-17A and IL-17F in response to TGF-β and IL-6 or IL-21(303, 307, 311). RORγT/RORC2 mediates its activity through the binding to consensus DNA elements in gene promoters named nuclear receptor-responsive elements (NRREs) via its ligand-binding domain (LBD)(312). RORγT/RORC2 does not act alone but also depends on a complex network of transcription factors such as STAT2, MAF, BATF, and IRF4.

Similar to RORC2, STAT3 is unconditionally required for Th17 development. Mice that lack the expression of STAT3 in T-cells showed defective Th17 differentiation and were protected against EAE(313). In contrast, mice that overexpressed a hyperactive form of STAT3 had a potentiated Th17 development(314). Additionally, IL-6 regulation of Th17 differentiation is mediated by STAT3(303, 314, 315), and downstream, its signaling pathway STAT3 modulates the upregulation and stabilization of RORγT/RORC2 expression(314). Strengthen the relevance of STAT3 on Th17 differentiation; it has also been shown that STAT3 initiates the transcriptional program of Th17 cells and then is tuned by RORγT/RORC2(312).

RORα, another transcription factor that binds to NRREs, is also highly expressed in Th17 cells and showed redundant and similar functions compared to RORγT/RORC2(316). However, RORα deficiency only partially reduces IL-17 expression.

The importance of RORC2 and STAT3 in regulating the role of Th17 cells in the maintenance of mucosal surfaces is well highlighted by the study of people with mutations in Rorc and Stat3 genes(317-319). Indeed, mutations in STAT3 are presented clinically as the autosomal dominant hyper-IgE syndrome (HIES, 'Job's syndrome). People with HIES syndrome present recurrent pulmonary infections, staphylococcal abscesses, and mucocutaneous candidiasis. CD4+ T-cells of HIES individuals failed to produce IL-17 upon mitogenic stimulation with staphylococcal enterotoxin B (SEB) or upon antigen stimulation with *Candida albicans*. Additionally, *in vitro* experiments have shown that isolated naïve T-cells cannot differentiate into Th17 cells and have lower expression of RORC2. Compared to healthy individuals, people with STAT3 mutations did not show a decreased number of IFN-γ CD4+ T-cells after SEB stimulation(319). Furthermore, 7 individuals carrying loss-of-function mutations in RORC2(RORC2-/-) suffered from infections with *Candida albicans* and *Mycobacterium tuberculosis.* These observations coincided with CD4+ T-cells of RORC2-/- unable to produce IL-17 or differentiate into Th17 cells. These results underscore the importance of Th17 cells in the response against pathogens such as *S. aureus*, *C. albicans, and Mycobacterium tuberculosis* at mucosal surfaces(317) .

The transcription factors IRF4 and BATF are fundamental in the initial chromatin changes necessary for Th17 cell differentiation. After TCR activation, IRF4 and BATF form a complex with STAT3 that permits the recruitment of RORγT/RORC2 to the promoter of Th17-related genes contributing to Th17 differentiation(312).

2.1.3. Effector functions of Th17 cells

The effector functions of Th17 cells are mediated by their secreted cytokines. The non-pathogenic or homeostatic program, which is driven by TGF-β and IL-6 stimuli, promotes the concomitant expression of IL-17, IL-22, and IL-10. Of note, these Th17 cells are enriched in tissue barriers such as the intestine, skin, lung, and oral cavity and protect the mucosal barriers(298, 299, 320). The pathogenic program, on its side, depends on IL-23 and induces the expression of IL-17, GM-CSF, and IFN-γ.

Th17 cells located in the intestine differentiate in response to the microbiota and play an important role in maintaining the gut barrier. In mice, homeostatic Th17 cells that reside in the intestine are induced in response to segmented filamentous bacteria (SFB) and mainly produce IL-17 and IL-22. SFB Th17 cells expressed genes involved in oxidative phosphorylation, (OXPHOS) similar to resting memory cells and an indication of a less inflammatory profile(321). In contrast, the colonization of mice with pathogenic bacteria such us *Citrobacter rodentium* induced a more proinflammatory profile of Th17 cells. *Citrobacter rodentium* Th17 cells produce large amounts of IFN-γ and express the transcription factor T-bet. These cells have a different metabolic profile and are highly glycolytic(321).

In humans, in the context of inflammatory bowel disease (IBD), non pathogenic Th17 cells were identified as CCR6+CCR4+AhR+MAF+IL-10+, whereas pathogenic Th17 cells were identified as CCR6+CXCR3+T-bet+GM-CSF+. These pathogenic Th17 cells expressed the xenobiotic transporter multidrug resistance 1 protein (MDR1), which gives resistance to glucocorticoids(322).

The effect of cytokines secreted by Th17 cells on neighboring cells determines their protective or inflammatory function. An example is the effect that IL-17 exerts on intestinal epithelial cells to maintain gut barrier integrity. IL-17A signals through the receptors IL17RA and IL-17RC and induce the expression of tight junctions such as claudin-1, ZO-1, and occludin on intestinal epithelial cells to maintain the structure of the epithelial monolayer(323). IL-17A also induces chemokines CXCL1, CXCL2, CXCL5, and IL-8 to recruit neutrophils(324). Furthermore, IL-17A reinforces the Th17 response through the expression of CCL20, which induces the migration of CCR6+ Th17 cells(325). IL-17A also mediates the defense against extracellular pathogens by secretion of antimicrobial peptides such as defensins and cathelicidins from intestinal epithelial(324). In line with this, IL-22 has similar functions to IL-17 and promotes epithelial cell proliferation and induces the secretion of antimicrobial peptides to limit bacterial dissemination(324).

2.2. Th17 cells and HIV pathogenesis

During acute and chronic phases of HIV/SIV infection, CD4+ T-cells are predominantly depleted in the gastrointestinal tract(195). A severe loss of CD4+ T cells was observed in the intestinal lamina propria of people living with HIV (PLWH) with chronic HIV infection, indicating this anatomical compartment as a preferential site of HIV replication(326). In SIV-infected rhesus macaques, it was observed that this depletion occurred very early after infection(197). Later studies also showed a depletion of CD4+ T cells in the sigmoid mucosa of PLWH during hyperacute HIV infection, which then progresses over time(327). Supporting the hypothesis that the intestinal mucosa is a major site of HIV replication, it was found that CD4+ T cells of the gastrointestinal tract harbor more HIV-1 DNA and RNA than CD4+ T cells from peripheral blood(328). Additionally, CD4+ T cells in the gastrointestinal tract express CCR5, which is an HIV-1 co-receptor and permissive factor for infection, making them a target of HIV-1(329).

For the first time, in 2008, it was reported that the Th17 response was impaired in the intestine of SIV-infected rhesus macaques (RM). Briefly, after exposure to Salmonella Typhimurium, SIV-infected RM have a diminished expression of IL-17 and IL-22 in the intestine compared to uninfected RM(330). Similarly, SIV-infected RM also had lower frequencies of Th17 cells in the large and small bowel and negatively correlated with plasma viremia, indicating their importance in viral control(331). Supporting the significance of Th17 cells in viral control, it was observed that SIV-infected sooty mangabeys (SM), which do not develop chronic immune activation or progress to AIDS, have the pool of Th17 cells preserved in the intestine(200).

In parallel, it was also found that Th17 cells were lost in the gastrointestinal tract of PLWH during the infection's acute, chronic, and AIDS phases(332). Of note, within Th17 cells, there was a preferential loss of CCR5+ Th17 cells suggesting that the virus played a direct role in their depletion(200). Pathogen-specific Th17 cells were also affected through the course of HIV infection. During acute infection, HIV-specific Th17 cells are expanded and subsequently depleted during chronic HIV infection. Th17 cells specific for other pathogens, such as CMV, were also not found during chronic disease(333). Since Th17 cells play a crucial role in maintaining the gut mucosal barrier, the depletion of Th17 cells is attributed as a cause of the observed microbial translocation and chronic immune activation in PLWH(334).

Multiple mechanisms can explain the preferential depletion of Th17 cells in the intestine. Peripheral blood Th17 cells that exhibit gut homing potential expressed higher levels of CCR5 and were highly permissive to HIV replication in vitro compared to other Th subsets(335-337). Peripheral and gut Th17 cells are also enriched in HIV dependency factors (HDFs) acting at post-entry levels such as PI3K/Akt/mTORC1 that can be targeted to limit HIV replication(338, 339). Therefore, mTOR inhibitors blocked HIV replication in vitro and viral reactivation in CD4+ T-cells from PLWH receiving ART. Th17 cells also have a diminished expression of the RNase A superfamily, which has been reported to inhibit HIV replication(340).

The impairment of Th17 cells migration from the blood to the intestine is another mechanism contributing to the scarcity of Th17 in the gut during HIV infection. The CCR6/CCL20 and CCR9/CCL25 chemotaxis axis, which are determinants for the migration of Th17 cells into the intestine, is altered in PLWH under ART with intestinal epithelial cells producing diminished amounts of CCL20 and CCL25(341). Notably, the defective gut homing of Th17 cells correlates with increased concentrations of markers of microbial translocation and systemic T cell activation(342).

IDO-1 metabolizes tryptophan and generates catabolites that enhance the proportion of CD4+CD25 T-cells expressing FoxP3 and deplete Th17 cells. IDO-1 activity is elevated in PLWH compared to HIV-uninfected individuals(343), and this is linked to the diminished host's capacity to generate Th17 cells and favored the generation of Regulatory T cells (Tregs). Furthermore, Treg cells play an immunosuppressive role that limits anti-HIV responses(344).

Studies by our group and others demonstrate that Th17 cells contribute to the latent HIV reservoir. In the blood and colon of PLWH memory CD4+ T-cells expressing CCR6, which is the Th17 hallmark phenotypic surface marker, were enriched in HIV DNA compared to CCR6- memory CD4+T-cells(345). Similarly, CCR6+ CXCR3+ CD4+ T-cells had a more significant contribution of integrated HIV DNA in rectal tissue than other CD4+ T-cell subsets(346).

Overall, the involvement of Th17 cells in HIV pathogenesis and persistence makes them an attractive therapeutic target to improve mucosal immunity and persistence during HIV infection. This topic was extensively reviewed in a review article recently published by our group(347).

2.3. Intestinal Epithelial Cells and HIV

The crosstalk between intestinal epithelial cells (IECs) and Th17 CD4+ T-cells is critical for mucosal homeostasis. Among anatomic sites targeted by HIV/SIV infection, the GALT plays a unique role

during primary viral acquisition, as well as chronic infection(195, 348). Indeed, the rectal mucosa composed of a monolayer of intestinal epithelial cells (IEC) facilitates HIV acquisition(349, 350), while GALT-infiltrating Th17-polarized CD4+ T-cells are enriched at portal sites of viral entry and represent the first targets for viral acquisition and persistence during acute and chronic infection, respectively(345, 351-353). By its capacity to target both IEC and Th17 cells, HIV causes profound alteration of the GALT physical and biological barrier functions(195, 351). Such alterations are observed during the very early acute phases of infection and are not fully restored by viral-suppressive ART, even with early ART initiation(327, 354-356).

Studies by multiple groups led to the establishment of an HIV transmission model in which the virus crosses the rectal epithelial cell monolayer by transcytosis and infects subjacent immune cells, such as dendritic cells, CD4+ T-cells or macrophages(357-360). Of note, primary IEC selectively transfer R5 tropic HIV to T-cells via the process of transcytosis(361, 362), with the formation of virological synapses between T-cells and IEC being visualized(358). During transcytosis, HIV first binds to surface molecules on the rectal epithelium such as the heparan sulfate proteoglycan "agrin" and galactosylceramide (GalCer) and then is internalized into endosome-like structures to cross the epithelium and be released in the subjacent space as a result of transcytotic vesicle fusion with the basolateral membrane(363-365). Experiments in vitro have shown that cytokines such as TNF-α and IFN-γ trigger the re-organization of the cortical actin cytoskeleton, thus increasing the release of viral particles through the basal and apical membrane and facilitating HIV transmission to CD4+ T-cells. Such cytoskeleton re-organization triggered by IEC interaction with T-cells is mediated by the adhesion molecules ICAM-1 and LFA-1(366, 367).

Multiple in vitro models allowed us to better understand the permissiveness of IEC to HIV-1 infection. In the context of IEC lines, it was reported that the permissiveness to HIV-1 infection is influenced by the viral titer they are exposed to. A high titer will induce a productive infection, whereas a low titer will induce a latent infection (367-373). In addition, the co-culture of latent IEC lines with T-cells will result in a productive infection(374). Distinctively, when primary

epithelial cells are used, studies showed that they were not permissive to infection with primary HIV-1 isolates but only with laboratory-adapted strains(362, 375).

HIV-1 cause a direct dysregulation of pathways involved in the epithelial barrier maintenance and a down-regulation of barrier structure proteins and tight junctions such as zonulin, claudins, and occludins(355, 376-378). Recent studies demonstrated that such alterations occur before the depletion of Th17 cells in the intestinal mucosa of SIV-infected macaques(379). In addition, IEC apoptosis was observed at high levels in untreated PLWH with high viremia and to a lesser extent in virally-suppressed ART-treated PLWH(380-382). Of particular note, the degree of epithelial cell damage and apoptosis correlated to systemic inflammation and microbial translocation(383).

3. Cardiovascular Disease and HIV

Cardiovascular diseases (CVDs) include a group of conditions that affect the heart in addition to the blood vessels and mainly refer to the following entities: coronary artery disease (CAD), cerebrovascular disease, peripheral artery disease, and aortic atherosclerosis. Coronary artery disease is caused by a diminished myocardial perfusion that results in angina, myocardial infarction, and/or heart failure. As for cerebrovascular diseases, they include both strokes and transient ischemic attacks. Regarding the peripheral arterial diseases, they result mainly in the claudication of arteries in the limbs. Finally, aortic atherosclerosis involves thoracic and abdominal aneurysms and can cause embolies. The underlying cause of CVDs regardless of their manifestation is atherosclerosis(384).

Atherosclerosis of the coronary artheries and stroke are the two leading causes of death in the world. Most deaths from CVD (75%) occur in low-income and middle-income countries(385). This in part due to limited access to healthcare and detection of cardiovascular disease at late stages. Although there is an overall decrease in cases of heart disease and stroke due to interventions aimed at decreasing risk factors (tobacco use, obesity, hyperlipidemia, hypertension and alcohol use) CVD places a heavy burden on the economy of developed and developing countries(385).

3.1. The physiological state of arteries

A normal artery has three layers that are distinct in their function and architecture: the innermost intima, which is in contact with the bloodstream, the tunica media, and the outer layer called the adventitia(386). The adventitia consists of elastin and collagen fibers, and fibroblasts whereas the tunica media comprises collagen, elastin, other elastic fibers, and smooth muscle cells. As for the intima layer, it is composed of endothelial cells and connective tissue.

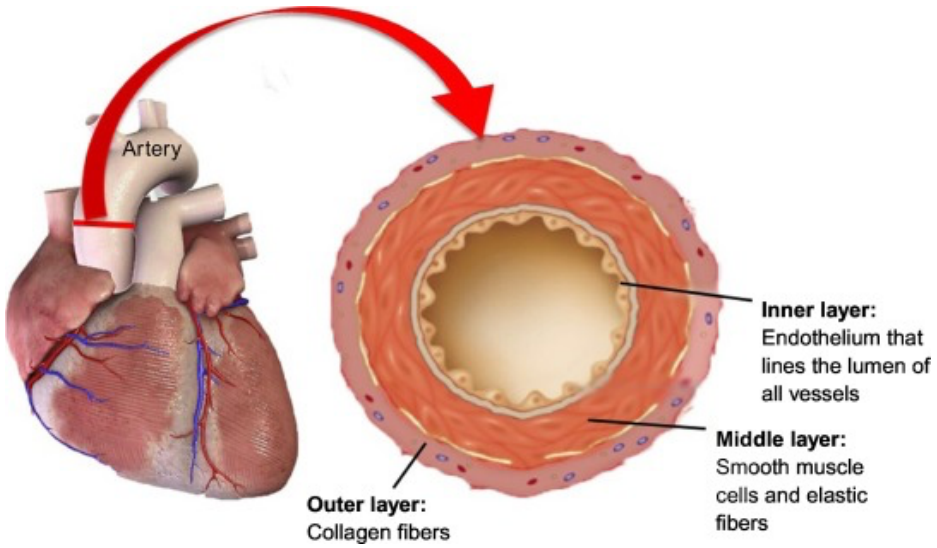

Figure 6: Aorta Artery structure

The aorta artery is divided into three layers, the outer layer or adventitia, the middle layer or tunica media and the inner layer or innermost intima, which all differ structurally and functionally from one another. Reprinted by permission from Elsevier, License Number 5411030088643, A. Taki, A. Kermani, S.M. Ranjbarnavazi, A. Pourmodheji, Chapter 4 - Overview of Different Medical Imaging Techniques for the Identification of Coronary Atherosclerotic Plaques, Editor(s): Simone Balocco, Maria A. Zuluaga, Guillaume Zahnd, Su-Lin Lee, Stefanie Demirci, In The Elsevier and MICCAI Society Book Series, Computing and Visualization for Intravascular Imaging and Computer-Assisted Stenting, Academic Press, 2017, Pages 79-106, ISBN 9780128110188, https://doi.org/10.1016/B978-0-12-811018-8.00004-7.

The endothelium of the intima layer is a continuous cellular lining that separates the vascular from the interstitial compartment and regulates the transport of macromolecules and fluids. It produces and degrades vasoactive mediators, buffers reactive oxygen species, transports

lipoproteins, and produces growth factors, cytokines, and hormone-like substances(387). Moreover, in a healthy context, the endothelium has anticoagulant and fibrinolytic properties(387). By producing this variety of factors, endothelial cells impact on cells within the vessel wall, such as smooth muscle cells and circulating cells such as leucocytes(388).

Furthermore, endothelial cells have the capacity to metabolize L-arginine to form Nitric oxide (NO) using the enzyme endothelial NO synthase (eNOS)(389). The synthesis of NO by endothelial cells influences other cell types through the activation of guanylate cyclase(390). NO inhibits the activation, adhesion, and aggregation of platelets and decreases the adhesivity of leukocytes(391-393). Importantly, the antagonist of NO increases vascular resistance and blood pressure(394).

Under homeostatic conditions, the endothelium does not congregate leukocytes. However, In response to bacterial products such as gram-negative endotoxins, pathogen-associated molecular patterns (PAMPs), oxidized LDL (lysophosphatidylcholine), damage-associated molecular patterns (DAMPs), or cytokines including TNF, IL-1 or IFN-γ, the endothelium activates the NF-kB pathway(395-397). Their engagement into this pathway induces the expression of pro-thrombotic mediators (Tissue factor, von Willebrand Factor and plasminogen activator inhibitor), adhesion molecules (VCAM-1 and ICAM-1), and chemokines (MCP-1 and fractalkine) that promote the adherence and migration of leukocytes (monocytes and lymphocytes) into the intima(398-403). Monocytes that have migrated into the intima mature into macrophages and engulf lipoproteins to become foam cells(404). The transmigration of monocyte is mediated by chemokine receptor families (CCR2, CCR5, and CX3CR1)(405, 406). Smooth muscle cells can also migrate from the tunica media into the intima. This is orchestrated by the secretion of growth factors from recruited leukocytes and activated endothelial cells. Smooth muscle cells can behave like macrophages and engulf lipoproteins to give rise to foam cells(407).

3.2. Overview of the pathophysiology of atherosclerosis

In the context of atherosclerosis, an accumulation of fatty and/or fibrous material injunction with immune cells infiltration is observed in the intima layer of mid-and large-sized arteries. This deposit, called plaque, can progressively calcify and, as it grows, can impair the blood flow resulting in tissue ischemia. A second, more acute path to ischemia is the rupture of an atherosclerotic plaque which causes thrombus formation and arterial lumen obstruction. The development process of atherosclerosis can be divided into initiation and progression(385, 408).

3.2.1. Initiation

Atherosclerosis is driven by different pathophysiological stimuli such as hypercholesterolemia, systemic inflammation (pro-inflammatory cytokines), hypertension, oxidative stress, infectious agents, environmental toxins, and hemodynamic forces, among the most relevant. Those stimuli contribute to endothelial cell dysfunction in the intima and initiation of atherosclerosis(387).

Although still not fully understood nowadays, low-density lipoprotein particles- cholesterol (LDL-C) is one of the particles that, in excess (hypercholesterolemia), leads to the initiation of atherosclerosis. Shaped as a sphere, it is composed of a monolayer of phospholipids, a surface membrane made of unesterified cholesterol forms, and a hydrophobic core made of fatty acid esters of cholesterol. In addition, apolipoprotein (apo) B surrounds the equatorial region(409). The accumulation of LDL-C in the intima prevents the antioxidants from acting on it, and the oxidation of this molecule grants it pro-inflammatory and immunogenic faculties(410). Other studies have suggested that native instead of oxidized LDL induces the activation of the adaptive immune response(411). In either case, its accumulation is mediated by the retention or trapping of apoB lipoproteins in the subendothelial compartment and not by an increased permeability or flux of LDL particles(412).

Another important component in the development of atherosclerosis is the alterations in the endothelium caused by inflammation(413). Inflammation-inducing conditions that have been

described as risk factors are hypertension, tobacco use, and metabolic syndrome(414). The contribution of hypertension to this phenomenon is seen through its activation of angiotensin II and the NF-κB pathway(414). For its part, Tobacco consumption induced inflammatory pathways in the lungs (increased oxidative stress, and activation of neutrophils, monocyte, platelets, and T-cells) that can also trigger damage in the endothelium(415). As for the metabolic syndrome, it is associated with visceral adipose tissue, which can produce inflammatory mediators (TNF-α, IL-1 and IL-6)(416). Additionally, inflammation interferes with the production of nitric oxide by endothelial cells contributing to the development of atherosclerosis(417).

Finally, hemodynamics can also influence the development of atherosclerosis(387). Early atherosclerotic lesions occur in regions with disturbed laminar flow patterns. There are shear stress response elements in promoters of genes such as eNOS and VCAM-1(418, 419). Disturbed flow increases oxidative stress, alters the cytoskeleton and junctional proteins organization, and induces IL-8 and VCAM-1(420). Laminar flow or atheroprotective waveform induces the expression of eNOS and has an antithrombotic, antiadhesive, anti-inflammatory, and antioxidant effect on endothelial cells, platelets, leukocytes, and vascular smooth muscle cells. Laminar flow induces the activation of the transcription factor Kruppel-like factor 2 (KLF2), which is an antagonist of the NF-κB pathway and provides to endothelial cells atheroprotective properties(421, 422).

3.2.2. Progression

The progression is defined by the accumulation of lipids and cells containing lipids in the atherosclerotic plaque(385). This dynamic process intercalates periods of quiescence and periods of fast growth(423).

The different cells that migrate and form part of the plaque contribute in various manners to the atherosclerotic plaque progression. For example, after having migrated to the intima and engulfed lipid particles, smooth muscle cells can proliferate or go through metaplasia to become macrophage like-cells. The ones that reside in the intima produce extracellular matrix

macromolecules such as interstitial collagen, elastin proteoglycans, and glycosaminoglycans, entrap lipoproteins leading to lipid accumulation and progression of atherosclerosis(407). Under normal circumstances, macrophages and smooth muscle participate in the clearance of dead cells by efferocytosis. During the progress of atherosclerosis, the alteration of this process leads to the accumulation of dead cells and the formation of a central necrotic core in the atherosclerotic plaque(424).

In addition to macrophages, leukocytes that have migrated into the plaque can proliferate and be retained inside the plaque by semaphorins and netrin, which are retention factors secreted by endothelial cells(425-427). Among those leukocytes, are the T lymphocytes which can have a positive or negative effect on the evolution of atherosclerosis. Indeed, by producing IFN-γ Th1 cells promote atherosclerosis(428, 429), whereas the Th2 cells and the Tregs that respectively produce the anti-inflammatory cytokine IL-10, and TGF-b limit inflammation and smooth cell proliferation(430, 431).

Most recently, a new condition called *Clonal hematopoiesis of indeterminate potential* (CHIP) has been associated with excess mortality attributed to CVD causes and atherosclerosis. This condition could contribute to the evolution of atherosclerosis in certain individuals(432, 433). This condition is characterized by an accumulation, with age, of somatic mutations in genes such as DNMT3A, TET2, ASXL1, and JAK2, which provide a proliferative advantage and give rise to myeloid clones(434). These mutations also change the methylation profile of DNA and alter the expression of pro-inflammatory genes such as IL1B. In addition, a mutation in JAK2 gives leukocytes an increased capacity to form extracellular traps and promote thrombosis(435).

Furthermore, as briefly mentioned previously, during the progression of atherosclerosis, some regions within the atherosclerotic plaque will calcify. These calcifications result from dysregulation of deposition and impaired clearance(385, 436). The calcifications can be microscopic or large accumulations of calcium. The microscopic ones have more instability and

are more prone to rupture, whereas the extensive calcifications are more stable and have a lower probability of rupture(437, 438).

3.3. Role of Innate and adaptive immunity in atherosclerosis

Innate immune cells such as monocytes and macrophages play a key role in atherosclerosis progression. As briefly described earlier, monocytes have the ability to migrate to the plaque and differentiate into macrophages, where the latter will engulf lipids and be determinant in the development of the plaque. On their side, CD4+ T-cells accumulate in the plaque and drive the inflammatory process.

3.3.1. Monocytes

Monocytes originate from bone marrow precursors. One of the main functions of monocytes is to patrol the body for microbial cells and organize an immune response during inflammation and infection. In humans, monocytes can be classified into three subsets based on the expression of the CD 14 and 16: classical (CD14++CD16-), which represent 90% of circulating monocytes, whereas intermediate (CD4++CD16++) and non-classical (CD14+CD16++) accounts for the remaining 10%(439-441). CD14 is part of the LPS receptor complex whereas CD16 (FcγRIIIa) is a low-affinity IgG receptor mediating antibody-dependent cellular cytotoxicity(441-444). In addition to these two markers, it was later discovered that the sugar structure 6-sulfo LacNac (slan), which is attached to the P-selectin glycoprotein ligand (PSGL-1) and recognized by the monoclonal antibody M-DC8, defined most non-classical monocytes as slan+ and the intermediate monocytes as slan-(445, 446). Monocyte subsets also express differentially the chemokine receptors CCR2 and CX3CR1, known to regulate their trafficking and recruitment to tissues. More precisely, CCR2 interaction with its ligand CCL2/MCP-1 is central for monocyte emigration from the bone marrow(447-449). Classical monocytes highly express CCR2 and no CXC3CR1, intermediate monocytes exhibit both CCR2 and CX3CR1, and non-classical monocytes present high levels of CX3CR1 and no CCR2 at their surface(440, 448, 450, 451). In mice, the equivalent of classical monocytes expresses CX3CR1int, Ly6Chi, CCR2+ and are called Ly6Chi

classical monocytes, whereas the non-classical monocytes express CX3CR1hi Ly6Clo, CCR2- CD62L- CD43hi. The CX3CR1 GFP knockin mouse model allowed major discoveries in the field of monocyte heterogeneity, trafficking and division of labor and allowed the understanding of the origin and differentiation fate of monocyte subsets(440, 452, 453).

Using *in vivo* human deuterium labeling, it was shown that the generation and differentiation of monocytes follow a sequential transition from classical to intermediate and then, to non-classical profile. More precisely, classical monocytes emigrate from the bone marrow and circulate in the bloodstream for 24 hours. At that time, they can disappear by death or migrate or differentiate into intermediate monocytes. Intermediate monocytes have a lifespan in the periphery of four days and as the previous subset, they can afterward die, migrate into tissues, or differentiate into non-classical monocytes. The latter has a lifespan in the circulation of approximately seven days(454).

The development of high-throughput technologies allowed the determination of additional features of the different monocyte subsets. Early studies found that 87% of the expressed genes in intermediate monocytes were at an intensity between that of classical and non-classical monocytes in congruence with the sequential differentiation of these cells. Furthermore, they had the highest expression of MHCII and CLEC10A. Of note, CLEC10A is a surface receptor known to induce cytokine production. Intermediate monocytes are good at antigen presentation in congruence with higher expression of HLA-DR and CD74. They also vividly express CD40, which is a co-stimulatory molecule that induces T cell proliferation(439). In response to LPS, intermediate monocytes are the main producers of TNF-alpha and produce IL-6 and CCL3(455).

On their side, non-classical monocytes present an enriched expression of genes related to cytoskeletal rearrangement, migration, and inflammatory cytokine production along with a sustained expression of CD294 and Siglec10. CD294 promotes the chemotaxis of leukocytes during allergic responses, while Siglec10 is an inhibitory receptor of innate immune cells.

Regarding their migration, non-classical monocytes depend on CX3CL1 binding of its cognate receptor CX3CR1 while in circulation for trafficking, homeostasis, and survival. CX3CL1 also inhibits apoptosis and oxidative stress(451, 456). Additionally, non-classical monocytes showed a crawling behavior on the endothelium. CD16+ monocytes are more mobile than classical monocytes and survey the luminal side of vascular endothelium in an LFA-1 and integrin-alpha4-dependent manner and using the chemokine receptor CX3CR1(457). In regards to their inflammatory profile, these monocytes have a poor response to Toll-like Receptor (TLR) 1, TLR-2, and TLR-4 ligands, but produce pro-inflammatory cytokines TNF-alpha, IL-1β, and CCL3 in response to the virus via TLR-7 or TLR-8 stimulation(458). They also play a crucial role in vascular homeostasis by recognizing and clearing endothelial cells in a TLR7-dependent manner. These features give them the name of "patrolling" monocytes in humans(458), by analogy to "patrolling" monocytes initially described in mice(457).

As for classical monocytes, they showed preferential expression of transcription factor AP-1 genes and IL-13Rα1 surface expression(439). Classical monocytes have high phagocytic capabilities (phagocyte pathogens), produce reactive oxygen species (ROS), secrete cytokines, and orchestrate the recruitment of other immune cells(447). These cells are weak producers of TNF-alpha *in vitro* and are reportedly producers of IL-6, IL-8, and CCL2 in response to LPS(458). During infection, they emigrate from the bone marrow using CCR2(449). Mice studies have also shown that classical monocytes survey steady-state tissues such as the lungs or skin without becoming macrophages or dendritic cells and transport antigens to the lymph nodes(459).

Comparing classical monocytes (CD16- monocytes) with total CD16+ monocytes (non-classical and intermediate), it was shown that CD16+ monocytes exhibited an up-regulation of transcripts related to dendritic cells (SIGLEC10, CD43, RARA), macrophages (CD115, CD97), DC-T cell interaction (ICAM-2, LFA-1), and cell activation markers, whereas CD16- monocytes had a profile of transcripts related to myeloid (CD14, MNDA, TREM1) and granulocyte (S100A8-9/12) markers. These results suggested a more differentiated state of CD16+ monocytes towards a dendritic cell macrophage program(460).

Most recently, blood monocytes underwent single-cell RNA seq after cell sorting of classical, intermediate, and non-classical monocytes. Based on their gene signatures, blood monocytes clustered in four different populations. Two of these populations contained classical and non-classical monocytes, with most non-classical monocytes distributed among both populations. These results support the transitional differentiation state of intermediate monocyte between classical and non-classical monocytes. Interestingly a third and fourth populations were identified with unique gene signatures within the sorted intermediate monocytes. One cluster expressed a combination of genes with the potential to affect cell cycle, differentiation, and trafficking (e.g., MXD1, CXCR1, CXCR2, VNN2), and the other cluster expressed a cytotoxic gene signature like previously reported: “natural killer dendritic cells”(461).

3.3.2. Monocytes in atherosclerosis

As previously described, blood monocytes are phagocytes that participate in the innate response to fungal, bacterial, parasitic, and viral infection. Monocytes also contribute to the pathogenesis of autoimmunity, cancer, and atherosclerosis(462-465). In the context of atherosclerosis, population studies determined that monocyte counts could be a predictive factor for atherosclerotic plaque formation in people without comorbidities or pre-existing carotid atherosclerosis(466, 467). In line with this observation, a reduction of circulating monocytes in animal models limited plaque development(468, 469). The contributions of monocyte subsets to atherosclerosis have been associated with their differential expression of chemokine receptors. More precisely, in population studies, polymorphisms in CX3CR1 were associated with a reduced risk of coronary artery disease or acute coronary events(469, 470). Similarly, polymorphisms in CCR2 were associated with a reduced coronary artery calcification(471).

These reported observations on polymorphism drove the interest in studying genetic deletions of chemokine receptors in mice models of atherosclerosis. Indeed, studies using genetic deletion of chemokine receptors or chemokines in mice have shown their importance in the adhesion and

trafficking of monocytes to the endothelium in the early stage of the disease. One of the frequently used models in that aspect is the ApoE knock-out ($ApoE^{-/-}$) mice which spontaneously develop atherosclerosis(472). In these mice, the lack CX3CR1 induced a reduction of lipid lesions and a decrease of 50% of macrophage infiltration in the aorta compared to $ApoE^{-/-}$ mice. Of note, a part of the intralesional macrophages is derived from blood-born macrophages(473). Similar results were obtained with mice depleted fpr CCR2 and ApoE ($CCR2^{-/-}/ApoE^{-/-}$) with an approximate 50% reduction of the arterial macrophage infiltration(474).

Another well-described mouse model of atherosclerosis is the LDL-receptor (LDL-R) deficient ($LDL\text{-}R^{-/-}$) animals. As the LDL-R is responsible of removing LDL from the blood, these mice have high LDL levels and accumulate considerable fat in their aortas when fed with high cholesterol diets(475). Mice deficient for CCL2 and LDLR-R ($CCL2^{-/-}LDL\text{-}R^{-/-}$) exhibited an 80% reduction in macrophage infiltration in the atherosclerotic lesions(476). Other chemokines receptor such as CCR5 was also proposed to affect the development of atherosclerosis in mice models(477). The combined inhibition of CCL2, CX3CR1, and CCR5 in $ApoE^{-/-}$ mice led to an additive reduction in atherosclerosis which was nearly absent(405). A subsequent study in mice confirmed that the migration of classical monocytes was dependent on CCR2, whereas non-classical monocytes migration relied, at least in part, on CCR5 rather than on CX3CR1. These studies postulated that the lack of these chemokines' receptors might prevent the adhesion and trafficking of monocytes and its subsequent differentiation in monocyte-derived cells(477).

Most recently it was observed in mice that non-classical monocytes could also play an atheroprotective role. Using cell live imaging system, it was observed that non-classical monocytes rather remain within the blood compartment and crawl into blood vessels with plaque formation(478). Mice that lacked non-classical monocytes (Nr4a1/ApoE-/-) had increased apoptotic damage in the vessel wall in the early stages of atherosclerosis(464, 479). The patrolling of non-classical monocytes is driven by the scavenger receptor CD36, which mediates the uptake of Oxidized LDL (OxLDL)(480).

In humans, an elevated frequency of circulating non-classical monocytes (CD14dimCD16++) was associated with an amplified carotid intima-media thickness (IMT) over 10 years in a prospective cohort study. The baseline levels of non-classical monocytes did correlate with baseline carotid IMT meaning that non-classical monocytes could be considered as a biomarker of early, preatherosclerotic lesions. Additionally, classical monocytes were associated with IMT regression. These observations were only applicable for men and not for women(481). Another study that used high-dimensional single-cell mass cytometry to determine the heterogeneity of human blood monocytes subsets found that participants with coronary artery disease had an expansion of Slan+CXCR6+ non-classical monocytes, and it was positively correlated with disease severity. Macrophages in plaques secrete CXCL16 and attract this population which is probably involved in the clearance of apoptotic cells from the necrotic core(482-484). Of note, among human monocyte subsets, the CD16+ monocytes are the source of CXCL16(460). Intermediate monocytes (CD14+CD16+) independently predicted cardiovascular events in subjects referred for elective coronary angiography. In contrast, classical monocytes (CD14+CD16-) were not associated with vulnerable plaque phenotype and did not predict secondary events in patients with severe atherosclerosis(485, 486).

Monocytes are also involved in how extramedullary hematopoiesis contributes to atherosclerosis. Stem cells can also exit the bone marrow for immunosurveillance and either come back to the bone marrow or, under specific circumstances, settle in secondary lymphoid organs and give rise to their progeny. This process, named extramedullary hematopoiesis, can occur in the spleen under stressful circumstances and, as a result, can allow the production of myeloid cells at this site. The chronic inflammation observed in atherosclerosis promotes a continuous mobilization of stem and progenitor cells to the spleen. Splenic monocytes proliferate robustly during hypercholesterolemia. Additionally, fate-mapping studies show that extramedullary-derived monocytes migrate to the atheroma, becoming lipid-laden foam cells(487).

3.3.3. Macrophages

Macrophages are myeloid cells distributed throughout all the tissues, where they ingest and degrade dead cells and debris and organize inflammatory processes. Eli Mechnikov discovered macrophages in the 19th century and was awarded the Nobel Prize in 1908 for this discovery. Macrophages can play antagonistic roles as an anti-inflammatory mediator of tissue repair and a pro-inflammatory mediator in infection and inflammation(488). Those innate immune cells have organ-specific functionalities and receive a different name according to their location. In the liver, they are called Kupffer cells; in the lung, alveolar macrophages; and in the central nervous system, microglia. Macrophages in different tissues share more differences in their transcriptional program than similarities(489).

The knowledge of the origin of macrophages has evolved over the last decades. Initially, the paradigm was that the sole source of macrophages was monocytes exiting from the circulation and differentiating in the tissues. This population was referred to as monocyte-derived macrophages (MDM)(490). Most recently, it has been shown that macrophages residing in tissues in steady-state homeostasis (tissue-resident macrophages, TRM) derive from two precursors during embryonic development(491). Macrophages originated from the yolk sac (YS) give rise to microglia(492) and the ones derived from the fetal liver and generated from erythro-myeloid progenitors produce macrophages located in the liver, brain, lung, and skin. These macrophages have long-lasting and self-renewing properties(493-496). In other tissues such as the gut, tissue-resident macrophages coexist with monocyte-derived macrophages(497-499). In homeostasis, tissue-resident macrophages clear apoptotic cells and proteins or either clear or respond to toxins and pathogens. They also crosstalk with other immune cells, epithelial cells, endothelial cells, and fibroblasts.

TRM and MDM have different roles in tissue repair of an injury. During an injury, both subsets have similar qualitative responses related to gene expression, but these responses are more robust in monocyte-derived macrophages. During injury resolution, tissue-resident macrophages

can die or proliferate through self-renewal to repopulate the niche. MDM could sometimes gain self-renewal capacity or undergo apoptosis(491).

3.3.4. Macrophages in atherosclerosis

As described before, the accumulation of lipoproteins in the intima triggers the activation of tissue-resident macrophages and the migration of monocytes that will subsequently differentiate into monocyte-derived macrophages. The clearance of lipoproteins by macrophages does not have negative feedback of uptake. Consequently, macrophages become congested with lipids, transforming into foam cells. In mice, macrophages in the atherosclerotic plaque can have inflammatory or anti-inflammatory features. In the first case, they contribute to atherosclerosis progression, whereas in the latter scenario, they inhibit plaque progression or promote its regression(482, 500).

Inflammatory macrophages are characterized by the expression of MHC class II molecules, CD64 (Fcy receptor I), co-stimulatory molecules CD80 and CD86, inducible Nitric Oxide Synthase (iNOS) or NOS2, inflammasome components, as well as the cytokines IL-6, TNF, and IL-1b. Additionally, inflammatory macrophages are mainly derived from monocytes and are not observed in healthy arteries(501, 502). In addition to the inflammatory subset, other populations of macrophages were described in atherosclerosis. For example, it was reported that Type I IFN-inducible macrophages derived from monocytes are uniquely found during atherosclerosis. These cells express the IFN-related genes such as Ifit3, Irf7, and Isg15(503, 504). In advanced atherosclerotic plaques, TREM2hi macrophages, which can be derived from either monocytes or embryonic precursors, are observed. It was described that they express genes related to lipid metabolism and oxidative phosphorylation(505) and that they contribute to the attenuation of the inflammation. Moreover, Mac air macrophages were documented to play a pro-atherogenic role in the early phases of atherosclerosis. Derived from monocytes, Mac air macrophages express-Itgax, MHC class II, and CCR2(506). Lastly, healthy arteries and atherosclerotic plaques contain resident macrophages which express CX3CR1 and are originated from embryonic precursors.

These cells express macrophage mannose receptor C type I (MRC1) and MHC class II. Additionally, these macrophages can limit collagen production and rigidity.

In humans, the gene expression signature of macrophages in atherosclerotic plaques was explored on carotid endarterectomy plaque specimens from patients with recent cardiovascular events or asymptomatic patients by mass cytometry and single-cell RNA-sequencing. Macrophages in atherosclerotic plaques of individuals with current cardiovascular events expressed genes associated with plaque instability, such as the pro-inflammatory chemokines CCL5 or IL-8 and the Hedgehog and Wnt signaling pathways. Macrophages in plaques of asymptomatic individuals expressed cytokines such as IL-1 and genes related to the production of IL-1β (NLRP3, NFKBIA and IL-1 receptor accessory protein)(507).

3.3.5. CD4+ T-cells in atherosclerosis

The adaptive immune response is vital in the modulation of atherosclerosis progression. So far, most of the functional studies exploring the role of CD4+ T cells in the pathogenesis of atherosclerosis were performed in mice for which the depletion of CD4+ T-cells protected them from developing atherosclerosis(508, 509).

As observed with macrophages, the contribution of CD4+ T-cells subsets to the modulation of atherosclerosis can either result in immune activation or immune suppression depending on the cascade of interactions between CD4+ T-cells and antigen-presenting cells(510). For example, atherosclerosis is halted with the overexpression of co-inhibitory protein CTLA4 in Apoe-/- mice(510). Similarly, the activation of the inhibitory PD1-PDL1 pathway limited T cell responses that promote atherosclerosis(511). In line with this, the blockade of co-stimulatory signals such as CD40-CD40L and CD80/CD86-CD28 interactions between APCs and T-cells attenuated the development of atherosclerosis(512, 513).

Among the CD4+ T-cells that have a role in atherosclerosis, it was reported that Th1 cells promote atherosclerosis and are abundant in the atherosclerotic plaque. In animal models of atherosclerosis, Th1 cells are observed in atherogenic plaques of mice expressing T-bet and secreting IFN-gamma(514, 515). Additionally, LDL-/- mice deficient in T-bet showed a significant decrease in atherosclerosis compared with LDL-/- and a switch to a Th2 protective response(516). Mice lacking IFN-γ or its receptor were also protected against atherosclerosis(429, 517). In line with this observation, the administration of IFN-γ increased the development of atherosclerosis in Apoe-/- mice compared with untreated mice(428). It is reported that IFN-γ mediates plaque instability by inhibiting vascular smooth muscle cells (VSMC) proliferation(518).

Despite this beneficial role of Th2 cells in atherosclerosis, their overall impact in the disease remains unclear in certain aspects. Th2 cells are characterized by the expression of the transcription factor GATA3 and secrete the cytokines IL-4, IL-5, and IL-13. On one side, ApoE(-/-) mice deficient in IL-4 showed a reduction in 27% of their plaque area compared to Apo(-/-) mice(519). In LDL (-/-) mice, the deficiency of IL-4 decreased the extension of the atherosclerotic lesion in the arch and thoracic aorta, but not in the aortic root. These results indicate that IL-4 deficiency reduced atherosclerotic lesion formation in a site-specific manner(520). On the other side, IL-5 and IL-13 were shown to be atheroprotective. Indeed, the administration of IL-13 reduced the plaque macrophage content in atherosclerotic mice by decreasing the expression of VCAM-1. IL-13 also favored the induction of anti-inflammatory macrophages(521). For IL-5, it is described that it protects from the establishment of atherosclerosis by producing protective antibodies against OxLDL(522). Furthermore, the administration of anti-IL-5 prevented the reduction in plaque size(523). These studies suggest that Th2 cells in mice could play an ambivalent role in atherosclerosis dependent on the cytokines secreted. In humans, an increased number of Th2 cells was associated with a decreased carotid intima thickness and reduced risk of acute myocardial infarction. Also, an increased release of IL-4 was associated with a reduced risk of CVD(524) and IL-5 showed an inverse correlation with carotid intima-media thickness(525). Further studies are needed to better understand the role of Th2 cell in atherosclerosis.

As for Th2 cells, the role of Th17 cells during atherosclerosis is not well determined(526). As described in section 2.1, due to the complexity of Th17 cells, some studies have suggested that they can play atherogenic, atheroprotective or be indifferent in the establishment of the disease. For example, Il17a$^{-/-}$Apoe$^{-/-}$ mice and Il17ra$^{-/-}$Apoe$^{-/-}$ mice presented smaller plaques in the aortic arch and aortic roots than Apoe$^{-/-}$ mice. No differences were observed in the plaque burden of thoracoabdominal aorta among those groups(527). In another mice model, the loss of TRIM21 in LDR$^{-/-}$ mice augmented IL-17 production and was accompanied with larger atherosclerotic lesions rich in type I collagen(528). However, in the same study it has been shown that IL-17 promotes the stabilization of atherosclerotic plaques. Furthermore, the administration of IL-17 to Ldlr$^{-/-}$ mice diminished the plaque burden in aortic roots compared to control mice(529).

In population studies, increased plasma levels of pro-inflammatory cytokines IL-17, IL-6, IL-8 and C-reactive protein were observed in patients with unstable angina and acute myocardial infarction, whereas IL-10 concentration was diminished(530). In line with these observations, patients with acute coronary syndrome had increased peripheral Th17 cells, Th17 cytokines (IL-17, IL-6 and IL-23) and transcription factor RORgt and decrease in Treg number, T reg related cytokines and transcription factor FOXP3 compared with controls(531). In organ culture system of human arteries with different grades of atherosclerosis, arteries with gross signs of atherosclerosis produced higher levels of IL-17 and IFN-γ. Further studies should address more deeply the contribution of IL-17 in the pathogenesis and development of atherosclerosis(532).

3.4. CVD, a major non-AIDS co-morbidity

The implementation of ART has significantly diminished AIDS-related mortality and increased life expectancy of PLWH(533). Despite the decreased mortality, PLWH under ART age and develop non-AIDS comorbidities such as CVD earlier than HIV negative individuals. It is noteworthy to mention that CVD is the main cause of morbidity and mortality among PLWH under ART(534, 535).

The increased risk of CVD in PLWH is mediated by traditional risk factors and HIV-related risk factors(536). PLWH have a high prevalence of traditional risk factors such as dyslipidemia, hypertension and smoking(537-539). However, there is an undertreatment of PLWH for these conditions which contributes in part to the increased prevalence of CVD(540). For example, only the third part of PLWH with a high CVD risk and therefore eligible to take statins received this treatment(537).

Among HIV-related risk factors, we have the use of antiretroviral drugs and chronic immune activation(536). One of the mechanism by which ART augments the risk of CVD is through altering lipid levels(536). As an example, protease inhibitors (ritonavir) used in older ART regimens induce hypertriglyceridemia and long exposure to nucleoside reverse transcriptase inhibitors was associated with an increased risk of myocardial infarction(536, 541, 542). Another mechanism by which ART increases the risk of atherosclerosis is by inducing lipodystrophy and insulin resistance(536) as well as platelet reactivity and endothelial dysfunction(543, 544). More precisely, this endothelial dysfunction is specifically associated with the use of abacavir(543, 544). On a more positive note, newer ART regimens have been reported to have a lower contribution to CVD development(536).

On an immune point of view, considering the importance of monocytes in the establishment and progression of atherosclerosis, their alteration in HIV infection can even amplify this disease. Indeed, pioneering studies demonstrated that CD14+CD16++ monocytes were expanded during HIV infection and in patients with AIDS. CD14+CD16++ monocytes also expressed higher levels of IL-1 alpha and TNF-alpha than CD14++CD16+ monocytes(545). Most recently, studies by our group demonstrated that CD16+ monocytes preferentially differentiate into DC that produce TNF-a and express retinaldehyde dehydrogenase (RALDH) activity(546). Of particular interest, exposure to TLR2 and TLR4 ligands promotes RALDH activity in CD16+ monocyte-derived DC (MDDC), which compared to CD16- MDDC mediate efficient HIV-1 trans infection in a RALDH-dependent manner(546). Together, these results emphasize the deleterious consequences of an

altered CD16-/CD16+ monocyte ratio during HIV-1 infection. The contribution of various subsets to HIV-1 infection was the subject of a previous review from our laboratory(136). The study of monocyte dynamics in the context of CVD and HIV-1 infection is explored in Manuscript #3.

Chapter 2: Hypotheses and Objectives

The main objective of my thesis is to determine the role of Th17 cells in HIV pathogenesis. More precisely, my goal was to study the interplay between Th17 cells and epithelial cells at the intestinal level, to evaluate their involvement in atherosclerotic cardiovascular disease, and to investigate potential therapeutic strategies targeting them. My investigations led to the preparation of three manuscripts, of which two are now published and one is in preparation for submission.

1. Manuscripts #1

Th17 and IEC crosstalk is determinant for the maintenance of mucosal homeostasis. Th17 cells promote mucosal integrity by producing the lineage-specific cytokine IL-17A(316, 547, 548). IL-17A induces the secretion of antimicrobial peptides and the expression of tight junction proteins in IECs(325, 549-551). In HIV infection, there is a significant disruption of intestinal immunity in PLWH despite ART. For example, HIV-1 diminishes the expression of tight junction proteins in IEC; therefore, HIV causes a direct dysregulation of the intestinal epithelial barrier(355, 376-378, 552). Since IL-17 is an essential regulator of IEC function, we hypothesize that IL-17A acting on IEC can alter HIV-1 dynamics. The objective of manuscript#1 is to investigate the effect of IL-17A on the crosstalk between IEC and CD4+ T-cells in the context of HIV transmission as well as during ART-treated chronic infection. These results are published in iScience 2021(553).

2. Manuscript #2

Previous research of our lab demonstrated that (i) Th17 cells have a unique molecular profile that allows efficient HIV replication and that (ii) a fraction of these cells are long-lived and contribute to viral reservoir persistence during ART(339, 351). We also presented evidence of HIV-DNA persistence in memory CCR6+T-cells from the blood and colon of HIV-infected individuals after many years of ART(338, 345). Therefore, these observations suggest preferential HIV infection of CCR6+ T-cells and/or preferential survival of these infected cells under therapy. The unique molecular profile associated with HIV permissiveness in Th17 cells included the master regulator

of Th17 polarization encoded by the human rar-related orphan receptor c (RORC) gene(345). We hypothesize that RORC expression is a critical molecular target that regulates the permissiveness of Th17 cells to HIV infection and maintains the HIV reservoir. The objective of manuscript 2 is to determine the effect of RORC antagonism on HIV expression and persistence during ART in human Th17 cells in vitro. These results are published in PNAS 2021(554).

3. Manuscript #3

The depletion of Th17 cells in PLWH is a significant cause of microbial translocation, chronic immune activation, and disease progression. Chronic immune activation has been associated with establishing cardiovascular disease in PLWH, but the underlying mechanism involving Th17 cells is not fully elucidated. We hypothesize that the chronic immune activation observed in PLWH is caused by the exposure to bacterial products following the microbial translocation in the intestine, resulting from the depletion of Th17 cells. The objective of manuscript 3 is to identify an immunological signature of atherosclerotic cardiovascular diseases in PLWH. These results are included in a manuscript in preparation for submission.

Chapter 3

Manuscript #1: IL-17A REPROGRAMS INTESTINAL EPITHELIAL CELLS TO FACILITATE HIV-1 REPLICATION AND OUTGROWTH IN CD4+ T CELLS

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Authors' contributions

TRWS contributed to the study design, performed all experiments, analyzed results, prepared figures, and wrote the manuscript. JPG and OT performed RNA-Seq analysis. OT contributed with figures from RNA-seq analysis. HT generated preliminary results for Figure 1A. AG, YZ, LRM, EMG and DV performed experiments. JPR and CT allowed access to biological samples and study participants' clinical information. MEF, NC, and AL contributed to the study design and provided protocols for IEC culture and HIV quantification. MGB and EAC kindly provide reagents and expertise for BST2 experiments. PA designed the study, contributed to data analysis and figure preparation, and wrote the manuscript.All authors reviewed and accepted the manuscript.

IL-17A Reprograms Intestinal Epithelial Cells to Facilitate HIV-1 Replication and Outgrowth in CD4+ T-cells

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Running title: IL-17A hinders anti-viral responses in epithelial cells

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ABSTRACT

The crosstalk between intestinal epithelial cells (IEC) and Th17-polarized $CD4^+$ T-cells is critical for mucosal homeostasis, with HIV-1 causing significant alterations in people living with HIV (PLWH) despite antiretroviral therapy (ART). In a model of IEC and T-cell co-cultures we investigated the effects of IL-17A, the Th17 hallmark cytokine, on IEC ability to promote *de novo* HIV infection and viral reservoir reactivation. Our results demonstrate that IL-17A acts in synergy with TNF to boost IEC production of CCL20, a Th17-attractant chemokine, and promote HIV *trans*-infection of $CD4^+$ T-cells and viral outgrowth from reservoir cells of ART-treated PLWH. Importantly, the Illumina RNA-Sequencing revealed an IL-17A-mediated pro-inflammatory and pro-viral molecular signature, including a decreased expression of type I interferon (IFN-I)-induced HIV restriction factors. These findings point to the deleterious features of IL-17A and raise awareness for caution when designing therapies aimed at restoring the paucity of mucosal Th17 cells in ART-treated PLWH.

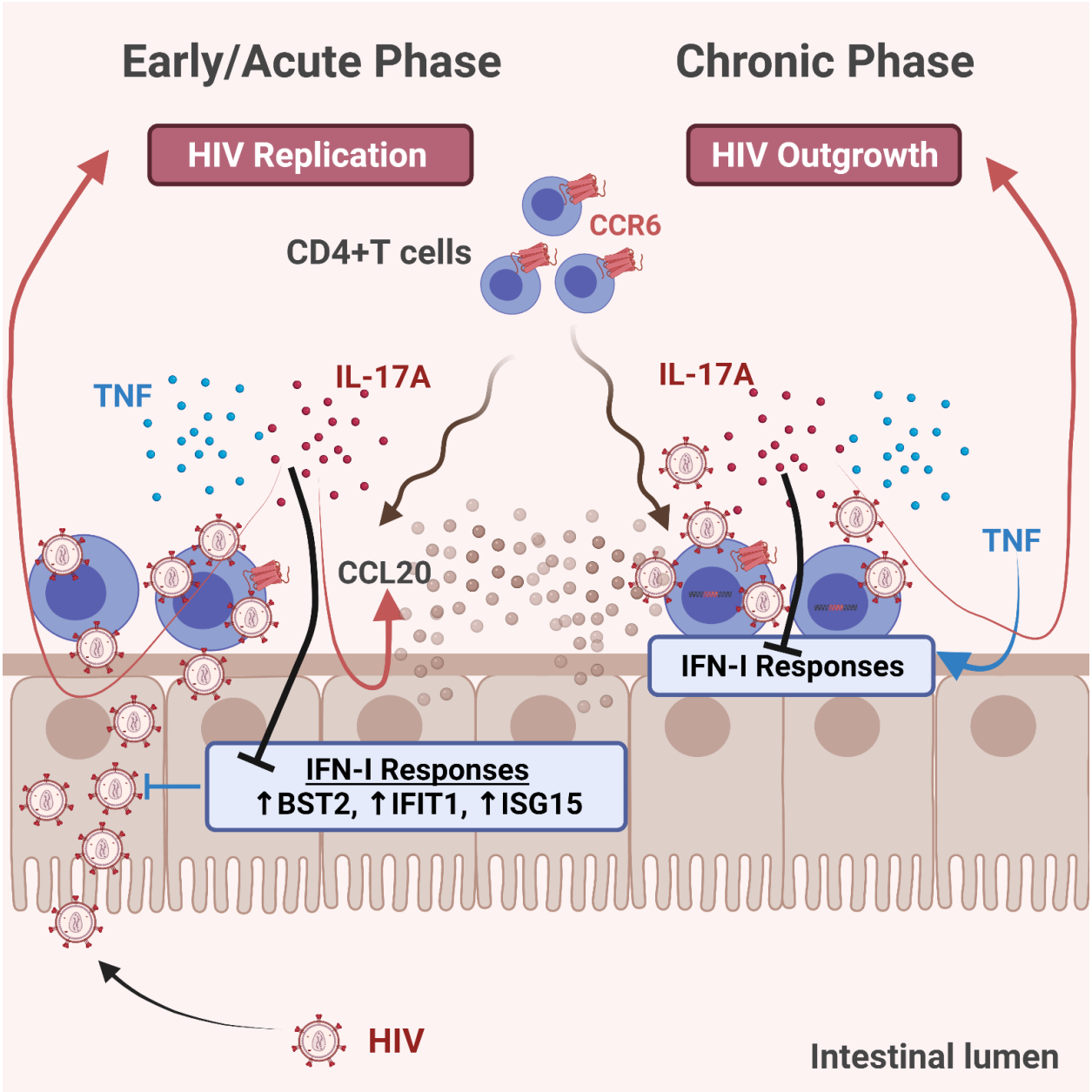
HIGHLIGHTS AND GRAPHICAL ABSTRACT

IL-17A acts in synergy with TNF to enhance CCL20 production in IEC exposed to HIV.

IL-17A/TNF-activated IEC efficiently promote HIV trans-infection of CD4+ T cells.

IL-17A reprograms IEC to boost HIV outgrowth from CD4+ T cells of ART-treated PLWH.

IL-17A decreases the expression of IFN-I-induced HIV restriction factors in IEC.



INTRODUCTION

The implementation of viral-suppressive antiretroviral therapy (ART) transformed the HIV-1 epidemics into a manageable chronic viral infection (Barre-Sinoussi et al., 2013; Ghosn et al., 2018). However, ART is unable to cure infection in people living with HIV (PLWH) (Cohn et al., 2020; Lederman et al., 2016; Margolis et al., 2020; Sengupta and Siliciano, 2018) and to reverse the state of chronic immune activation that leads to premature aging and non-AIDS co-morbidities (Clifford and Ances, 2013; El-Far and Tremblay, 2018; Gabuzda et al., 2020). This justifies the need for the identification of new therapeutic interventions toward an HIV cure (Deeks et al., 2016).

The gut-associated lymphoid tissues (GALT) play a unique role during primary HIV/SIV transmission, but also during chronic infections (Brenchley et al., 2006; Grossman et al., 2006). Although it is well-established that GALT-infiltrating CD4+ T-cells are rapidly targeted by infection and massively depleted during the early acute phases of HIV/SIV infections (Brenchley et al., 2004; Mehandru et al., 2004; Veazey, 2019), intestinal epithelial cells (IEC) are the first cells to interact with viral particles during rectal transmission (Gonzalez et al., 2019). Indeed, rectal IEC monolayers facilitates HIV/SIV acquisition and dissemination (Gonzalez et al., 2019; Real et al., 2018; Ribeiro Dos Santos et al., 2011), in part via transcytosis, a process involving virion capture, intracellular trafficking, and trans-infection of subjacent target cells (e.g., CD4+ T-cells) in the absence of integrative infection in IEC (Alfsen et al., 2005; Bomsel, 1997). In addition to transcytosis, the capacity of IEC to support productive HIV infection has been studied on multiple models, including cell lines and primary cells. Studies on IEC lines demonstrated that latent and productive infection occurs depending on the use of low and high HIV titers, respectively (Bouhlal et al., 2002; De Mareuil et al., 1995; Fantini et al., 1991a; Fantini et al., 1992b; Fantini et al., 1991b; Faure et al., 1994; Gauthier and Tremblay, 2010; Kagnoff and Roebuck, 1999; Omary et al., 1991). In contrast to IEC lines, primary IEC were reported to be permissive to integrative infection when exposed to laboratory-adapted HIV strains, but not primary isolates (Chenine et al., 1998; Meng et al., 2002; Ribeiro Dos Santos et al., 2011). Finally, primary IEC were documented to selectively transfer CCR5-tropic HIV strains to subjacent CD4+ T-cells (Margolis and Shattock, 2006; Meng et al., 2002) via the formation of virological synapses (Real et al., 2018).

Thus, the interplay between IEC and CD4+ T-cells plays a key role in HIV/SIV mucosal transmission (Gonzalez et al., 2019; Margolis and Shattock, 2006).

Among CD4+ T-cells preferentially infiltrating the GALT, CCR6+ Th17-polarized cells represent the first targets of HIV/SIV infection during acute phases and contribute to viral reservoir persistence in ART-treated PLWH (Anderson et al., 2020; Gosselin et al., 2017; Haase, 2011; Kelley et al., 2017; Masson et al., 2015; Planas et al., 2019; Shanmugasundaram et al., 2014; Stieh et al., 2016; Wacleche et al., 2016; Wacleche et al., 2017). At homeostasis, Th17 cells maintain the integrity of the intestinal physical/biological barrier, in part by producing the lineage-specific cytokine IL-17A under the control of the transcription factor RORγt (Dong, 2008; Kelley et al., 2017; Yang et al., 2008). IL-17A acts on IEC to strengthen the expression of tight junction proteins and to induce the secretion of antimicrobial peptides and the production of chemokines specifically attracting Th17 cells (e.g., CCL20, CCL25) (Hirota et al., 2007; Liang et al., 2006; Sano et al., 2015; Wang et al., 2009; Yamazaki et al., 2008). It is noteworthy that IL-17A acts in synergy with other cytokines, such as TNF (Friedrich et al., 2014; Guo et al., 2014; Lee et al., 2008a), and represents an "alarmin" that promotes antibacterial immunity at mucosal sites (Minegishi and Karasuyama, 2009). In line with these observations, studies in a model of Rhesus macaques exposed to Salmonella showed an increased expression of IL-17A and TNF, as well as other IL-17A-regulated genes in the gut (Raffatellu et al., 2008). Similar conclusions regarding an enrichment of gene signatures associated with Th17 cells were reported in the rectal mucosa of HIV-negative men who have sex with men (MSM) that engaged in condomless receptive anal intercourse (CRAI), along with an increase of rectal CD4+ T cells that produced greater levels of IL-17A compared to HIV-negative men who had never engaged in anal intercourse (Kelley et al., 2017). HIV-negative MSM who engaged in CRAI also shown a microbiota enriched for the Prevotellaceae family. Finally, our most recent study indicates the expression of IL-17A mRNA in colon biopsies of ART-treated PLWH (Gabriel et al., 2021). These lines of evidence support the production of IL-17 and TNF in vivo, at the mucosal level, after exposure to different components of the microbiota.

The depletion of Th17 cells, together with their functional alterations observed in the blood and GALT of PLWH are not completely restored with ART (Brenchley et al., 2008; Schuetz et al., 2014). The paucity of Th17 cells is caused by multiple mechanisms, including infection per se and an altered Th17 trafficking into mucosal sites (Loiseau et al., 2016; Mavigner et al., 2012; Planas et al., 2019; Wacleche et al., 2017). The depletion of Th17 cells is the main cause of gut barrier dysfunction leading to microbial translocation and chronic immune activation in ART-treated PLWH (Mudd and Brenchley, 2016; Somsouk et al., 2015; Tincati et al., 2016). In this context, the restoration of Th17-mediated mucosal immunity in the GALT represents an important research priority for HIV remission (Planas et al., 2019; Wacleche et al., 2017). For example, the administration of IL-21, a cytokine essential for Th17 survival, increased the frequency of Th17 cells in SIV-infected rhesus macaques and induced the expression of genes related to antimicrobial and anti-inflammatory functions (Micci et al., 2015; Pallikkuth et al., 2013). This intervention also reduced the residual plasma viremia and decreased the size of SIV-DNA reservoirs in ART-treated SIV-infected animals (Micci et al., 2015; Pallikkuth et al., 2013). Taking into consideration the importance of improving Th17-mediated immunity in HIV-infected ART-treated PLWH, it is highly relevant to evaluate the impact of IL-17A on HIV reservoir establishment and persistence at mucosal sites.

In this manuscript, we used the HT-29 IEC line and primary CD4+ T-cells from uninfected individuals, as well as T-cells from untreated and ART-treated PLWH, to investigate the effects of IL-17A on the interplay between IEC and CD4+ T-cells, with relevance for early HIV transmission events, as well as viral reservoir dynamics during ART-treated chronic infection. Our results support a model in which IL-17A, despite its beneficial effects on mucosal homeostasis, acts on IEC to promote HIV dissemination and viral outgrowth from reservoir cells by compromising the type I IFN-mediated anti-viral immunity at mucosal sites. These findings raise awareness for caution when designing therapies aimed at restoring the paucity of mucosal Th17 cells in ART-treated PLWH.

RESULTS

IL-17A acts in synergy with TNF to boost CCL20 production by IEC in the presence of HIV-1. The chemokine CCL20 attracts CCR6+ Th17 cells at mucosal sites (Wang et al., 2009; Yamazaki et al., 2008), with CCL20 expression in IEC being induced by the synergic action of IL-17A and TNF (Friedrich et al., 2014). HIV/SIV infections were previously associated with an altered CCL20 expression in IEC (Loiseau et al., 2016; Mavigner et al., 2012; Raffatellu et al., 2008), a deficit likely contributing to an impaired recruitment of Th17 cells into the GALT. This prompted our initial hypothesis that HIV exposure alters the capacity of IEC to secrete CCL20. To address this hypothesis, HT-29 IEC were activated with IL-17A or IL-17F in the presence or the absence of TNF, and exposed to two different CCR5-tropic HIV strains, HIVTHRO (a transmitted founder (T/F) molecular clone (Ochsenbauer et al., 2012) and HIVNL4.3BaL [a molecular clone containing the env- NL4.3 backbone, with an CCR5-tropic env from an HIV strain isolated from the bronchoalveolar lavage (BaL) of an individual with chronic infection (Bobardt et al., 2008). We used CCR5-tropic HIV considering the essential role of such strains in HIV transmission (Parrish et al., 2012). It is noteworthy that HT-29 cells are documented to support low level integrative infection (Gauthier and Tremblay, 2010), as a consequence of galactosyl ceramide (GalCer)-mediated HIV entry (Delezay et al., 1997; Fantini et al., 1993), despite the fact that HT-29 IEC express low to undetectable levels of the main HIV receptor CD4 and co-receptors CCR5 and CXCR4 (Dwinell et al., 1999; Jordan et al., 1999) (data not shown). In the absence of HIV, IL-17A acted in synergy with TNF to induce the production of CCL20 (Figure 1A, left panel). The production of IL-8/CXCL8, a chemokine ligand of CXCR1-2, mainly mediating the migration of neutrophils (Murphy, 1997), was induced by TNF, but was not influenced by IL-17A (Figure 1A, right panel). In contrast to IL-17A, IL-17F did not affect the production of CCL20 or IL-8 (data not shown). In contrast to our initial hypothesis, exposure to HIVTHRO did not decrease, but rather significantly increases CCL20 production by IEC in response to TNF alone, as well as TNF+IL-17A (Figure 1B, left panel), with no major impact on IL-8 production (Figure 1B, right panel). A similar effect was observed when cells were exposed to HIVNL4.3BaL (data not shown). Levels of CCL20 produced by cytokine-activated and HIV-exposed HT-29 IEC remained stable in the presence of the integrase inhibitor Raltegravir or the gp120/CD4-mediated entry inhibitor BMS-806 (Supplemental Figure 1). This suggests that CCL20 production was not dependent on HIV entry

and integration in IEC. Overt CCL20 production may contribute to the abundant recruitment of CCR6+ Th17 cells at mucosal sites during the initial steps of HIV transmission.

IL-17A-activated IEC efficiently transmit HIV infection to CD4+ T-cells. The ability of IEC to capture and disseminate HIV to subjacent T-cells is well-documented (Alfsen et al., 2005; Bomsel, 1997; Margolis and Shattock, 2006; Meng et al., 2002; Real et al., 2018). Cytokines such as TNF and IFN-γ were reported to facilitate HIV transmission from IEC to CD4+ T-cells (Fantini et al., 1992a; Yasen et al., 2017). To investigate the effects of IL-17A on IEC-mediated HIV transmission, cytokine-activated IEC were exposed to HIVTHRO and co-cultured with CD3/CD28-activated CD4+ memory T-cells from uninfected individuals, as depicted in Figure 2A. The highest levels of HIVTHRO replication were observed when CD4+ T-cells were co-cultured with HIV-exposed TNF+IL-17A-activated (IECTNF+IL-17A) compared to inactivated (IECMedium) and TNF-activated IEC (IECTNF), as measured by ELISA (Figure 2B-C). Similar results were obtained when HIV-p24+ T-cells were quantified by flow cytometry, indicative that IECTNF+IL-17A highly efficiently transmit HIV infection to T-cells (data not shown).

Robust HIV replication in T-cells co-cultured with HIV-exposed IECTNF+IL-17A coincided with the highest levels of CCL20 (Figure 2D), a chemokine reported to promote HIV latency (Cameron et al., 2010), but also involved in HIV resistance through the induction of APOBEC3G (Lafferty et al., 2010). To determine whether CCL20 modulates HIV replication, CD3/CD28-activated CD4+ T-cells were infected with HIV in vitro and cultured in the presence or the absence of recombinant human CCL20 (Supplemental Figure 2A). Results in Supplemental Figure 2B-E demonstrate that CCL20 does not directly influence HIV replication in CD4+ T-cells.

To determine whether cytokine-activated IEC differ in their ability to harbor HIV after exposure, integrated HIV-DNA, as well as early (RU5) and late (Gag) reverse transcripts were quantified 24 hours post HIV exposure (Supplemental Figure 3A), using published protocols (Cattin et al.,2019). Integrated HIV-DNA, together with early and late reverse transcripts were increased in IECTNF+IL-

17A and IECIL-17A (Supplemental Figure 3B). In addition, cell-associated HIV-p24 levels, quantified in cell lysates by crude HIV-p24 ELISA 48 hours post HIV exposure (Supplemental Figure 3A), were increased in both IECTNF and IECTNF+IL-17A compared to IECMedium. (Supplemental Figure 3C).

Thus, HIV-exposed IECTNF+IL-17A favor HIV transmission/replication to/in CD4+ T-cells by mechanisms involving an augmented level of HIV uptake and an increased integrative infection in an environment rich in CCL20. The finding that recombinant CCL20 exerts no direct effects on HIV replication in vitro suggests that in vivo, this chemokine rather contributes to the recruitment of CCR6+ cells, including Th17 cells, at mucosal sites, thus facilitating their infection.

IL-17A-activated IEC promote HIV replication in HIV-infected memory CD4+ T cells. HIV reservoirs persist in colon-infiltrating CCR6+CD4+ T-cells of ART-treated PLWH (Anderson et al., 2020; Gosselin et al., 2017; Haase, 2011; Kelley et al., 2017; Masson et al., 2015; Planas et al., 2019; Shanmugasundaram et al., 2014; Stieh et al., 2016; Wacleche et al., 2016; Wacleche et al., 2017). To further explore the effect of IL-17A on the interplay between IEC and CD4+ T-cells during HIV infection, we studied the ability of cytokine-activated IEC to promote viral replication in memory CD4+ T-cells infected with HIVTHRO in vitro (Figure 3A). HIV-infected T-cells were identified by flow cytometry as CD3+CD4lowHIV-p24+ at day 9 post-infection (Figure 3B-C), with CD4 downregulation being indicative of a productive infection (Richard et al., 2018). Also, HIV-p24 levels were measured in cell-culture supernatants by ELISA (Figure 3D). For HIVTHRO-infected CD4+ T-cells, the frequency of CD3+CD4lowHIV-p24+ T-cells was similar in all experimental conditions (Figure 3B), HIV-p24 levels in cell-culture supernatants, indicative of productive viral replication, were the highest when HIVTHRO-infected T-cells were co-cultured with IL-17A-activated IEC (IECIL-17A) compared to IECTNF (Figure 3D). The viability of cells at day 9 post-coculture was around 80%, without major differences between conditions (Figure 3E).

To determine whether cytokines used to activate IEC may act directly on T-cells to modulate HIV replication, CD3/CD28-activated memory CD4+ T-cells of HIV-uninfected individuals were infected with HIVTHRO in vitro and cultured in the presence or the absence of IL-17A and/or TNF. The addition of these cytokines had no effect on HIV replication measured by flow cytometry and ELISA in T-cells cultured alone (Supplemental Figure 4), indicating that IL-17A acts via IEC to exert its pro-viral effects in T-cells.

IL-17A acts on IEC to decrease Tetherin/BST2 expression. In an effort to identify molecular mechanisms used by IL-17A to promote HIV dissemination and replication, in the context of the IEC:T-cell interplay, we first evaluated the expression of BST2, an HIV restriction factor counteracted by HIV Nef and Vpu, with BST2 documented to play a crucial role in HIV release and cell-to-cell transmission (Casartelli et al., 2010; Neil et al., 2008). We used flow cytometry to quantify first the surface expression of BST2 on IEC upon exposure to cytokines and cultured alone (as in Figure 1A-B), as well as on memory CD4+ T-cells infected with HIVTHRO in vitro (as in Figure 3A).

The expression of BST2 was significantly decreased in IECTNF+IL-17A compared to IECMedium, with no significant differences between IECMedium and IECTNF or IECIL-17A (Figure 4A-B). Considering the possibility that covert productive HIV infection occurs in HT-29 cells (Chenine et al., 1998), a decreased BST2 expression may facilitate the release of virions produced by HIV-infected IECTNF+IL-17A, compared to IECMedium for an efficient transmission to T-cells, as observed in Figure 2.These results suggest the increased dissemination potential of IECTNF+IL-17A compared to IECTNF(Figure 2), despite similar HIV capture capacity (Supplemental Figure 3C).

The expression of BST2 was also significantly decreased on total T-cells and CD4lowHIV-p24+ T-cells in co-cultures performed with memory CD4+ T-cells infected with HIVTHRO in vitro and IECIL-17A compared to IECTNF (Figure 4C-D). Noteworthy, the BST2 expression on total T-cells

and CD4lowHIV-p24+ T-cells negatively correlated with levels of productive HIV replication in cell-culture supernatants (Figure 4E).

In addition to CD4 downregulation, another way to identify productively HIV-infected T-cells is based on their downregulation of BST2 (Van Damme et al., 2008). Consistent with HIV replication results in Figure 3D, the highest frequency of BST2lowHIV-p24+ cells was observed in the presence of IECIL-17A compared to IECMedian (Supplemental Figure 5A top panels and 5B).

Together, these results point to the BST2 downregulation on IEC as well as CD4+ T-cells as a mechanism facilitating HIV infection in IEC: T-cell co-cultures upon IL-17A exposure in the presence or the absence of TNF.

IL-17A counteracts the TNF-mediated type I IFN expression. To further investigate mechanisms underlying the pro-viral effects promoted by IECTNF+IL-17A and IECIL-17A, bioactive type I IFN levels were quantified in cell culture supernatants at day 3 of co-culture between HIVTHRO-infected T-cells with cytokine-activated IEC. The highest levels of type I IFN were detected in IECTNF:T-cell co-cultures, while the lowest levels were detected in T-cells co-cultured with IECIL-17A and IECMedium (Figure 5A). Interestingly, IL-17A decreased type I IFN levels in co-cultures of T-cells with IECTNF+IL-17A compared to IECTNF (Paired t-Test p=0.0065, Figure 5A). It is noteworthy that type I IFN levels positively correlated with the MFI expression of BST2, a known IFN stimulated gene (ISG) that limits HIV release from infected cells (Liberatore and Bieniasz, 2011), on T-cells from all experimental conditions at day 3 post-co-culture (Figure 5B). Furthermore, a co-culture between HIVTHRO-infected T-cells and cytokine-activated IEC in the presence of soluble IFN alpha/beta receptor (B18R), which prevents IFN signaling, was performed as depicted in Figure 6A. The presence of B18R in co-cultures of T-cells with IECTNF counteracted the effect of TNF and increased HIV replication to a similar extent of what is observed in co-cultures of T-cells with IECTNF+IL-17A (Figure 6B). In addition to BST2, the other ISG ISG15 (Freitas et al., 2020) and IFIT1 (Fensterl and Sen, 2015) were upregulated in co-cultures with IECTNF

(Figure 6C) and this upregulation was abrogated when T-cells were cultured with IECTNF+IL-17A as observed in Figure 4C-D. B18R blocked the upregulation of ISG in co-cultures of T-cells with IECTNF as compared to the ones with IECTNF+IL-17A. These results reveal that the proviral features of IL-17A are mediated, at least in part, by its capacity to downregulate type I IFN production and responses induced by TNF.

IL-17A activated IEC promote HIV outgrowth in memory CD4+ T cells of PLWH

To further explore the effect of IL-17A on the interplay between IEC and CD4+ T-cells during chronic HIV infection, we studied the ability of cytokine-activated IEC to promote viral outgrowth from memory CD4+ T-cells of both suppressed ART-treated PLWH (ART+ PLWH) and untreated PLWH (ART- PLWH) (Figure 7A), with detectable plasma viremia (Table 1). Similar results to memory CD4+ T-cells infected with HIVTHRO in vitro were obtained with T-cells of ART- PLWH (Figure 7B top panels and C), with an increased viral outgrowth measured by ELISA observed in the presence of IECIL-17A versus IECTNF (Figure 7E). Remarkably, when co-cultures were performed with T-cells of ART+ PLWH, the highest levels of HIV outgrowth, as measured by both FACS and ELISA, were achieved in the presence of IECIL-17A (Figure 7B bottom panels, D and F). An increased frequency of BST2lowHIV-p24+ cells was observed when memory CD4+ T-cells from ART- and ART+ PLWH were co-cultured with IECIL-17A in comparison with IECTNF (Supplemental Figure 5A middle and bottoms panels, C and D). Additionally, a decrease in BST2 expression on total T-cells and/or CD4lowHIV-p24+ T-cells was also noticed for memory CD4+ T-cells isolated from ART- (Supplemental Figure 6A-B), as well as ART+ PLWH (Supplemental Figure 6D-E) upon co-culture with IECIL-17A compared to IECTNF, with a negative correlation between BST2 expression and levels of HIV outgrowth (Supplemental Figure 6C and F). The cell viability decreased in co-cultures of IECIL-17A with T-cells of ART- PLWH in line with a higher HIV outgrowth (Figure 7H). In conclusion, these results show that the activation of IEC with IL-17A promotes viral outgrowth from CD4+ T-cells isolated from ART- and especially ART+ PLWH.

Transcriptional reprogramming associated with IL-17A-mediated pro-viral features. To further explore in an unbiased manner mechanism underlying the pro-viral features of IL-17A, the

Illumina RNA sequencing technology was used on RNA extracted from cytokine-activated IEC cultured alone (Figure 8A-B; Supplemental Files 1-2) and co-cultured with CD4+ T-cells isolated from ART+ PLWH (Figure 8C-D; Supplemental Files 3-4), using culture/co-culture protocols described in Figures 1 and 3, respectively.

For cytokine-activated IEC cultured alone, large sets of differentially expressed genes (DEG) were identified using a fold-change (FC) cutoff of 1.3. We found 6,639, 5,429, and 3,983 transcripts upregulated, along with 5,297, 3,925, and 6,437 transcripts downregulated in IECTNF, IECIL-17A+TNF, and IECIL-17A compared to IECMedium, respectively (Supplemental File 1). To gain insights into gene sets modulated by IL-17A and TNF alone or in combination, we used a hierarchical clustering based on the scaled FC of each condition compared to medium. We identified 3 main clusters (MC) characterized by specific patterns of response in IECTNF, IECIL-17A+TNF, and IECIL-17A, that were subdivided into 8 distinct transcriptional signatures with slight variations within each of the 3 MC (Figure 8A; Supplemental File 1). Clusters 2, 5, 7, and 8 (MC I) included gene sets expressed at higher levels in IECIL-17A+TNF compared to IECTNF or IECIL-17A (Figure 8A), thus reflecting transcriptional changes in the expression of different effector molecules prompted by the synergic action of IL-17A and TNF (e.g., the cytokines IL-4 and IL-1β, and the chemokines CCL20, CXCL2 and CXCL3; Supplemental File 1). Clusters 4 and 6 (MC II) included gene sets with higher expression levels in IECIL-17A compared to IECTNF or IECIL-17A+TNF (Figure 8A) (e.g., genes involved in the monocarboxylic and organic acid transport: SLC16A14, PPARD, and SLC10A5; Supplemental File 1). Finally, Clusters 1 and 3 (MC III) included gene sets with higher expression levels in IECTNF compared to IECIL-17A+TNF (Figure 8A), thus revealing transcripts down regulated by IL-17A in TNF-activated IEC (e.g., the cytokine IL-32 and the interferon stimulated genes IFITM1, IFIT1, ISG15, IFI6 and OAS2; Supplemental File 1).

We further used a gene set enrichment analysis (GSEA), focusing on the Gene Ontology: Biological Processes database. Each of the gene modules was compared to a reference background consisting of all transcripts measured (28,083). The graphical representation of enriched GO terms that reached statistical significance (false discovery rate, FDR, <5%) was

generated using ClueGO on Cytoscape software. Only clusters 1, 3, and 4 were enriched in GO terms with statistical significance and a functional trend (Supplemental File 2). Cluster 3 was associated with immune response processes such as “Defense response to virus” and “Response to Type I Interferon” (Figure 8B). Other gene sets were upregulated in IECTNF but downregulated with the addition of IL-17 in IECIL-17A+TNF; noteworthy, some of those genes were also downregulated in IECIL-17A (Supplemental Figures 7A-B). For example, enriched pathways in IECTNF versus IECIL-17A+TNF and/or IECIL-17A included genes with a demonstrated activity against HIV, such as the viral sensor MB21D1 (Gao et al., 2013) and the restriction factors APOBEC3G, IFI16, IFITM1, SAMHD1, IFIT1, IFITM3, ISG15, MX2, RSAD2 and TRIM22 (Colomer-Lluch et al., 2018; Hotter et al., 2019; Hotter and Kirchhoff, 2018; Nasr et al., 2017; Nasr et al., 2012) (Supplemental Figure 7A-B). While cluster 3 was associated with an upregulation of viral response associated transcripts, genes associated with the GO term “Defense response to virus” were also included in Cluster 1; those genes were downregulated in IECIL-17A+TNF compared to IECTNF and they included the viral sensor TMEM173 (Trotard et al., 2016), and the HIV restriction factors BST2, APOBEC3D, and IFITM2 (Neil et al., 2007; Qian et al., 2015; Sato et al., 2014) (Supplemental Figure 8C).

A similar analysis was performed in cytokine-activated IEC co-cultured with CD4+ T-cells isolated from ART+ PLWH (Supplemental File 3). This analysis allowed us to identify similar patterns of gene expression also grouped in 3 MC and categorized in 8 clusters (Figure 7C, Supplemental File 3). Only Cluster 1, which mainly included genes that were upregulated in co-cultures performed with IECTNF and downregulated in co-cultures performed with IECTNF+IL-17A and IECIL-17A, showed statistical significance for the immune response process “Defense to virus” and “Response to Type I Interferon” (Figure 7D; Supplemental File 4). Among cluster 1 genes, some were known HIV restriction factors such as APOBEC3F, BST2, HERC5, IFIT1, IFITM1, IFITM2, ISG15, MX2, RSAD2, TRIM22, and TRIM5 (Supplemental Figure 8A-B).

These results demonstrate that IL-17A, in the presence or the absence of TNF, abrogates the anti-viral type I IFN-mediated responses in IEC cultured alone and co-cultured with CD4+ T-cells

carrying HIV reservoirs in ART+ PLWH. This abrogation of anti-viral responses highly likely explain the pro-viral features of IL-17A.

IL-17 acts on primary human IEC to modulates CCL20 production and ISGs expression

To extend our results obtained on the HT-29 cells in a more physiological setting, we performed experiments with commercially available primary human IEC (phIEC) isolated from one HIV-uninfected individual. We confirm here that TNF acts in synergy with IL-17A in promoting CCL20 production in phIEC (Figure 9A). Furthermore, the expression of the ISGs ISG15 and IFIT1 mRNA was increased in phIECTNF and decreased in phIECTNF+IL-17 and phIECIL-17 compared to phIECMedium (Figure 9B-C). These results support a model in which IL-17A can increase the production of pro-inflammatory chemokines such as CCL20, while interfering with antiviral mechanisms such as those mediated by ISGs in vivo.

DISCUSSION

In this study, we provide evidence supporting the deleterious role of the Th17 hallmark cytokine IL-17A in the context of HIV-1 infection by its capacity to reprogram the pro-inflammatory and pro-viral features of IEC. Briefly, we demonstrate that IL-17A in the presence of TNF stimulation and/or HIV exposure, increases the capacity of IEC to produce CCL20, a chemokine that recruits CCR6+ Th17 cells into mucosal sites (Wang et al., 2009; Yamazaki et al., 2008). We revealed the capacity of IL-17A to enhance HIV trans-infection from IEC to CD4+ T-cells and to promote HIV outgrowth from CD4+ T-cells of ART-treated PLWH. By using an RNA-Sequencing approach, we identified pro-inflammatory and anti-viral gene sets modulated by IL-17A in IEC alone and in IEC:T-cell co-cultures, with the decrease in type I IFN production/responses (i.e., BST2, IFIT1, ISG15) representing a common pattern of IL-17A exposure. In this context, the dual role played by IL-17A in controlling mucosal immunity versus HIV infection should be considered when seeking to implement new therapeutic interventions aimed at the restoration of mucosal Th17 cells in ART-treated PLWH.

By its capacity to target both IEC and Th17 cells, HIV causes profound alterations of the GALT physical and biological barrier functions (Mudd and Brenchley, 2016; Tincati et al., 2016). Such alterations are observed during the very early acute phases of infection and are not resolved by viral-suppressive ART, even with early ART initiation (Ananworanich et al., 2012; Deleage et al., 2016; Epple et al., 2009; George et al., 2005; Hensley-McBain et al., 2018; Schuetz et al., 2014). Nevertheless, recent studies reported IEC alterations even before the depletion of Th17 cells occurs in the intestinal mucosa of SIV-infected macaques (Hensley-McBain et al., 2018), thus emphasizing the key role played by IEC in HIV/SIV pathogenesis.

It is well established that Th17 cells, via their production of IL-17A, represent essential players in the defense against fungal (e.g., Candida albicans) and bacterial (e.g., Staphylococcus aureus) pathogens at mucosal and skin barrier surfaces (Henry et al., 2010; Li et al., 2018). Nevertheless, IL-17A-producing Th17 cells play a pathogenic role in the context of auto-immunity (e.g., psoriasis and ankylosing spondylitis) (Burkett and Kuchroo, 2016) and viral infections, such as the 2009

influenza A (H1N1), Respiratory Syncytial Virus (RSV) (Li et al., 2012; Niwa et al., 2018; Ryzhakov et al., 2011), and the new SARS-CoV-2 infection (De Biasi et al., 2020; Wiche Salinas et al., 2020). However, whether IL-17A plays a deleterious role during HIV/SIV remains unknown. The purpose of the current study was to investigate the pro-viral features of IL-17A in the context of IEC and CD4+ interactions.

Previous reports demonstrated that IL-17A acts in synergy with TNF to promote CCL20 production by IEC (Friedrich et al., 2014; Lee et al., 2008b). This synergy is mechanistically explained by the TNF-dependent upregulation of IL-17 receptors on IEC, as well as the stabilization of messenger RNA (Herjan et al., 2018). Here, we confirmed these findings in HT-29 IEC and further demonstrated that IL-17A but not IL-17F increases the TNF-mediated CCL20 production in IEC. The chemokine CCL20 binds on its receptor CCR6 and regulates trafficking of CCR6+ cells, including dendritic cells (DC) and Th17 cells (Wang et al., 2009; Yamazaki et al., 2008). Noteworthy, studies by the group of A.T. Haase demonstrated that glycerol monolaurate, an antimicrobial compound, inhibits the production of CCL20 and prevented SIV infection of rhesus macaques by interfering with the trafficking on CCR6+ DC into the female genital tract (Li et al., 2009; Shang et al., 2017). Thus, the CCL20/CCR6 axis plays a key role in immune cell trafficking into mucosal sites, as well as in the distal dissemination of HIV/SIV infection from the portal sites of viral entry. A deficit in CCL20 expression was reported in the intestine of PLWH and SIV-infected macaques (Loiseau et al., 2016; McGary et al., 2017; Raffatellu et al., 2008), and was suggested to represent one mechanism explaining the paucity of mucosal Th17 cells during HIV/SIV infection. In contrast to our initial hypothesis, we found that HIV exposure in vitro does not reduce, but rather increases CCL20 production by IEC in response to TNF+IL-17A. In line with these findings, HIV/SIV challenge in vivo was reported to induce the secretion of multiple chemokines promoting the migration of CD4+ T cells that are preferential infection targets (e.g., CCR6+ T-cells) (Li et al., 2009; McGary et al., 2017; Nazli et al., 2010; Sankapal et al., 2016; Shang et al., 2017; Zhou et al., 2018). Thus, during the initial steps of HIV/SIV infection, IL-17A may contribute to the recruitment of CCR6+ Th17 cells at mucosal sites. In contrast, during the chronic phase, when HIV/SIV-infected Th17 cells are massively depleted (Brenchley et al., 2008; Loiseau

et al., 2016), an alteration in CCL20 expression at the intestinal level may be the consequence of an altered IL-17A production.

Previous studies demonstrated that CCL20 acts on T-cells to increase their resistance to infection via the induction of the HIV restriction factor APOBEC3G (Lafferty et al., 2017). Other studies reported on the capacity of CCL20 to promote HIV latency (Cameron et al., 2010). In our experimental settings, we found that high levels of HIV replication coincided with the highest levels of CCL20 production in IEC:T-cell co-cultures, and exogenous CCL20 does not interfere with HIV replication in memory CD4+ T-cells. Therefore, the effects of CCL20 on HIV replication remain controversial.

IEC are the first cells to interact with HIV/SIV during the initial phases of viral transmission. HIV capture, transcytosis, and release from EC is positively and negatively regulated by multiple host factors (Gonzalez et al., 2019). In non-rectal EC, HIV is internalized and retained in vacuoles or multi-vesicular bodies (Yasen et al., 2017). TNF can modulate the retention of HIV into female genital tract EC and can increase their capacity to release HIV to infect CD4+ T cells (Yasen et al., 2017). IEC release exosomes that contain HIV restriction factors and miRNAs after stimulation with TLR-3 ligand. Those factors restrict HIV replication in macrophages infected in vitro (Guo et al., 2018). We observed that IL-17A, in combination with TNF, increased the capacity of IEC to capture and transmit HIV to CD4+ T-cells. These results attribute to IL-17A a direct role in promoting HIV dissemination from the portal sites of entry. This idea is in line with results emerging from population studies where individuals with increased levels of IL-17A or IL-17A-induced chemokines exhibited an increased risk for HIV acquisition (Arnold et al., 2016; Chege et al., 2012; Gosmann et al., 2017). Of note, it is established that HIV-Tat protein induces the production of IL-17A on CD4+ T-cells (Cafaro et al., 2016; Johnson et al., 2013). The latter finding, in addition to the facts that Th17 cells have a superior permissiveness to HIV infection (Gosselin et al., 2010; Monteiro et al., 2011) and are located at portal sites of HIV entry (Kelley et al., 2017; Masson et al., 2015; Stieh et al., 2016), highlight the relevance of evaluating the Th17 responses

and their correlation to protection versus susceptibility to HIV acquisition in vaccination trials (Perreau et al., 2008; Stephenson et al., 2020).

One important finding of our study is that IL-17A-stimulated IEC exhibit an increased capacity to promote/facilitate viral replication when co-cultured with primary CD4+ T-cells infected with HIVTHRO in vitro or CD4+ T-cells from viremic and ART-treated PLWH. Although solid evidence supports that Th17 cells are depleted during HIV/SIV infections (Brenchley et al., 2008; Schuetz et al., 2014), IL-17A can be produced by other cell types of the innate and adaptive immune system, including neutrophils (Hu et al., 2017) and NKT cells (Campillo-Gimenez et al., 2010), which massively infiltrate mucosal sites during HIV/SIV infections (Somsouk et al., 2015). Whether IL-17A-activated IEC may contribute to HIV reservoir reactivation in ART-treated PLWH, thus leading to a state of chronic immune activation in the GALT, remains an important question to address in future studies. This knowledge is highly relevant in the context of therapies aimed to Th17 cell restoration in the GALT. Noteworthy, the administration of IL-21 proved to be beneficial in reestablishing the frequency of Th17 cells and in decreasing the size of viral reservoir during ART in the gut of SIV-infected rhesus macaques (Micci et al., 2015). This is in line with the fact that IL-21, in addition to its effect on Th17 cell survival (Planas et al., 2019; Wacleche et al., 2017), it also exhibits anti-viral activities (Adoro et al., 2015) that may counteract the pro-viral activity of IL-17A. The pro-viral activity of IL-17A may also be neutralized in the presence of ART. However, ART does not target HIV transcription and therefore IL-17A may still facilitate HIV provirus reactivation, as in a “shock and kill strategy”. Noteworthy, studies reported that the administration of the TLR-9 agonist MGN1703 decreased the size of the gut HIV-reservoir and correlated with the intestinal expression of IL-17R (Krarup et al., 2018). Further studies should investigate better how IL-17A impacts HIV reactivation in the gut in ART-treated PLWH.

In search of molecular mechanisms underlying the pro-viral features of IL-17A, we performed RNA Sequencing and used bioinformatics tools for data mining. The GO enrichment analysis indicated that IL-17A dampens the response to type I IFN in IEC cultured alone and in the presence of CD4+ T-cells from ART-treated PLWH. Genes included in the GO term Response to

Type I IFN were essentially linked to HIV sensing and restriction and were upregulated by TNF but downregulated by IL-17A. One of the transcripts downregulated by IL-17A was BST2, an HIV restriction factor limiting the release of de novo produced virions (Neil et al., 2008). In line with this, we found the lowest levels of BST2 expression on IEC stimulated with TNF+IL-17A versus TNF or IL-17A alone. These results may explain the superior ability of IEC activated with TNF+IL-17A to transmit HIV compared to IEC activated with either TNF or IL-17A alone. Previous studies demonstrated covert productive HIV replication in HT-29 cells (Fantini et al., 1991b). Therefore, levels of BST-2 expression on IEC may determine their ability to transmit HIV infection to subjacent CD4+ T-cells. We also found that HIVTHRO-infected CD4+ T-cells expressed the lowest levels of BST-2 when co-cultured with IEC activated with IL-17A compared to TNF, in line with the highest levels of HIV replication in IECIL-17A:T-cell co-cultures. Consistent with the fact that BST-2 expression is regulated by type I IFN (Van Damme et al., 2008), we observed a positive correlation between BST-2 expression and type I IFN levels in cell culture supernatants. Our results also provide evidence that IL-17A promote a pro-inflammatory transcriptional program, while impairing the capacity of IEC to sense HIV via pathogen recognition receptors. Indeed, the RNA Sequencing results revealed that IL-17A exposure of IEC results in a decrease in TLR3 and RIG-I and an increase in the expression of multiple pro-inflammatory cytokines/chemokines, as well as a decrease in A20, a negative regulator inflammation (Chitre et al., 2018).

Previous studies demonstrated that early type I IFN supplementation can prevent the establishment of SIV infection, while a delay in the type I IFN production lead to an accelerated disease progression (Sandler et al., 2014). Accordingly, our results raise the hypothesis that increased IL-17A levels/responses prior to infection could dampen or delay the type I IFN responses, thus facilitating HIV/SIV acquisition. Indeed, in a model of SIV infection, mucosal type I IFN responses were detected at late time points post-infection and coincided with vanished IL-17A responses (Hensley-McBain et al., 2018). Further studies targeting the IL-17A pathway blockade to boost type I IFN responses during early HIV/SIV infection or before exposure should test this hypothesis.

In conclusion, our results provide evidence supporting the deleterious role of IL-17A during acute and chronic HIV infection by its capacity to transcriptionally reprogram IEC for an efficient HIV capture and transmission but also an increased capacity to promote replication/reactivation in subjacent infected CD4+ T-cells. Our results point to IL-17A as a potential therapeutic target to limit HIV acquisition at mucosal sites and reduce the deleterious effects of HIV reactivation in ART-treated PLWH. The successful use of IL-17A Abs for the treatment of specific autoimmune conditions (e.g., psoriasis) (Burkett and Kuchroo, 2016) opens the path for testing such Abs for HIV cure/remission.

Limitations of the study

We acknowledge that one major limitation of our study is the absence of experiments with polarized IEC. We indeed performed our co-culture experiments on plastic grown IEC which does not reflect the physiological interplay between T cells and IEC. *In vivo,* T cells have access to the serosal basolateral side of the epithelial cell barrier whereas in our study T cells had access to the apical side. A polarized distribution of chemokine receptors or polarized secretion of cell products in IEC could affect the interpretation of our experiments and the application of our conclusions to an *in vivo* scenario. Future experiments should validate our results performing co-cultures of polarized human primary IEC with T cells, as reported previously (Real et al., 2018).

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Authors' contributions

TRWS contributed to the study design, performed all experiments, analyzed results, prepared figures, and wrote the manuscript. JPG and OT performed RNA-Seq analysis. OT contributed with figures from RNA-seq analysis. HT generated preliminary results for Figure 1A. AG, YZ, LRM, EMG and DV performed experiments. JPR and CT allowed access to biological samples and study participants clinical information. MEF, NC and AL contributed to study design and provided protocols for IEC culture and HIV quantification. MGB and EAC kindly provide reagents and expertise for BST2 experiments. PA designed the study, contributed to data analysis and figure preparation and wrote the manuscript.

Declaration of Interests

The authors declare no competing interests.

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MATERIAL AND METHODS

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for reagents may be directed to and will be fulfilled by the Lead Contact, Petronela Ancuta (petronela.ancuta@umontreal.ca).

Material availability

This Study did not generate new unique reagents.

Data and code availability

- RNA-Sequencing data sets have been deposited in the Gene Expression Omnibus (GEO) database: GSE147045 and are publicly available as of the date of publication. Accession numbers are listed in the Key Resources Table.
- All original code has been included in Supplemental File 5 and is publicly available as of the date of publication.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Human subjects

HIV-uninfected individuals (HIV-), virally suppressed ART-treated PLWH (ART+PLWH) and untreated PLWH (HIV+PLWH) (Table 1), were recruited at the Montreal Chest Institute, McGill University Health Centre and Centre Hospitalier de l'Université de Montréal (CHUM, Montreal, QC, Canada). Peripheral blood mononuclear cells (PBMC) (109–1010 cells) were collected by leukapheresis and frozen until use, as previously described (Boulassel et al., 2003). Plasma viral

load in ART-treated PLWH was measured using the Amplicor HIV-1 monitor ultrasensitive method (Roche).

The collection of leukapheresis from HIV-uninfected individuals, untreated ART- PLWH and ART+ treated PLWH was conducted in compliance with the principles included in the Declaration of Helsinki. This study received approval from the Institutional Review Board of the McGill University Health Centre and the CHUM-Research Centre, Montreal, Quebec, Canada. Written informed consents were obtained from all study participants.

Epithelial cell line

The female human colorectal adenocarcinoma intestinal epithelial cell (IEC) line HT-29 (ATCC, Manassas, VA, USA) was seed in T75 culture flask (Corning, Tewksbury, MA, USA) with McCoy's 5A medium (ATCC, Manassas, VA, USA) containing 10% fetal bovine serum (FBS) and 1% Penicillin-Streptomycin (P/S; original solution at 10,000 U/ml) (Gibco, Carlsbad, CA, USA). Cells were split every 4-6 days when reaching 90-95% confluence using Trypsin-EDTA (Gibco, Carlsbad, CA, USA). No cell authentication was performed on the HT-29 cell line.

HEK293- Type I IFN reporter cell line

Bioactive human Type I interferons (IFN) were quantified using the Female Human HEK293- Type I IFN reporter cell line provided by Dr. Eric Cohen accordingly to the protocol described by the original provider (InvivoGen, San Diego, CA, USA). Briefly, cells were cultured with DMEM media (Gibco, Carlsbad, CA, USA) containing 10% FBS, 1% P/S, Blasticidin (InvivoGen, San Diego, CA, USA) and Zeocin (InvivoGen, San Diego, CA, USA). Cells were harvest every 4-6 days after reaching 90-95% confluence using Versene (Gibco, Grand Island, NY, USA). Experiments were performed with cells at passages 3-7. No cell authentication was performed on the HT-29 cell line.

Primary intestinal epithelial cells

Primary human IEC were purchased from Lonza (lot 0000751353; unknown sex as reported by the certificate of analysis) and seed in culture following the instructions of the provider. Prior to primary IEC culture, Rat-tail type 1 collagen was used to cover the surface of 48 well plates at a concentration of 30 ug/ml and incubated for 30 min at 37°C. Then, collagen was aspirated, and wells were washed with PBS twice. Primary IEC were cultured at density of ~100,000 cell/well with SmBM smooth muscle cell basal medium (SmBM-2™, Lonza USA) and SmGM™-2 Single Quots™ (Lonza, USA) formulates which contains insulin, hFGF-B, hEGF-B, FBS and gentamicin/amphotericin-B. Cells were incubated at 33 °C with CO2 5% for 5 days to reach 90% confluence and be used for subsequent experiments.

METHOD DETAILS

Cytokines, chemokines and proteins

Human recombinant TNF-α, IL-17A, IL-2, CCL20 and B18R Protein (R&D Systems) were used at concentrations indicated in the Results or Figure legends.

Primary CD4+ T-cell isolation and culture

Memory CD4+ T-cells were isolated from PBMCs of HIV-uninfected and ART-treated PLWH by negative selection using magnetic beads (Miltenyi Biotec, Auburn, CA, USA), as we previously described (Planas et al., 2017). T-cells (2x106 cells/ml per 48 well-plate well) were stimulated with immobilized CD3 and soluble CD28 Abs (1 µg/mL) for 3 days prior to infection and/or co-culture experiments with IEC. T-cells were cultured with RPMI1640 media (Gibco, Grand Island, NY, USA) containing 10% FBS and 1% P/S. After infection and/or co-culture with IEC, T-cells were maintained in culture in the presence of IL-2 (5 ng/ml; R&D Systems, Minneapolis, MN, USA) for up to 9 days, with 50% media being refreshed every 3 days.

HIV-1 exposure and infection in vitro

The molecular clones of CCR5-tropic HIV-1 strain used in this study were: transmitted Founder (T/F) THRO and the recombinant NL4.3BAL viruses. The T/F THRO HIV plasmid was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: pTHRO.c/2626 (cat# 11745) from Dr. John Kappes and Dr. Christina Ochsenbauer. The NL4.3BaL HIV plasmid was a gift from Dr. Dana Gabuzda (Dana-Farber Cancer Institute, Boston, MA, USA). HIV-1 plasmid was amplified by MiniPrep and MaxiPrep and viral stocks were produced and titrated, as we described it previously (Gosselin et al., 2010; Monteiro et al., 2011). HT29 IEC cultured in 48 well-plates (150,000 cells/well) until 80% confluence were exposed to HIV (50 ng HIV-p24/150,000 cells) for 3 hours. Unbound virions were removed by extensive washing. In a set of experiments, cytokine-activated HIV-exposed HT-29 IEC were cultured either alone or co-cultured with CD3/CD28-activated memory CD4+ T-cells from HIV-uninfected individuals. In another set of experiments, memory CD4+ T-cells from HIV-uninfected individuals were infected with HIV (20 ng HIV-p24/106 cells) and then co-cultured with cytokine-activated HT-29 IEC. Viral replication was measured by flow cytometry intracellular HIV-p24 staining and HIV-p24 ELISA in cell-culture supernatant, as previously described (Gosselin et al., 2017; Zhyvoloup et al., 2017). Briefly, the IEC:T cell co-cultures were harvested at day 9 post infection (for HIV infection in vitro) or day 9-12 post culture (for VOA). Cells were stained on the surface with fluorochrome-conjugated CD3, CD4, and BST2 Abs and intracellularly with anti-HIV-p24 Abs. The positivity gates were established using the fluorescence minus one (FMO) strategy. Acquisition was performed using the BD LSRFortessa flow cytometer (BD Bioscience, USA). In parallel, cell culture supernatants collected every 3 days were used for HIV-p24 ELISA.

Flow cytometry staining and analysis

The following fluorochrome-conjugated Abs were used for flow cytometry analysis: HIV-p24 FITC (KC57) (Beckman Coulter, Fullerton, CA, USA), CD3 Pacific blue (UCHT1), CD3 Alexa Fluor 700 (UCHT1), BST2 BV421 (Y129) (BD Biosciences, San Diego, CA, USA) and CD4 PerCP/Cy5.5 (RPA-T4) (Biolegend, San Diego, CA, USA). BST2 staining was also performed with homemade primary rabbit polyclonal BST2 Abs (provided by Dr. Éric Cohen) and secondary goat anti-rabbit IgG conjugated with Alexa Fluor 594 (Abcam, Cambridge, UK). Live/Dead Fixable Aqua Dead Cell Stain Kit (Vivid, Life Technologies, Burlington, Ontario, CA) was used to exclude dead cells. Cells were

analyzed with the BD-LSRII cytometer and BD-Diva (BD Biosciences) and FlowJo version 10 (Tree Star, Inc., Ashland Oregon, USA). The positivity gates were placed using fluorescence minus one (FMO) strategy (Roederer, 2002).

ELISA

HIV-p24 levels in cell culture supernatant or cell lysates were quantified using a homemade sandwich ELISA, as described previously (Bounou et al., 2002; Cattin et al., 2019; Gosselin et al., 2017). Prior to the ELISA, virions in cell supernatants and cells were lysed using a homemade buffer solution (PBS 1X, Tween 20 0.05%, Triton X-100 2.5%, Trypan Blue 1% and Thimerosal 0.02% in deionized water) for 1h at 37°C. Levels of CCL20 and IL-8 were measured in the cell culture supernatant, according to the manufacturer's protocols (R&D Systems, Minneapolis, MN, USA).

HIV-DNA quantification

Integrated HIV-DNA, as well as early (RU5) and late (Gag) reverse transcripts were quantified in cell lysates by nested real-time PCR using specific primers (Supplemental Table 1), as we previously described (Gosselin et al., 2010; Monteiro et al., 2011; Planas et al., 2017; Cattin et al., 2019). Briefly, integrated HIV-DNA, as well as early (RU5) reverse transcripts and total HIV-DNA (which detects both late reverse transcripts and integrated HIV-DNA by targeting the Gag gene) were quantified in cell lysates by nested real-time PCR using primers purchased at IDT. The CD3 gene was used to normalize the number of HIV-DNA copies per cell number (two CD3 copies per cell) (Planas et al., 2017; Cattin et al., 2019).

Real-time RT-PCR

Total RNA was extracted from the primary intestinal epithelial cells with the RNeasy Plus Mini kit (Qiagen). IFIT1 and ISG15 gene expression was evaluated by One step SYBR green RT-PCR (Qiagen) using a LightCycler 480. IFIT1 primers were purchased from Qiagen (QuantiTect Primer

Assay). ISG15 primers were purchased from IDT with the following sequence Forward 5′ -ACTCATCTTTGCCAGTACAGGAG-3′, Reverse: 5′ -CAGCATCTTCACCGTCAGGTC-3′ (Supplemental Table 1). The amplification cycles used for ISG15 RT-PCR was reverse transcription at 50°C for 30 min, 15 min at 95 °C and then 35 cycles each at 94 °C for 10 sec, 56 °C for 10 sec, and 72 °C for 10 sec. The relative expression of the target genes mentioned above were normalized relative to the internal control 28S rRNA. The sequence of primers used were the 28S rRNA forward primer 5′ -CGAGATTCCTGTCCCCACTA-3′ and 28S rRNA reverse primer 5′ GGGGCCACCTCCTTATTCTA-3′ (purchased from IDT) (Supplemental Table 1). Each reaction was performed in triplicates. Melting curve analysis was used to determine the uniformity of the thermal dissociation profile for each product amplified by real-time RT-PCR.

Illumina RNA sequencing and analysis

Total RNA was extracted from cytokine-activated IEC and cytokine-activated IEC co-cultured with CD4+ memory T-cells using RNeasy Plus mini kit (Qiagen, Germantown, Maryland, USA). Genome-wide transcriptional profiling was performed by Genome Québec (Montreal, Québec, Canada) using the Illumina RNA-Sequencing model NovaSeq S4 6000 PE100. Briefly, the paired-end sequencing reads were aligned to coding and non-coding transcripts from Homo Sapiens database GRCh 37 version75 and quantified with the Kallisto software version 0.44.0. The entire RNA-Sequencing data set and the technical information requested by Minimum Information About a Microarray Experiment (MIAME) are available at the Gene Expression Omnibus database under accession GSE147045. Log2-counts per million (log CPM) transformed transcript-level data of IECTNF, IECTNF+IL-17A and IECIL-17A alone or co-cultured with memory CD4+ T-cells of ART+PLWH was compared respectively with IEC Medium alone or co-cultured with memory CD4+ T-cells of ART+PLWH, to calculate the fold change (Supplemental File 6).

Gene set enrichment analysis

ClueGO application on the software Cytoscape 3.7.2 and a Fisher exact-t-Test was applied to find significant enrichments and produce networks of enriched pathways. The latest Gene Ontology:

Biological Processes database was used, as available at https://www.gsea-msigdb.org/gsea/msigdb/genesets.jsp?collection=BP. To stablish modules of genes with similar responses to cytokine-activations, hierarchical clustering was performed on the scaled fold-changes with the pheatmap package in R. Only genes with an absolute fold-change greater than 1.3 in at least one of the cytokine-activation conditions were considered, therefore focusing mainly on larger transcriptional changes. Two different resolutions of clustering were utilized, yielding either 3 main clusters or 8 clusters. Each of the gene modules was compared to a reference background consisting of all genes measured (28,083 genes). The R scripts used for analysis are available on Supplemental File 5. The matrix data base with fold change gene information used to run this analysis is available on Supplemental File 6.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analyses were performed with GraphPad Prism 10 software (GraphPad Software, Inc., La Joya, CA, USA). Paired t-Test were performed to assess the statistical difference between two conditions for the same donor and p values were added on the graph. Friedman along with Dunn's multiple comparisons test evaluated the statistical differences between more than two conditions (*, $p<0.1$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$). Spearman Correlation and Linear regression models were applied to explore the relationship between two quantitative variables. P-values of ≤ 0.05 were considered statistically significant. More details of the statistical analysis are included in Figure legends.

MAIN FIGURES

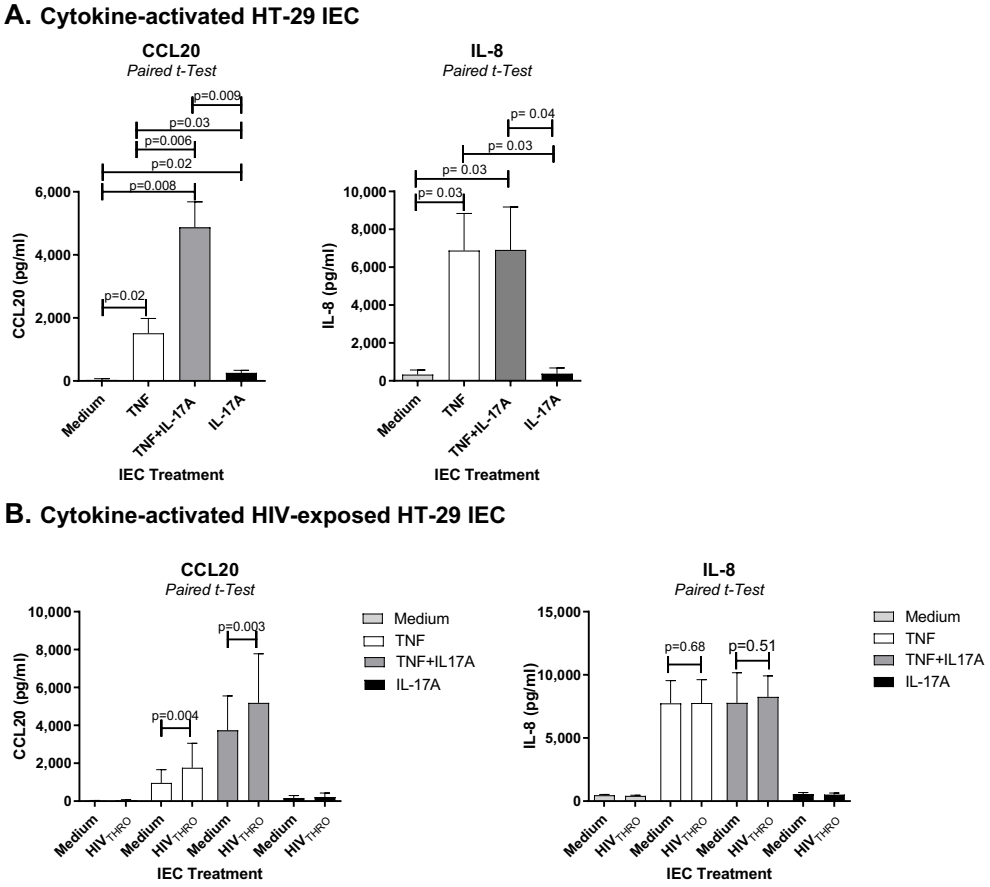


Figure 1: IL-17A acts in synergy with TNF to promote CCL20 production by IEC in the presence or the absence of HIV-1. (A) Intestinal epithelial cells (IEC; HT29 cell line) were cultured in 48 well-plates until 80% confluence (150,000 cells/well). IEC were activated with TNF (10 ng/ml) and/or IL-17A (10 ng/ml) for 24 h. (B) IEC cells were cultured in 48 well-plates wells until 80% confluence and activated with TNF (10 ng/ml) and/or IL-17A (10 ng/ml) for 3h and then exposed to T/F THRO (HIVTHRO) HIV strain (50 ng/well) for 24 h. Levels of CCL20 (A-B, left panels) and IL-8 (A-B, right panels) were quantified in cell culture supernatants by ELISA 24 h post-stimulation with cytokines (A) and/or HIV exposure (B). Paired t-Test evaluated the statistical differences. P-values are indicated on the graphs. Results are mean±SD from three independent experiments performed with HT-29 IEC.

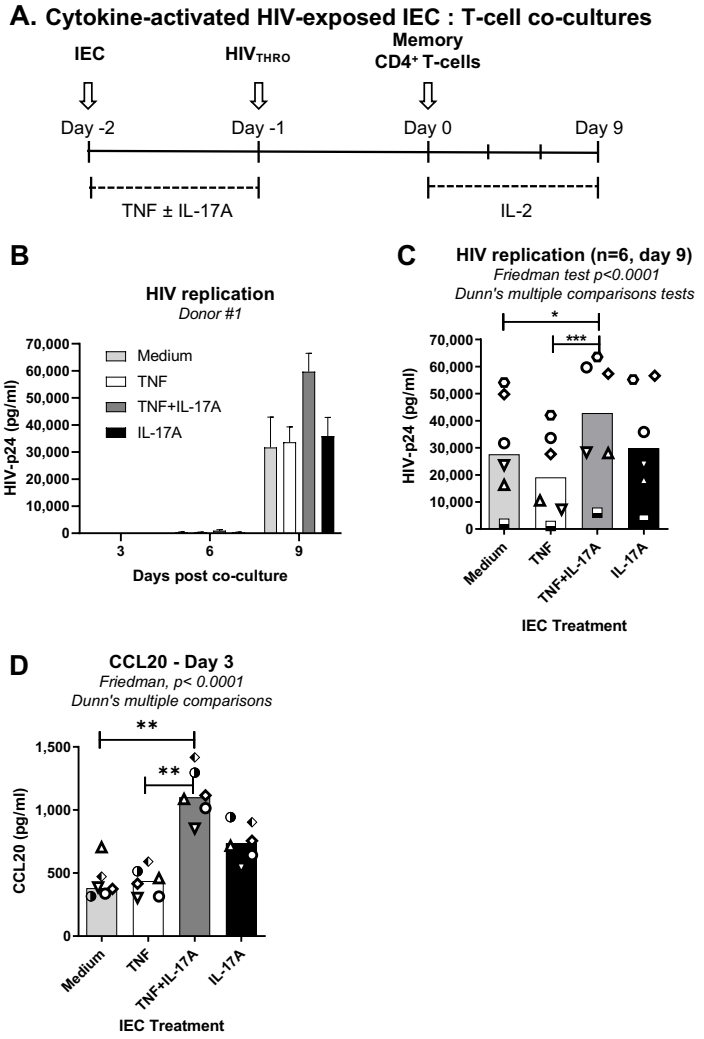


Figure 2: IL-17A-activated IEC efficiently transmit HIV infection to CD4+ T-cells. (A) Shown is the experimental flow chart. Briefly, IEC were cultured in 48 well-plates until they reached 80% confluence (150,000 cells/well). IEC were then, activated with TNF (10 ng/ml) and/or IL-17A (10 ng/ml) for 24 h before being exposed to HIVTHRO for 3 h. Unbound HIV was removed by extensive washing and cells were further cultured for 24 h. In parallel, memory CD4+ T-cells isolated from PBMCs of HIV-uninfected individuals were activated with CD3/CD28 Abs for 3 days. Cytokine-activated HIV-exposed IEC cells were co-cultured with CD3/CD28-activated memory CD4+ T-cells (500,000 cells/well) and media supplemented with IL-2 (5 ng/ml) was refreshed every 3 days for 9 days. (B-C) Levels of HIV-p24 in cell culture supernatant were quantified by ELISA at days 3, 6, and 9. Shown are the mean±SD of HIV-p24 levels between the different IEC

treatment from one representative donor at days 3, 6 and 9 (B) and from n=6 different individuals at day 9 post co-culture (C). Levels of CCL20 in cell culture supernatants were quantified at day 3 post-coculture (D). For Figure 2 C-D, each symbol represents results generated with memory CD4+ T-cells from a different individual (mean values of ELISA triplicate measurements). The bars represent the median value of all individuals. Friedman test with Dunn's post-test evaluated the statistical differences. P-values are indicated on the graphs (*, $p<0.1$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$).

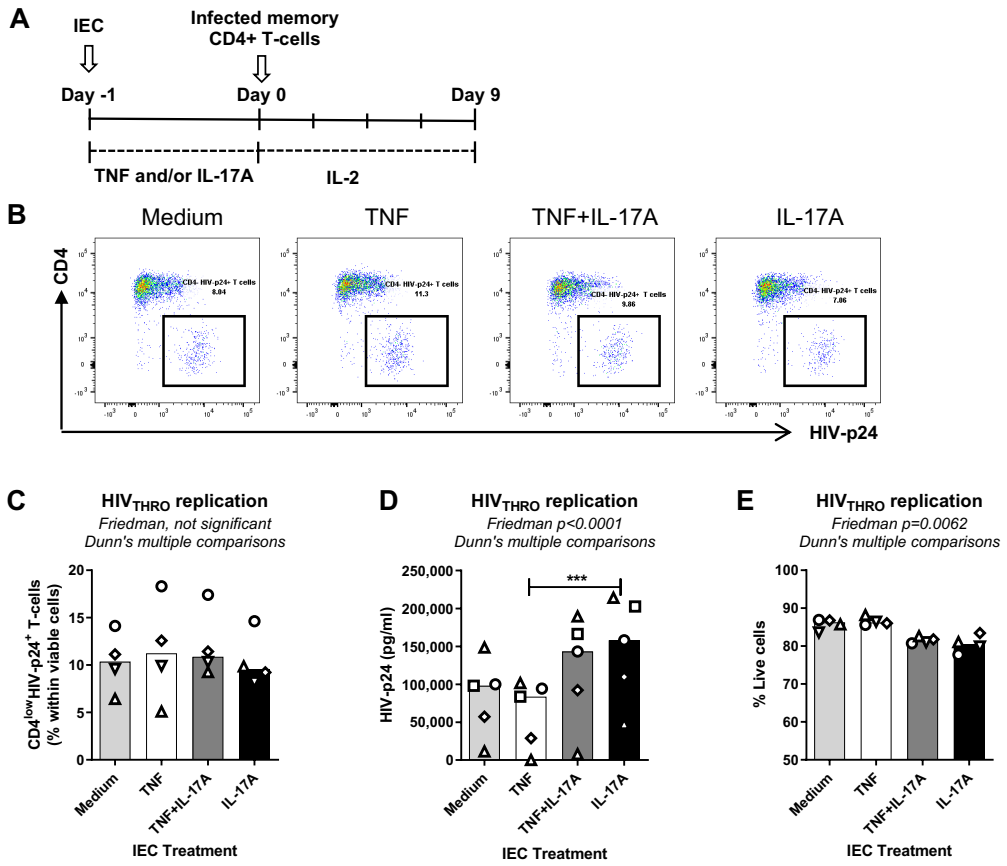


Figure 3: IL-17A-activated IEC promote HIV replication/outgrowth in HIV-infected memory CD4+ T-cells. (A) Shown is the experimental flow chart. Briefly, IEC were activated with TNF and/or IL-17A as described in Figure 1 A. In parallel, memory CD4+ T-cells of HIV-uninfected individuals were isolated and activated as in Figure 2. After activation, memory CD4+ T-cells of HIV-uninfected individuals were infected with HIVTHRO in vitro. Cytokine-activated IEC (150,000 cells/well) were co-cultured with in vitro infected memory CD4+ T-cells (500,000 cells/well). Media containing IL-2 (5 ng/ml) was refreshed every 3 days for 9 days. (B-C) The intracellular expression of HIV-p24 in memory CD4+ T-cells was quantified by flow cytometry upon staining with fluorochrome-conjugated CD3, CD4 and HIV-p24 Abs. Shown are dot plots presenting the frequency of CD4lowHIV-p24+ T-cells across the diferent experimental conditions for one representative donor (B) and the statistical analysis of results obtained with HIVTHRO-infected memory CD4+ T cells of n=4 different individuals at day 9 post co-culture (C). Levels of HIV-p24 in cell culture supernatant were quantified by ELISA at days 3, 6, 9 and 12 post-culture (D). Shown is the statistical analysis of results obtained with HIVTHRO-infected memory CD4+ T-cells of n=4 different individuals at day 9 post co-culture. (E) Cell viability was quantified by flow cytometry

upon staining with Live/Dead Fixable Aqua Dead Cell Stain Kit. Shown is the statistical analysis of the percentage of live cells obtained with HIVTHRO-infected memory CD4+ T-cells of n=4 different individuals at day 9 post co-culture. Each symbol represents results generated with memory CD4+ T-cells from a different individual (mean values of ELISA triplicate measurements). The bars represent the median value of all individuals. Friedman test with Dunn's post-test evaluated the statistical differences. P-values are indicated on the graphs (*, $p<0.1$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$).

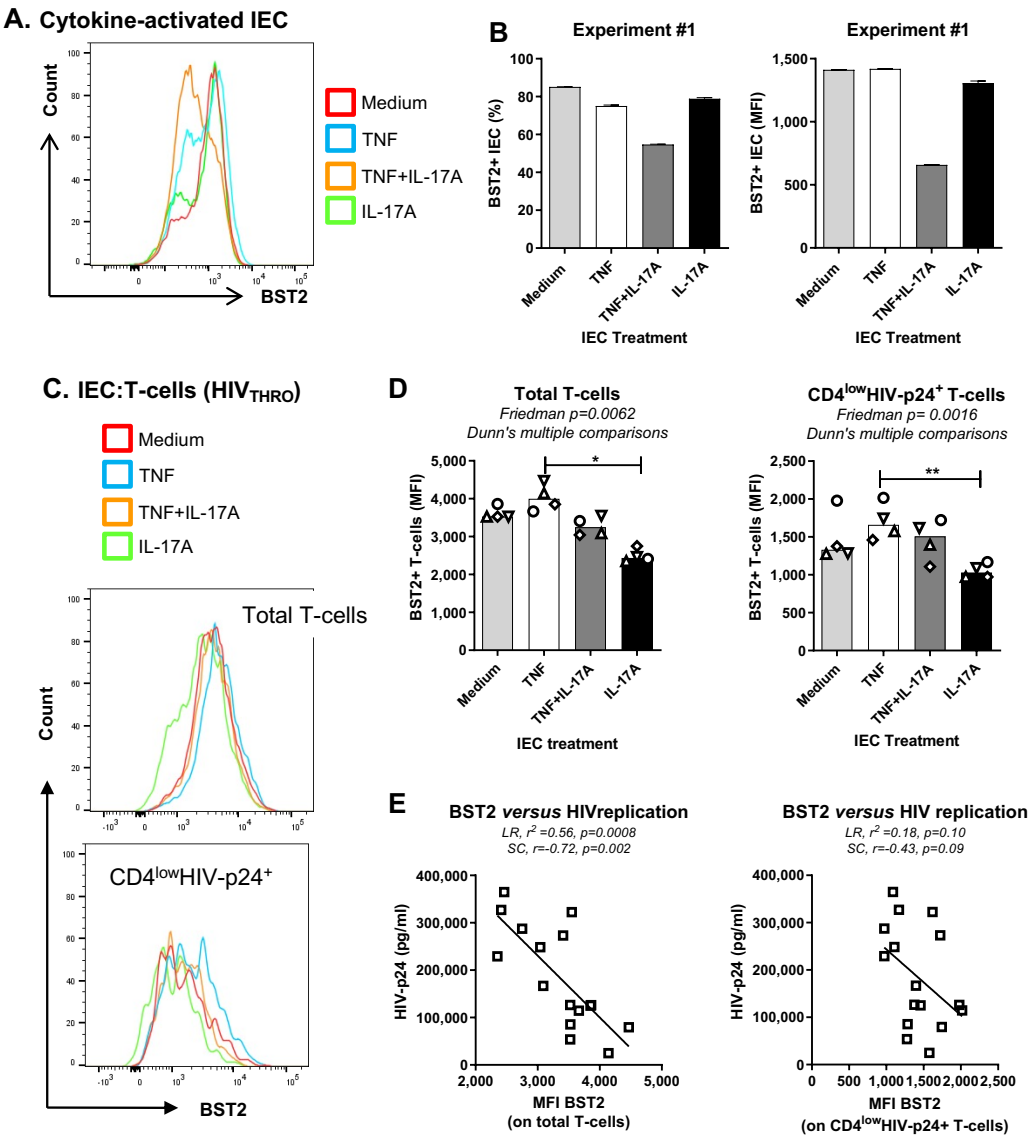


Figure 4: IL-17A acts on IEC to decrease Tetherin/BST2 expression. (A-B) IEC were activated as described in Figure 1. BST2 expression on IEC was quantified by flow cytometry on day 3 post-activation. Shown are histograms of BST2 expression on IEC (A), the frequency of BST2+ IEC (B, left panel) and MFI of BST2 expression (B, right panel) (mean±SD of triplicate wells) of one experiment performed with HT-29 cells; similar results were obtained in a second experiment. (C-E) Cytokine-activated IEC were co-cultured with memory CD4+ T-cells activated via CD3/CD28 and infected with HIVTHRO in vitro. The expression of BST2 in memory CD4+ T-cells was quantified by flow cytometry using fluorochrome-conjugated CD3, CD4, HIV-p24 and BST-2 Abs. Shown are histograms of BST2 expression (C) on total T-cells (CD3+ cells, C, upper panel) and CD4lowHIV-p24+ T-cells (C, lower panel). Shown is statistical analysis of MFI BST2 expression on

total T-cells (D, left panel) and CD4lowHIV-p24+ T-cells (D, right panel) in n=4 different individuals at day 9 post co-culture. The bars represent the median value of all individuals. Friedman test with Dunn's post-test addressed the statistical differences. P-values are indicated on the graphs (*, p<0.1; **, p<0.01; ***, p<0.001; ****, p<0.0001). (E) Correlation between the HIV-p24 levels in cell culture supernatant and the MFI of BST2 expression on total T-cells (E, left panel) and CD4lowHIV-p24+ T-cells (E, right panel) were calculated using. Spearman correlation (SC, p and r values) and linear regression (LR, p and r2 values) models for all individuals and experimental conditions tested. Each symbol represents results generated with T-cells from a different individual.

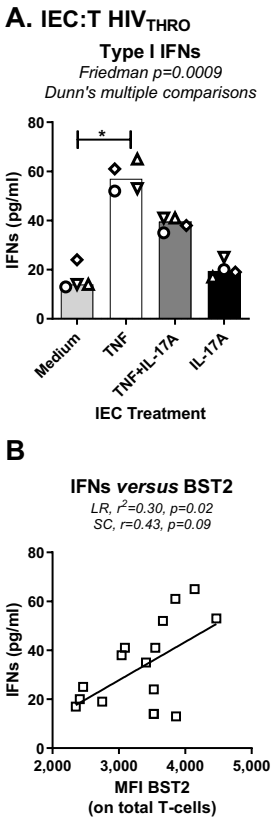


Figure 5: IL-17A-activated IEC decrease type I interferon levels in positively relationship with BST2 expression on HIV-infected T-cells. Cytokine-activated IEC were co-cultured with HIVTHRO-infected memory CD4+ T-cells, as described in Figure 3. Bioactive human Type I interferons (IFN) levels were quantified using the reporter cell line HEK-Blue IFN-α/β in cell culture supernatants collected at days 3 post-co-culture. (A) Shown are type I IFN levels in co-cultures performed with HIVTHRO-infected memory CD4+ T-cells from n=4 different individuals and the bars represent the median value of all individuals Friedman test with Dunn's post-test evaluated the statistical differences. P-values are indicated on the graphs (*, p<0.1; **, p<0.01; ***, p<0.001; ****, p<0.0001). (B) The correlation between type I IFN levels with the MFI of BST2 expression on total T-cells (B) was evaluated using SC and LR models in all donors and experiments conditions. Each symbol represents results generated with T-cells from a different individual.

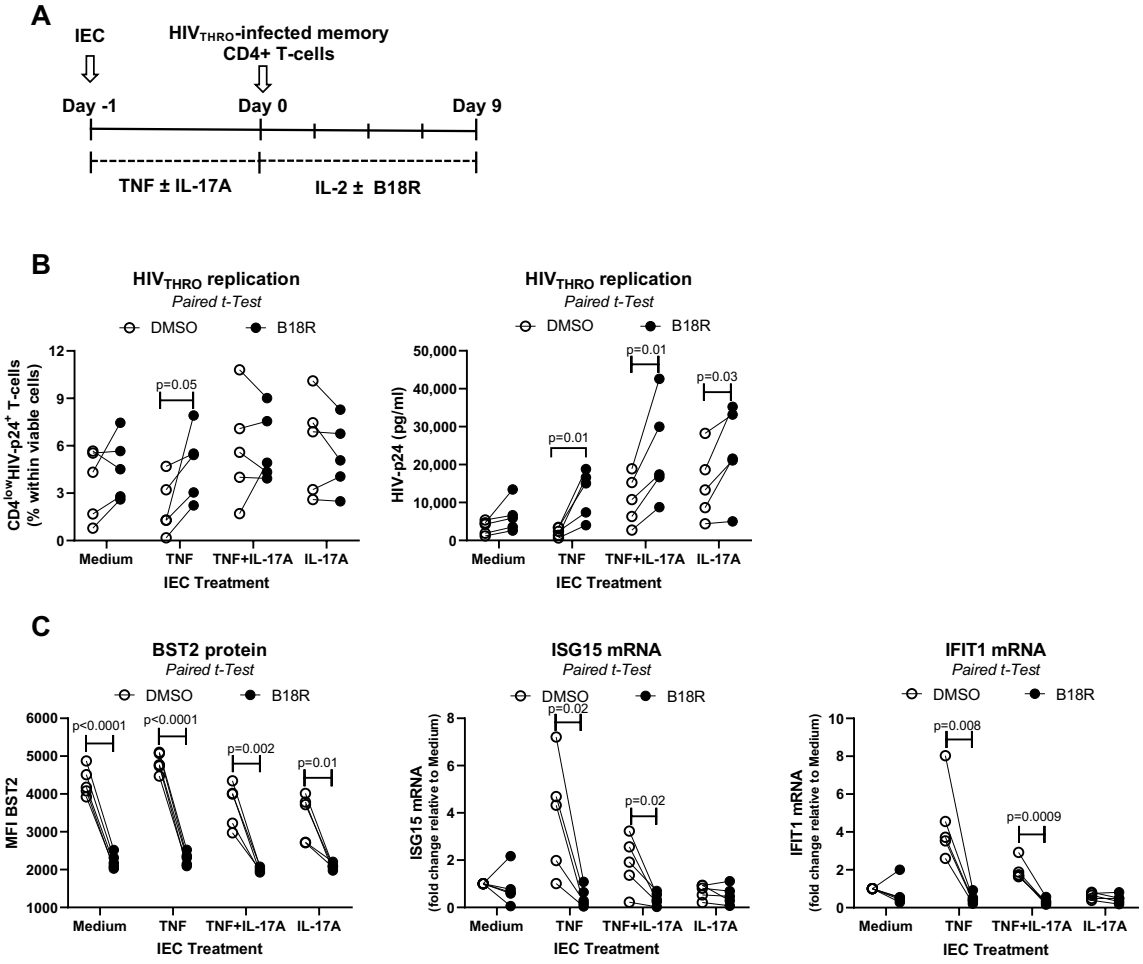


Figure 6: IL-17A acts on IEC to modulate HIV dissemination to T cells and the expression of IFN-stimulated genes. Shown is the experimental flow chart (A). Briefly, IEC were activated with TNF and/or IL-17A, as in Figure 1A. In parallel, memory CD4+ T-cells of HIV-uninfected individuals were isolated and activated, as in Figure 2. After activation, the memory CD4+ T-cells were infected with HIVTHRO. Cytokine-activated IEC (150,000 cells/well) were then co-cultured with in vitro infected memory CD4+ T-cells (500,000 cells/well) for 9 days. Media containing IL-2 (5 ng/ml) with or without B18R (100 ng/ml) was refreshed every 3 days. (B) The intracellular expression of HIV-p24 in memory CD4+ T-cells was quantified by flow cytometry upon staining with fluorochrome-conjugated CD3, CD4 and HIV-p24 Abs and levels of HIV-p24 in cell culture supernatants were quantified by ELISA. Shown is the statistical analysis of results obtained with HIVTHRO-infected memory CD4+ T-cells of n=5 different individuals at day 9 post co-culture by flow cytometry (left panel) and ELISA (right panel). (C) The expression of HIV-p24 in memory CD4+

T-cells was quantified by flow cytometry upon staining with fluorochrome-conjugated BST2 and the expression of ISG15 and IFIT1 were quantified by RT-PCR. Shown is the statistical analysis of results obtained with cells of n=5 different individuals at day 3 post co-culture. Each symbol represents results generated with memory CD4+ T-cells from a different individual. Paired t-Test evaluated the statistical differences among DMSO and B18R. P-values are indicated on the graphs.

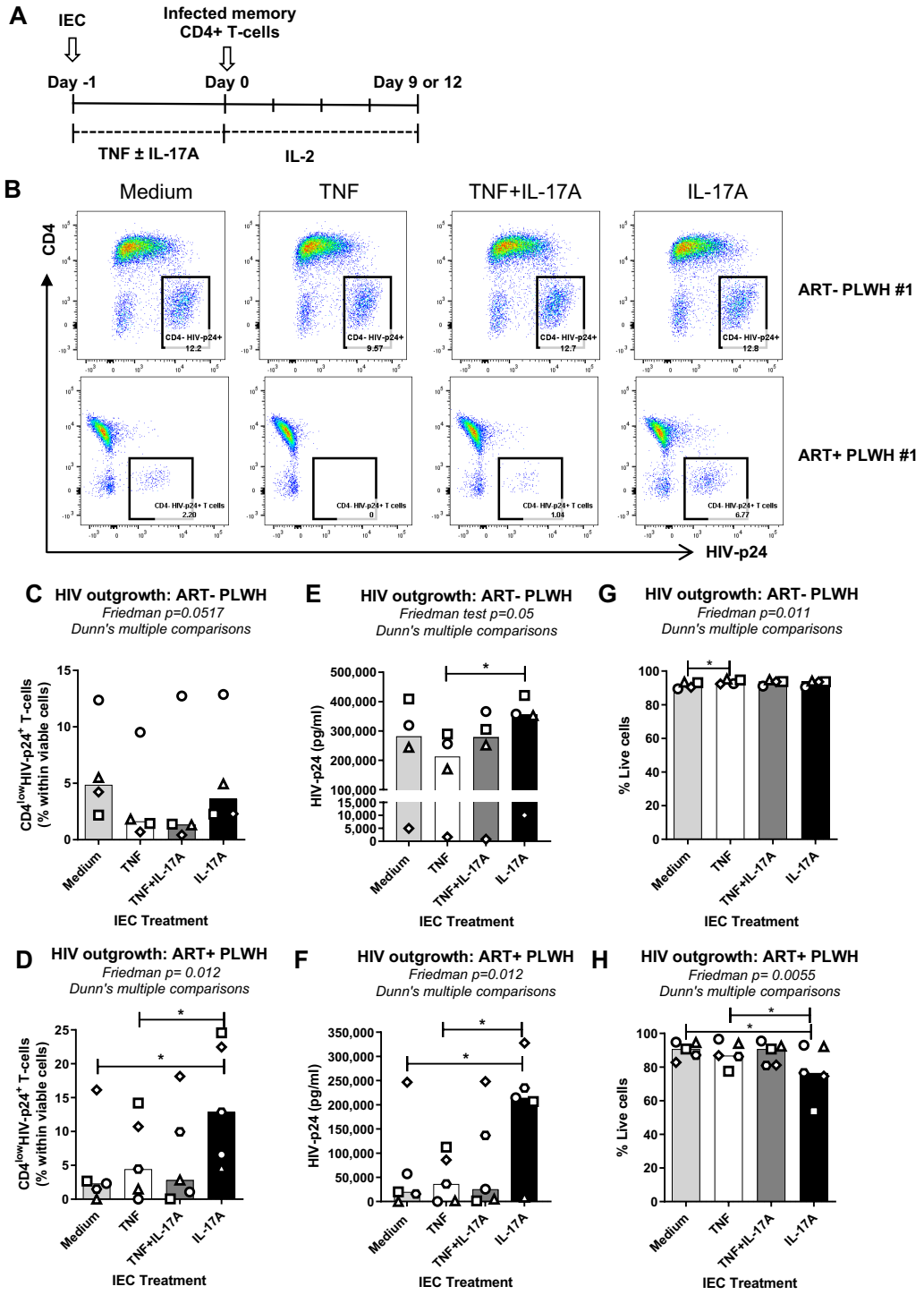


Figure 7: IL-17A-activated IEC promote HIV outgrowth in HIV-infected memory CD4+ T-cells. (A) Shown is the experimental flow chart. Briefly, IEC were activated with TNF and/or IL-17A, as in Figure 1A. In parallel, memory CD4+ T-cells of untreated (ART-) and ART-treated (ART+) PLWH were isolated and activated as in Figure 2. Cytokine-activated IEC (150,000 cells/well) were co-cultured with either memory CD4+ T-cells isolated from ART- PLWH (500,000 cells/well) or ART+

PLWH (1 000,000 cells/well). Media containing IL-2 (5 ng/ml) was refreshed every 3 days for 9 to 12 days. (B-D) The intracellular expression of HIV-p24 in memory CD4+ T-cells was quantified by flow cytometry upon staining with fluorochrome-conjugated CD3, CD4 and HIV-p24 Abs. Shown are dot plots presenting the frequency of CD4lowHIV-p24+ T-cells across the diferent experimental conditions for one representative donor of each group (B) and the statistical analysis of results obtained with memory CD4+ T-cells of n=4 different ART- PLWH at day 9 post co-culture (C), and memory CD4+ T cells of n=5 different ART+ PLWH at day 12 post co-culture (D). (E-F) Levels of HIV-p24 in cell-culture supernatant were quantified by ELISA at days 3, 6, 9 and 12 post-culture. Shown is the statistical analysis of results obtained with memory CD4+ T-cells of n=4 different ART- PLWH at day 9 post co-culture (E), and memory CD4+ T-cells of n=5 different ART+ PLWH at day 12 post co-culture (F). (G-H) Cell viability was quantified by flow cytometry upon staining with Live/Dead Fixable Aqua Dead Cell Stain Kit by flow cytometry. Shown is the statistical analysis of the percentage of live cells obtained in co-cultures with ART- (G) and ART+ PLWH (H). Each symbol represents results (mean values of ELISA triplicate measurements) generated with memory CD4+ T-cells from a different individual. The bars represent the median value of all individuals. Friedman test with Dunn's post-test evaluated the statistical differences. P-values are indicated on the graphs (*, $p<0.1$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$).

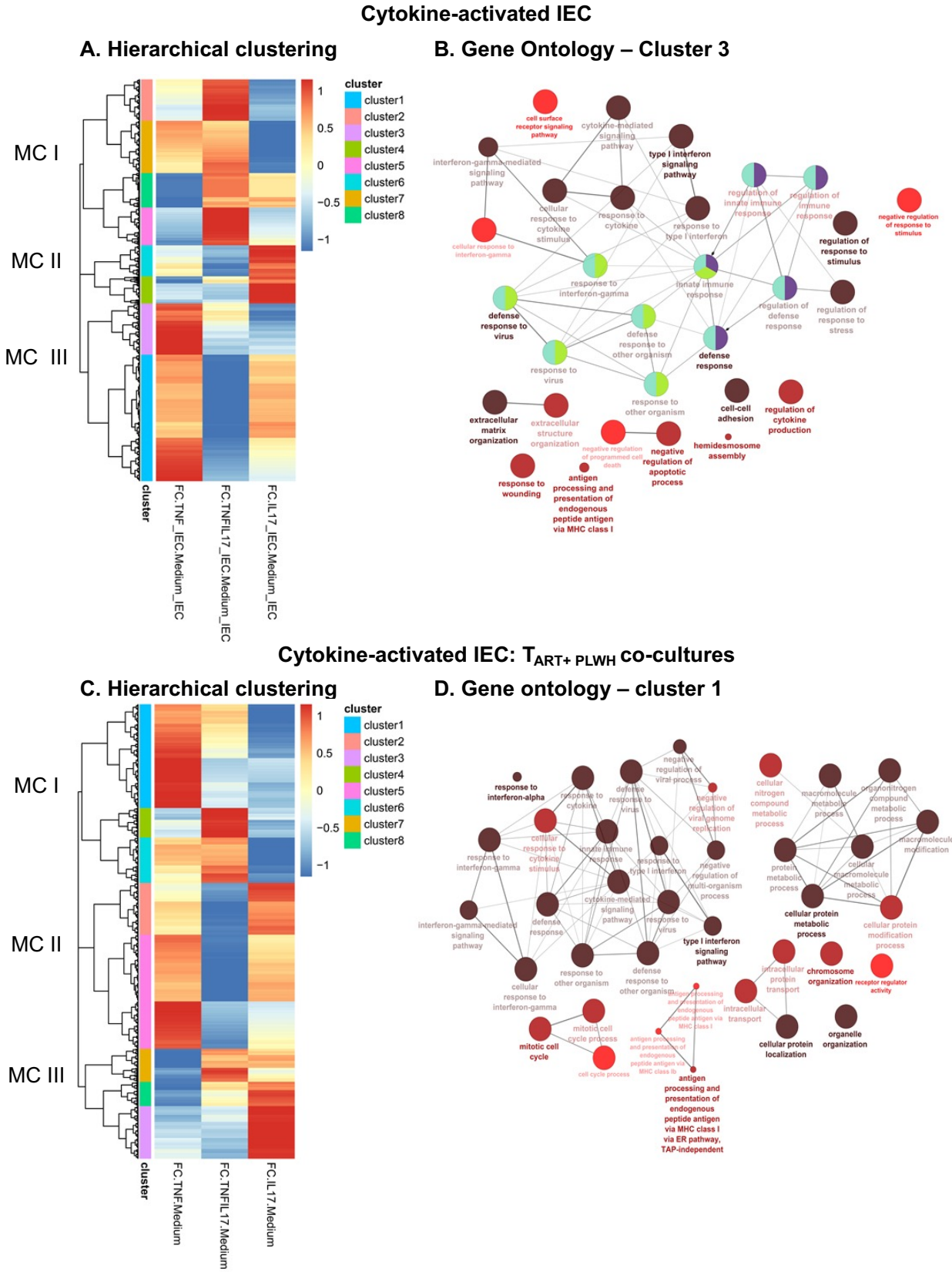


Figure 8: IL-17A modulates anti-viral responses in IEC cultured alone or co-cultured with memory CD4+ T-cells of ART+ PLWH. RNA sequencing was performed with RNA extracted from

IEC activated with TNF and/or IL-17A as described in Figure 1A and from cytokine-activated IEC (150,000 cells/well) co-cultured with CD3/CD28-activated memory CD4+ T-cells of n=3 different ART+ PLWH individuals (1 000,000 cells/well) for 3 days as described in Figure 3A. (A and C) Hierarchical clustering was performed on the scaled fold-changes to identify eight clusters of transcriptional signatures grouped in three major clusters (I, II, and III) associated to cytokine-activated IEC (A) and cytokine-activated IEC co-cultured with memory CD4+ T cells (C). (B and D) Gene set enrichment analysis was performed on each gene cluster using the Gene Ontology: Biological Processes database. The most significant differentially expressed pathways were within cluster 3 for IEC (B) and cluster 1 for cytokine-activated IEC co-cultured with memory CD4+ T-cells of ART+ PLWH individuals (D). Only pathways with FDR<0.01 are depicted. Nodes (pathways) are color coded with significance.

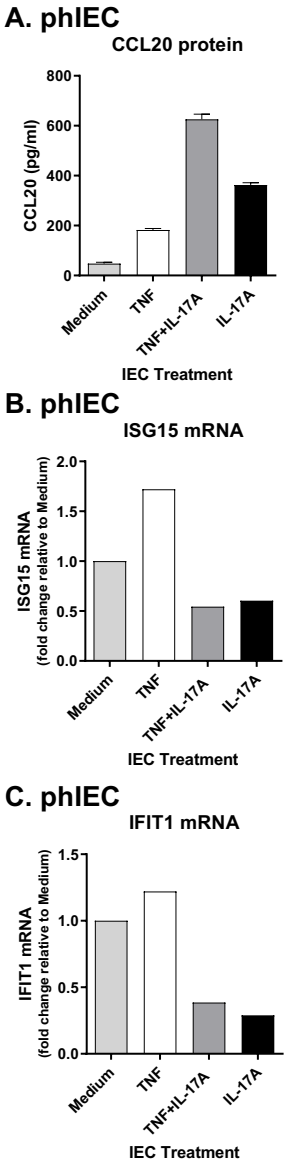


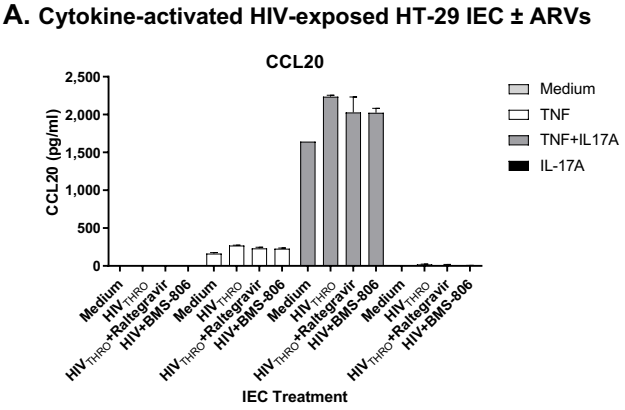
Figure 9: Effect of IL-17 on primary human intestinal epithelial cells. Primary human Primary human intestinal epithelial cells (phIEC) were cultured in 48 well-plates for 5 days until 90% confluence (150,000 cells/well). phIEC were activated with TNF (10 ng/ml) and/or IL-17A (10 ng/ml) for 24 h. (B) IEC cells were cultured in 48 well-plates wells until 80% confluence and activated with TNF (10 ng/ml) and/or IL-17A (10 ng/ml) for 24h. Levels of CCL20 were quantified in cell-culture supernatants by ELISA (mean±SD values of experimental triplicates) (A) and ISG15 and IFIT1 mRNA expression were quantified by RT-PCR (mean value of RT-PCR duplicate measurements) (B-C).

Table 1: Clinical parameters of untreated and ART-treated PLWH study participants.

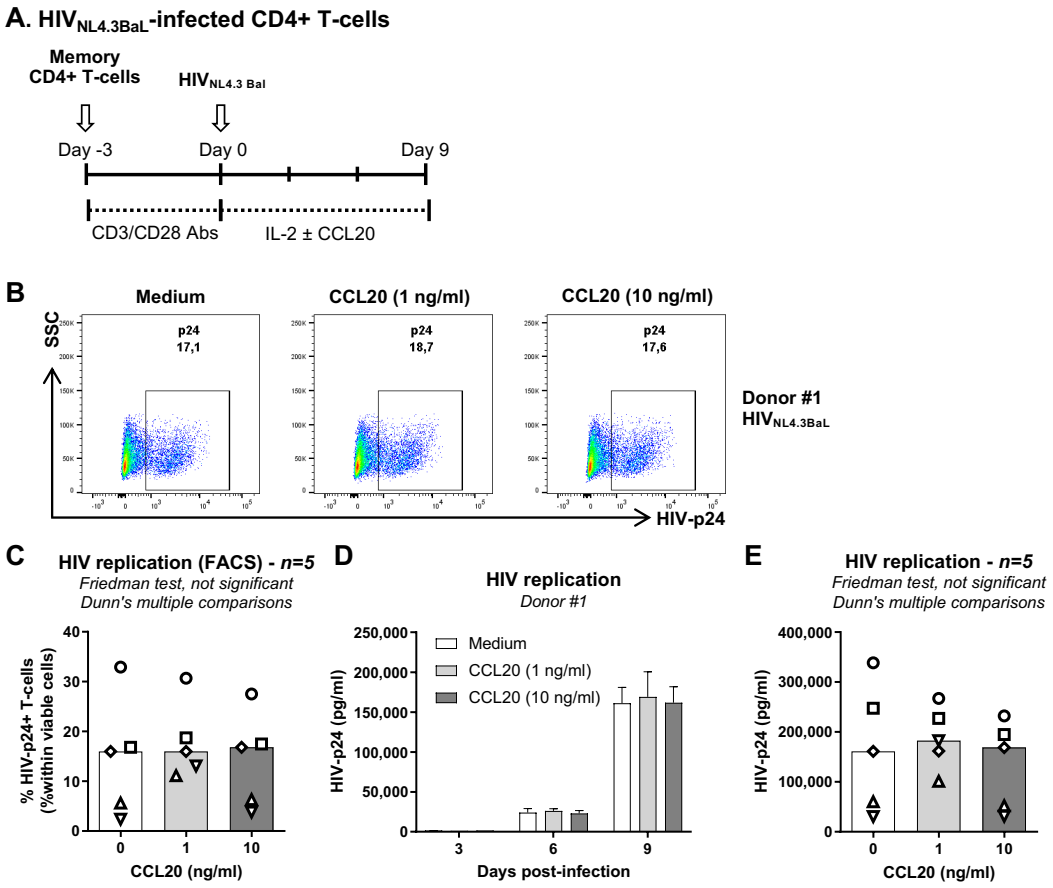
ID	Sex	Age″	CD4 counts#	CD4:CD8 Ratio	Viral load&	Time since infection*	ART	Time on ART*
ART+ #1	M	31	824	0.9	<40	58	Atripla	44
ART+ #2	F	28	535	1.8	<50	177	Viracept, Truvada	151
ART+ #3	M	50	569	1.2	<50	111	Darunavir, Raltegravir	101
ART+ #4	M	21	796	2.0	<40	8	Stribild	4
ART+ #5	M	50	827	2.3	<40	140	Complera	30
ART+ #6	M	26	331	N.A	<40	86	N.A	24
ART+ #7	M	42	795	1.1	<40	46	Tivicay/Edurant, Viread	33
ART+ #8	M	55	963	1.5	<40	124	Prevista, Kivexa, Norvir	107
ART- #1	M	40	1,068	1.2	22,812	2	None	N.A
ART- #2	M	47	529	1.2	3,189	110	None	N.A
ART- #3	M	39	946	0.7	8,809	11	None	N.A
ART- #4	M	24	316	0.5	9,496	55	None	N.A

*″ , years; #, cells/µl; &, HIV-RNA copies/ml plasma; *, months; N.A, information not available; M, male; F, female.*

SUPPLEMENTAL FIGURES

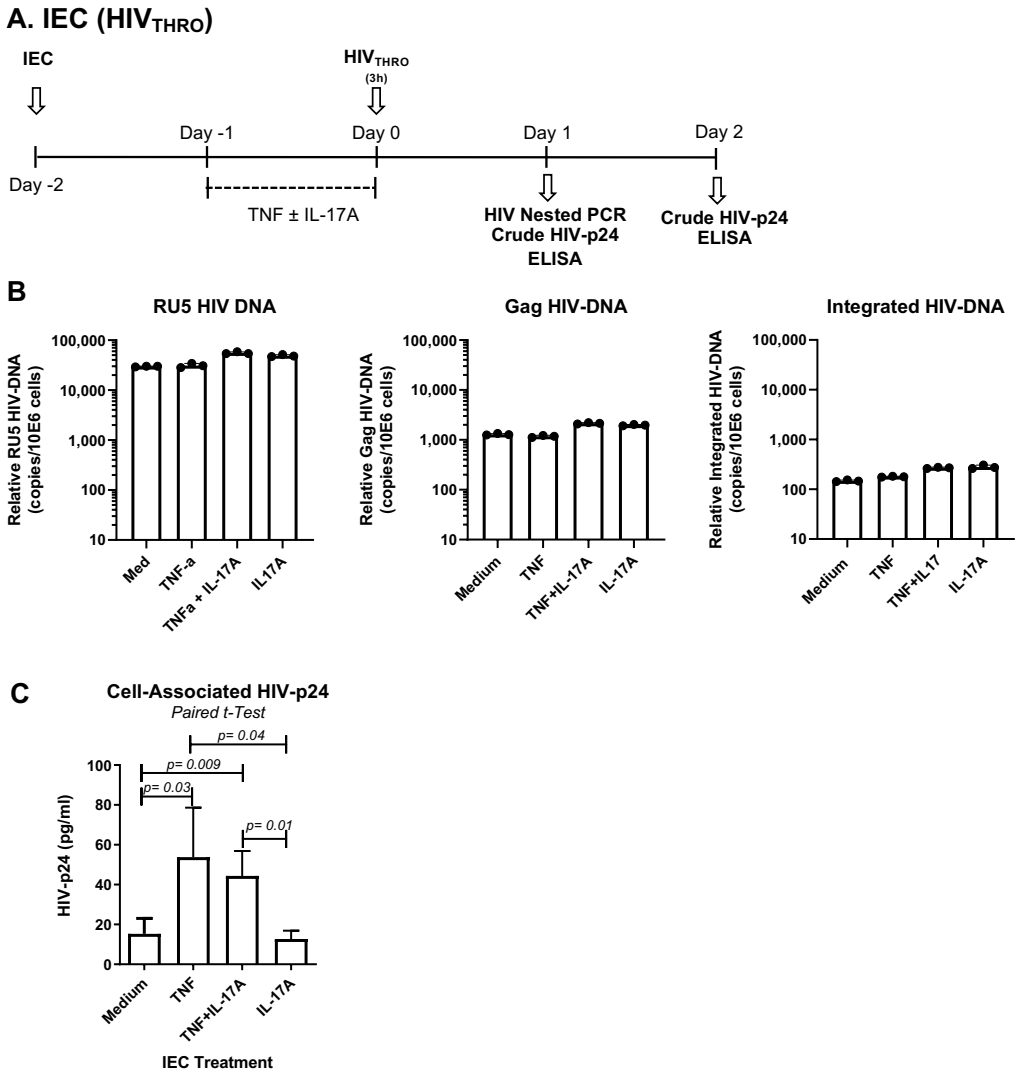


Supplemental Figure 1. **Related to Figure 1: Effects of TNF and IL-17A on HIV infection of HT-29 IEC.** (A) IEC cells were cultured in 48-well plates until reaching 80% confluence before being activated with TNF (10 ng/ml) and/or IL-17A (10 ng/ml) for 3h in the presence or absence of integrase inhibitor raltegravir (0.2 µM) or entry inhibitor BMS-806 (1 µM). They were then exposed to T/F THRO (HIV_{THRO}) (50 ng/well) for 24 h. Levels of CCL20 were quantified in cell culture supernatants by ELISA 24 h post-HIV exposure. Mean±SD values of ELISA triplicates are shown.

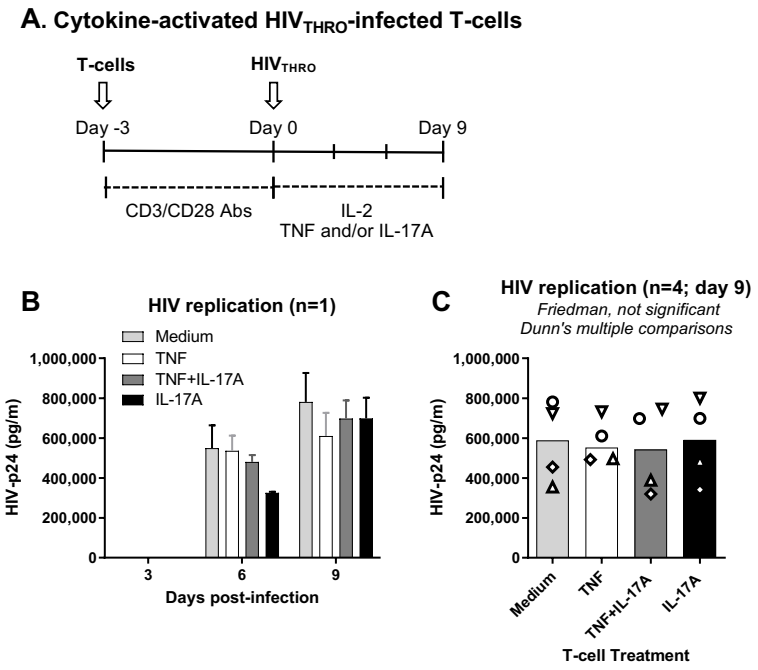


Supplemental Figure 2. Related to Figure 2: CCL20 does not influence HIV replication in memory CD4$^+$ T-cells. (A) Shown is the experimental flow chart. Briefly, memory CD4$^+$ T-cells from HIV-uninfected individuals were isolated and activated *via* CD3/CD28, as in Figure 3, and infected with HIV$_{NL4.3BaL}$ by for 3 h. HIV-infected memory CD4$^+$ T-cells were cultured in the presence of IL-2 (5 ng/ml) and in the presence or the absence of CCL20 (1 and 10 ng/ml) for 9 days. Media was refreshed with cytokines every 3 days. The intracellular expression of HIV-p24 was quantified, as in Figure 3, by flow cytometry at day 9 post-infection. Shown are flow cytometry results from one representative donor (B) and statistical analysis of results generated with cells from n=5 different individuals (C). In parallel, HIV-p24 levels in cell-culture supernatants were measured by ELISA. Shown are the mean±SD of HIV-p24 levels generated with cells from one representative donor at days 3, 6 and 9 (D), statistical analysis of results generated with cells from n=5 individuals at day 9 post-infection (E). For Figures 2C and 2E, each symbol represents results generated with memory CD4$^+$ T-cells from a different individual (mean values of ELISA triplicate wells. The bars

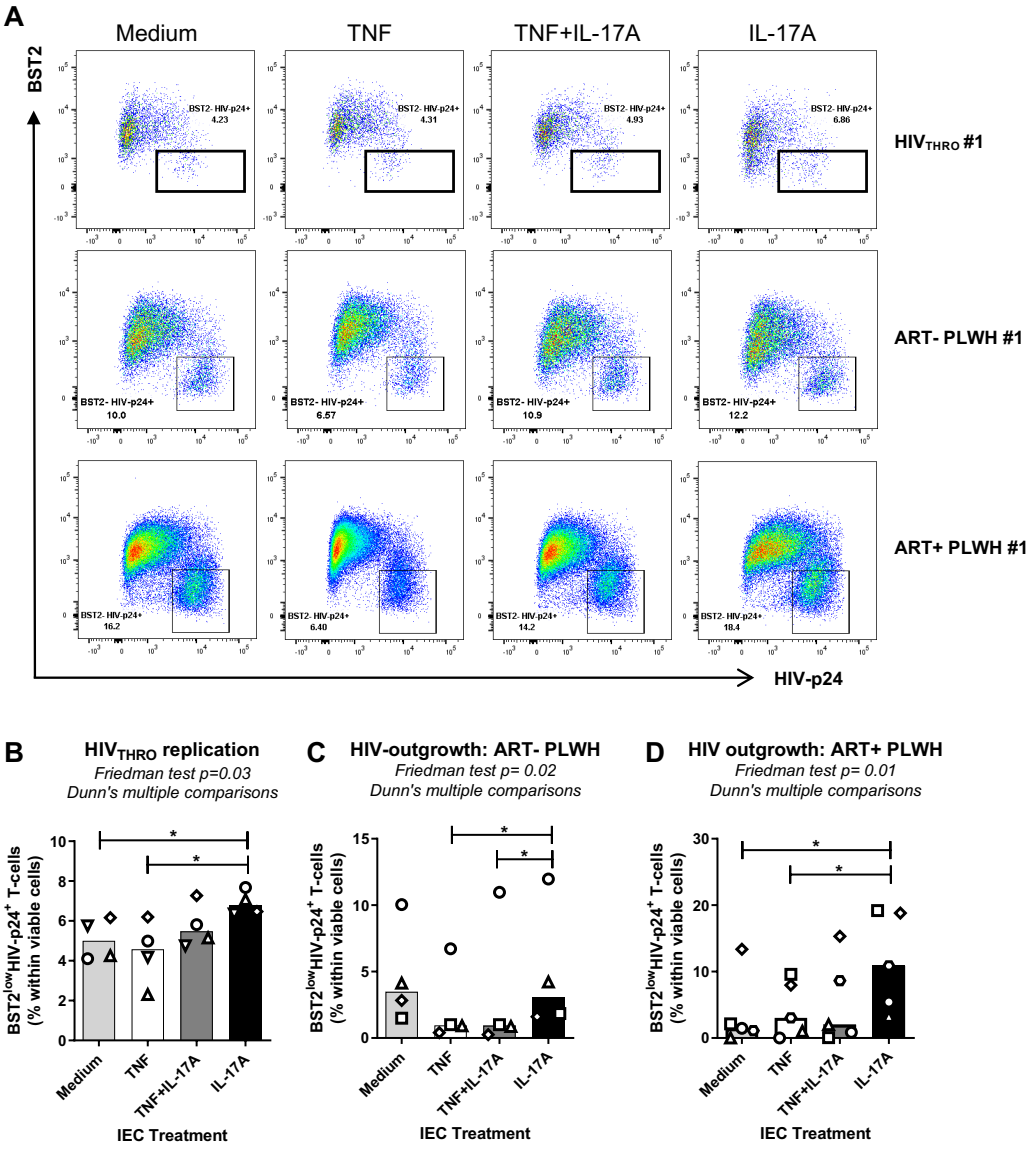
represent the mean value of all individuals. Friedman test with Dunn's post-test evaluated the statistical differences. P-values are indicated on the graphs (*, $p<0.1$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$).



Supplemental Figure 3. Related to Figure 2: HIV-1 capture and replication in cytokine-activated IEC. (A) Shown is the experimental flowchart. Briefly, HT-29 IEC were activated with TNF and/or IL-17A, and exposed to HIV_{THRO}, as described in Figure 2. Unbound HIV was removed by extensive washing and IEC were further cultured for 1 or 2 days. HIV-exposed IEC were lysed and integrated HIV-DNA, as well as early (RU5) and late (Gag) reverse transcripts, were quantified 1day post HIV exposure. Additionally, HIV-exposed IEC were also disrupted and cell-associated HIV-p24 levels were quantified by crude ELISA2 days post HIV exposure. (B) Shown are results (mean±SD) of early (RU5) and late (Gag) reverse transcripts, and HIV integrated DNA from on experiment. (C) Shown are results (mean±SD) for crude HIV-P24 ELISA from four independent experiments. Paired t-Test evaluated the statistical differences. P-values are indicated on the graphs.

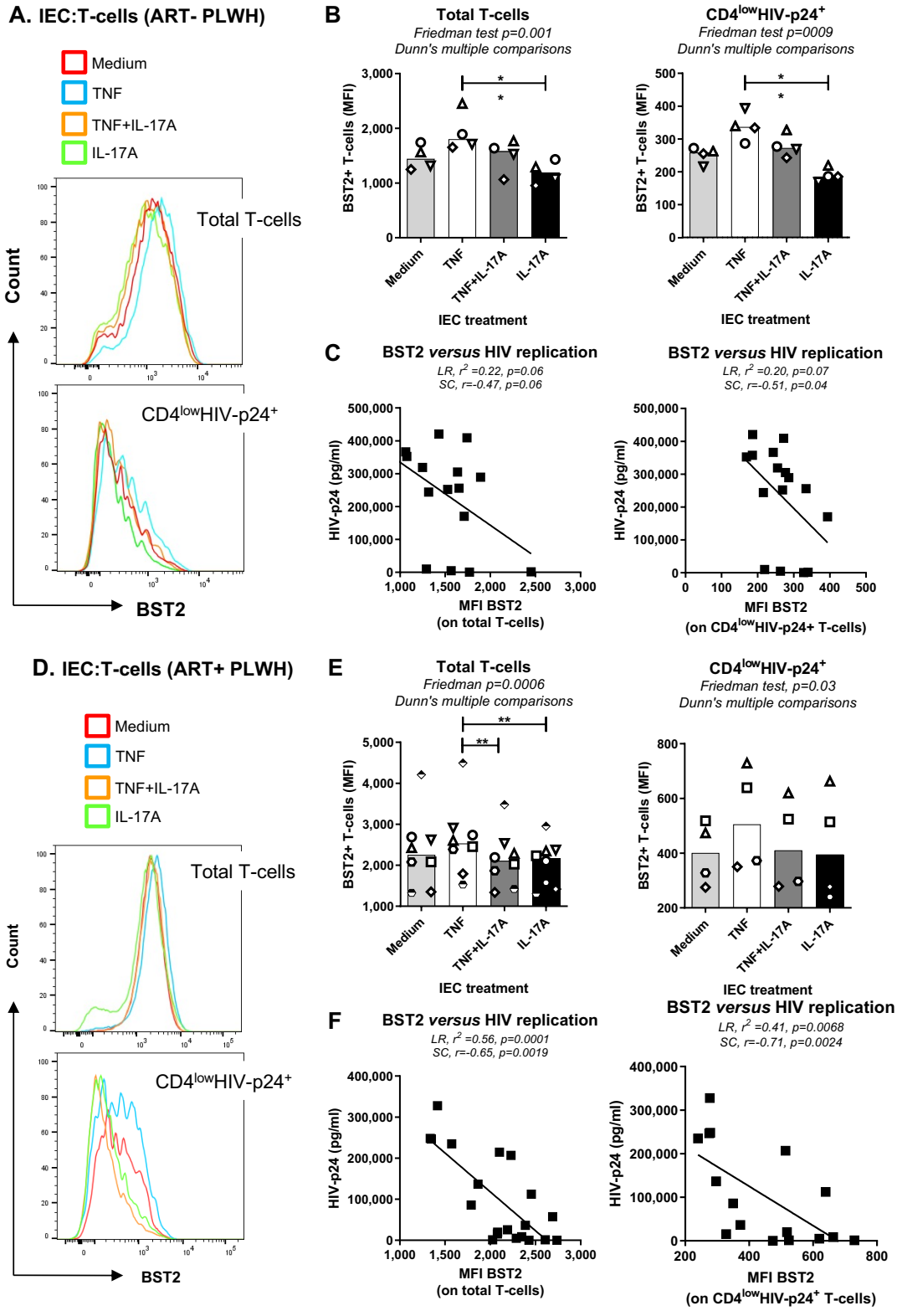


Supplemental Figure 4. Related to Figure 3: Effect of TNF and IL-17A on HIV-replication in CD4$^+$ T-cells. (A) Shown is the experimental flow chart. Briefly, memory CD4$^+$ T-cells from HIV-uninfected individuals were isolated, activated *via* CD3/CD28, as described in Figure 3, and infected with HIV$_{THRO}$ for 3 h. Memory CD4$^+$ T-cells were cultured with IL-2 (5 ng/ml) in the presence or the absence of TNF (10 ng/ml) and/or IL-17A (10 ng/ml) for 9 days. Media was refreshed with cytokines every 3 days. HIV-p24 levels in cell culture supernatants were quantified by ELISA at days 3, 6 and 9 post-infection. (B-C) Shown are the HIV-p24 levels (mean±SD) in one representative individual (B) and statistical analysis of results obtained with cells from n=4 different individuals (C). Each symbol represents results generated with memory CD4$^+$ T-cells from a different individual (mean values of ELISA triplicate wells). The bars represent the median of all individuals. Friedman test and Dunn's multiple comparisons evaluated the statistical differences. P-values are indicated on the graphs (*, $p<0.1$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$).



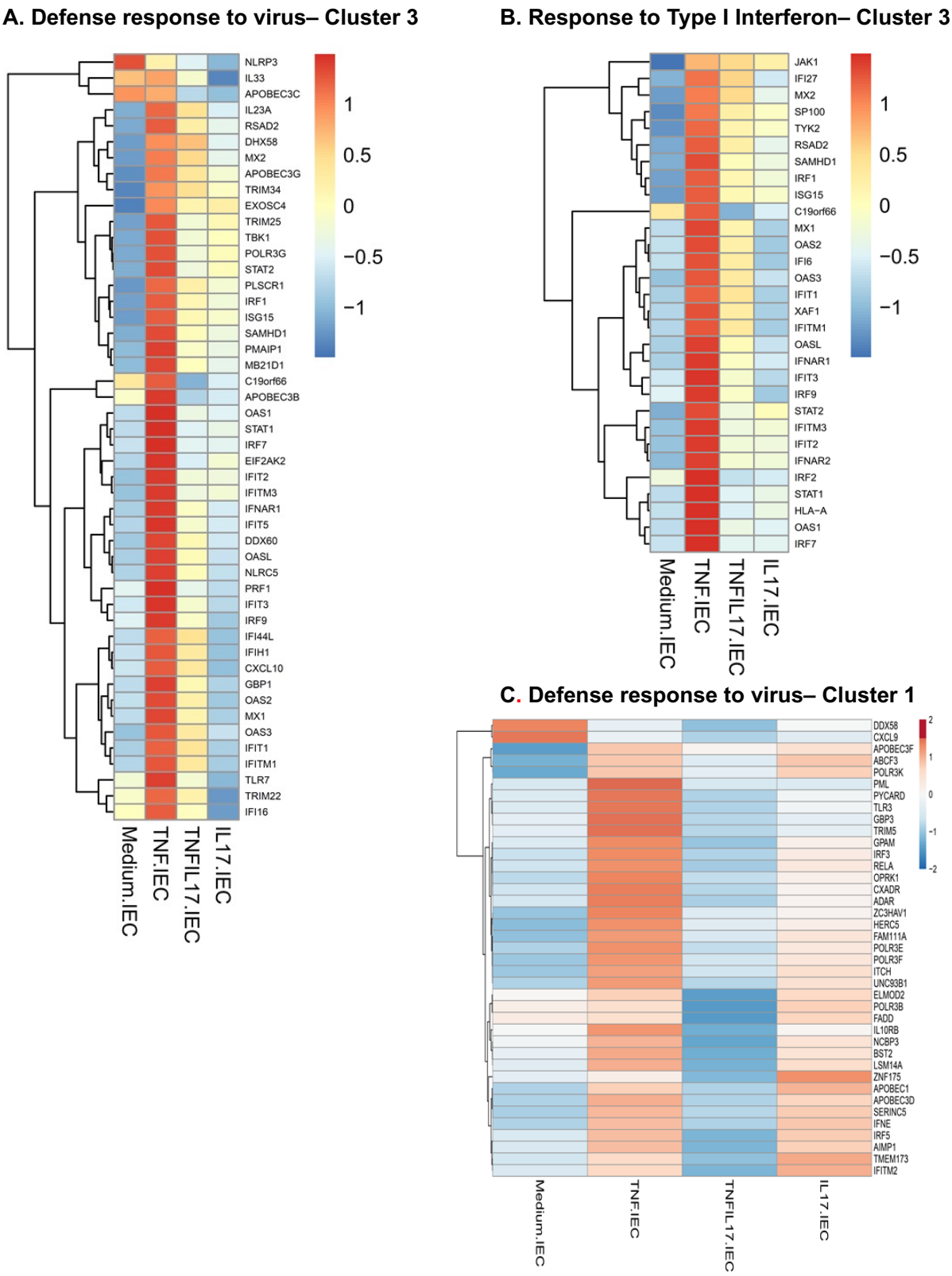
Supplemental Figure 5. Related to Figure 3 and 7: Frequency of BST2^{low} HIV-p24^{+} T-cells in cytokine-activated IEC co-cultured with HIV-infected memory CD4^{+} T-cells. BST2 and HIV-p24 expression was evaluated as depicted in Figure 4. (A) Shown is the frequency of BST2^{low}HIV-p24^{+} T-cells in representative individuals for cells form HIV-uninfected individuals infected with HIV$_{THRO}$ *in vitro* (upper panels), as well as ART- PLWH (middle panels) and ART+ PLWH (bottom panels). Shown are statistical analysis of HIV replication evaluated based on the frequency of BST2^{low}HIV-p24^{+} T-cells for results obtained with HIV$_{THRO}$-infected memory CD4^{+} T cells of n=4 individuals (B), memory CD4^{+} T-cells of n=4 ART- PLWH (C) and memory CD4^{+} T-cells of n=5 ART+ PLWH (D). Each symbol represents results generated with memory CD4^{+} T-cells from a different individual (mean values of ELISA triplicate wells). The bars represent the median value of all

individuals. Friedman test with Dunn's post-test evaluated the statistical differences. P-values are indicated on the graphs (*, $p<0.1$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$).



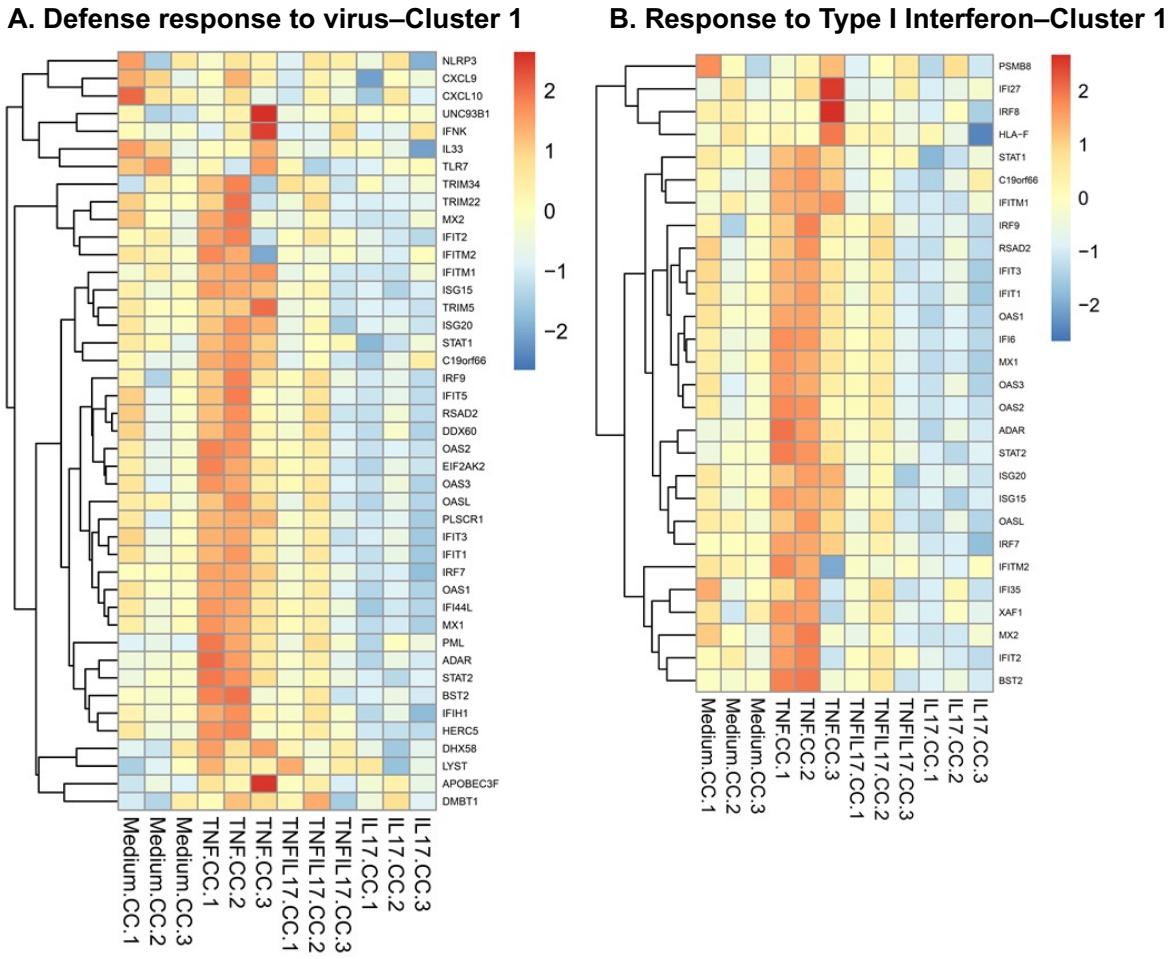
Supplemental Figure 6. Related to Figure 7: IL-17A-activated IEC decrease BST2 expression on T-cells of ART- and ART+ PLWH. Cytokine-activated IEC were co-cultured with memory CD4+ T-

cells isolated from ART- PLWH (n=4) (A-C) and ART+ PLWH (n=8) (D-F), as described in Figure 3. The expression of BST2 in memory $CD4^+$ T-cells was quantified by flow cytometry using fluorochrome-conjugated CD3, CD4, HIV-p24 and BST-2 Abs. Shown are histograms of BST2 expression on total T-cells ($CD3^+$, upper panels) and $CD4^{low}$HIV-$p24^+$ T-cells (bottom panels) (A and D); statistical analysis of MFI BST2 expression on total T-cells (B and E, left panels) and $CD4^{low}$HIV-$p24^+$ T-cells (B and E, right panels) at day 9 post co-culture. Each symbol represents results generated with memory $CD4^+$ T-cells from a different individual and the bars represent the median value of all individuals. Friedman test with Dunn's post-test evaluated the statistical differences. P-values are indicated on the graphs (*, $p<0.1$; **, $p<0.01$; ***, $p<0.001$; ****, $p<0.0001$). (C and F) Correlation between HIV-p24 levels in cell-culture supernatants and the MFI of BST-2 in total T-cells (left panels) and $CD4^{low}$HIV-$p24^+$ T-cells (right panels) were calculated using SC (p and r values) and LR (p and r^2 values).



Supplemental Figure 7. Related to Figure 8: Differentially expressed genes in cytokine-activated IEC. To evaluate differential gene expression, RNA-sequencing was performed in TNF and/or IL17

activated-IEC after 24 hours of activation. Shown are heatmaps with the transcripts belonging to immune response processes “Defense to virus” (A) and “Response to Type I interferon” (B) for Cluster 3 and genes belonging to the immune response process Defense response to virus for gene Cluster 1 (C) of Figure 6A.



Supplemental Figure 8. Related to Figure 8: Differentially expressed genes in cytokine-activated IEC co-cultured with memory CD4$^+$ T-cells of ART+ PLWH. To evaluate gene expression, RNA-sequencing was performed in TNF and/or IL17 activated-IEC co-cultured with ART+ PLWH memory CD4$^+$ T-cells of n=3 individuals, as described in Figure 6. Shown are heatmaps with the genes belonging to immune response process “Defense to virus” (A) and “Response to Type I interferon” (B) in Cluster 1 of Figure 6C.

Supplemental Table 1: Primers and probes used for PCR and RT-PCR. Related to STAR Methods.

Identification	Sequence	Provider	Identifier
Primer AA55	5'-CGT CTA GAG ATT TTC CAC AC-3'	IDT	N/A
Primer M667	5'-CTA ACT AGG GAA CCC ACT G-3'	IDT	N/A
CD3 external primer 1	5'-ACT GAC ATG GAA CAG GGG AAG-3'	IDT	N/A
CD3 external primer 2	5'- CCA GCT CTG AAG TAG GGA ACA TAT-3'	IDT	N/A
Primer GagR	5'- AGC TCC CTG CTT GCC CAT A-3'	IDT	N/A
Primer Alu1	5'- TCC CAG CTA CTG GGG AGG CTG AGG-3'	IDT	N/A
Primer Alu2	5'- GCC TCC CAA AGT GCT GGG ATT ACA G-3'	IDT	N/A
Primer LM667	5'- ATG CCA CGT AAG CGA AAC TCT GGC TAA CTA GGG AAC CCA CTG-3'	IDT	N/A
Primer LambdaT	5'- ATG CCA CGT AAG CGA AAC T-3'	IDT	N/A
Primer AA55M	5'- GCT AGA GAT TTT CCA CAC TGA CTA A-3'	IDT	N/A
Primer SK30	5'- GGT CTG AGG GAT CTC TAG-3'	IDT	N/A
Primer SK29	5'- ACT AGG GAA CCC ACT GCT-3'	IDT	N/A
CD3 internal primer 1	5'- CCT CTC TTC AGC CAT TTA AGT A-3'	IDT	N/A
CD3 internal primer 2	5'- GGC TAT CAT TCT TCT TCA AGG T-3'	IDT	N/A
Probe LTR-LC	5'-LC640-CACTCAAggCAAgCTTTATTgAggC-3'-phosphate	TIB MolBiol	N/A
Probe LTR-FL	5'- CACAACAgACgggCACACACTACTTgA-3'-Flurescein	TIB MolBiol	N/A
Probe P1	5'-ggCTgAAggTTAgggATACCAATATTCCTgTCTC-3'-Flurescein	TIB MolBiol	N/A
Probe P2	5'-LC640-CTAgTgATgggCTCTTCCCTTgAgCCCTTC-3'-phosphate	TIB MolBiol	N/A
Primer ISG15 Forward	5'-ACTCATCTTTGCCAGTACAGGAG-3'	IDT	N/A
Primer ISG15 Reverse	5'- CAGCATCTTCACCGTCAGGTC-3'	IDT	N/A
Primer 28S Forward	5'-CGAGATTCCTGTCCCCACTA-3'	IDT	N/A
Primer 28S Reverse	5' GGGGCCACCTCCTTATTCTA-3'	IDT	N/A
Primers IFIT1	N.A	Qiagen	GeneGlobe ID: QT01852466

N.A, information not available; N/A, Not applicable

Supplemental Files

Supplemental File 1. Transcriptional profiles of cytokine-activated IEC, related to Figure 8. https://www.cell.com/cms/10.1016/j.isci.2021.103225/attachment/131efc07-f4bd-45cc-9570-1fa869272081/mmc2.xlsx

Supplemental File 2. Gene Ontology terms enriched in cytokine-activated IEC, related to Figure 8. https://www.cell.com/cms/10.1016/j.isci.2021.103225/attachment/30e75abb-d795-410e-8902-61863fd08479/mmc3.xlsx

Supplemental File 3. Transcriptional profiles of cytokine-activated IEC co-cultured with CD4+ T cells of ART+PLWH, related to Figure 8. https://www.cell.com/cms/10.1016/j.isci.2021.103225/attachment/154dec99-198c-45b2-bc31-d93f501c7f41/mmc4.xlsx

Supplemental File 4. Gene Ontology terms enriched in cytokine-activated IEC co-cultured with CD4+ T cells of ART-treated PLWH, related to Figure 8. https://www.cell.com/cms/10.1016/j.isci.2021.103225/attachment/46e1215e-4979-41aa-a52e-6e52bda23119/mmc5.xlsx

Supplemental File 5. R codes used to generate heatmaps of differentially expressed genes and gene ontology enrichment analysis, related to STAR Methods. https://www.cell.com/cms/10.1016/j.isci.2021.103225/attachment/150e1599-914d-470a-b1d5-0c396693dfb8/mmc6.zip

Supplemental File 6. Transcriptional profiles of cytokine-activated IEC and in cytokine-activated IEC co-cultured with CD4+ T cells of ART+PLWH, related to Figure 8. https://www.cell.com/cms/10.1016/j.isci.2021.103225/attachment/7e9dbd0f-2969-4abf-9f53-6f1f312a6ab4/mmc7.xlsx

Chapter 4:

Manuscript #2: Th17 CELL MASTER TRANSCRIPTION FACTOR RORC2 REGULATES HIV-1 GENE EXPRESSION AND VIRAL OUTGROWTH

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Authors' contributions

# equal contribution

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TRWS and YZ designed and performed experiments, analysed data, prepared figures and contributed to write the manuscript.

DS, AZ, LRM, DP, AFert, ML, DC, KK, and SO designed and performed experiments, analysed data and prepared figures.

JPR provided access to clinical samples/information, set up clinical research protocols, and contributed to manuscript revision.

HA-M and DI provided critical reagents and expertise.

PA and AF conceived the research study, designed research, analysed data and wrote the manuscript.

All co-authors revised and approved the manuscript.

Th17 CELL MASTER TRANSCRIPTION FACTOR RORC2 REGULATES HIV-1 GENE EXPRESSION AND VIRAL OUTGROWTH

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Keywords: RORC2, HIV-1, Th17 cells, gene expression, hormone receptor

ABSTRACT

Among CD4+ T cells, T helper 17 (Th17) cells are particularly susceptible to HIV-1 infection and are depleted from mucosal sites, which causes damage to the gut barrier resulting in a microbial translocation-induced systemic inflammation, a hallmark of disease progression. Furthermore, a proportion of latently infected Th17 cells persist long-term in the gastro-intestinal lymphatic tract, where a low-level HIV-1 transcription is observed. This residual viremia contributes to chronic immune activation. Thus, Th17 cells are key players in HIV pathogenesis and viral persistence. It is however unclear why these cells are highly susceptible to HIV-1 infection. Th17 cell differentiation depends on the expression of the master transcriptional regulator RORC2, a retinoic acid-related nuclear hormone receptor that regulates specific transcriptional programs by binding to promoter/enhancer DNA. Here, we report that RORC2 is a key host-cofactor for HIV replication in Th17 cells. We found that specific inhibitors that bind to the RORC2 ligand-binding domain reduced HIV replication in CD4+ T cells. Depletion of RORC2 inhibited HIV-1 infection, whereas its overexpression enhanced it. RORC2 was also found to promote HIV-1 gene expression by binding to the nuclear receptor responsive element (NRRE) in the HIV-1 LTR. In treated HIV-1 patients, RORC2+ CD4 T-cells contained more proviral DNA than RORC2- cells. Pharmacological inhibition of RORC2 potently reduced HIV-1 outgrowth in CD4+ T cells from antiretroviral-treated patients. Altogether, these results provide a new explanation as to why Th17 cells are highly susceptible to HIV-1 infection and suggest that RORC2 may be a cell-specific target for HIV-1 therapy.

Significance statement

HIV-1 infects CD4 T cells and among these, Th17 cells are known to be particularly permissive for virus replication. Infection of Th17 cells is critical for AIDS pathogenesis and viral persistence. It is however not clear why these cells are highly permissive to HIV-1. We found that Th17 cell permissiveness depends on the expression of the hormone receptor RORC2, which is the master transcriptional regulator of Th17 cell differentiation. We identify RORC2 as a new, cell-specific host-dependency factor that can be targeted by small molecules. Our results suggest that RORC2 may be a cell-specific target to mitigate the loss of Th17 cells as a consequence of their preferential HIV 1 infection.

INTRODUCTION

A hallmark of HIV-1 infection is systemic inflammation, which can best predict disease progression (1, 2). A significant proportion of people living with HIV (PLWH) with undetectable plasma viral load during antiretroviral therapy (ART) have systemic inflammation, the severity of which correlates with the overall mortality, morbidity and co-morbidity (1, 2). This inflammation also supports viral persistence by promoting homeostatic proliferation and clonal expansion of memory CD4+ T cells carrying HIV-1 reservoirs (3, 4), by enhancing their migration into lymphatic organs and by inducing their activation, which stimulates local HIV-1 infection and reactivation from latency (5). The systemic inflammation observed in some virally suppressed individuals, indicates that the widespread and ongoing virus replication is not the main cause of this immune disorder; instead, substantial evidence suggests that inflammation has predominantly an indirect origin and is linked to alterations at mucosal sites (1, 6, 7).

T helper 17 (Th17) cells are a heterogeneous subset of CD4+ T cells that expresses the chemokine receptor CCR6 and produce lineage-specific cytokines such as IL-17A, IL-21 and IL-22 (8-10). They are mainly found in the intestinal lamina propria and vaginal cervix mucosa, where they maintain the immunological barrier to microbiota, including bacteria and fungi (9-11). Remarkably, Th17 cells, which are preferentially targeted by HIV-1 in vitro and in vivo (10, 11) are also among the very first cells infected upon Simian immunodeficiency virus (SIV) vaginal exposure in macaques (12). As a consequence, Th17 cells are depleted from the gut and vaginal mucosa during acute infection in both PLWH (10, 11) and in SIV-infected monkeys (13, 14). The depletion of Th17 cells from the gastro intestinal lymphatic tract (GALT) of HIV-1 infected individuals has critical consequences for disease progression and viral persistence (7). Studies in PLWH and in pathogenic models of SIV infection showed that the loss of Th17 cells correlates with systemic inflammation, putatively via the disruption of the immunological homeostasis at the mucosal barriers and the translocation of bacterial products from the mucosa into the circulation (15, 16). Bacterial and fungal products then trigger the release of pro-inflammatory cytokines by various immune cells, establishing and maintaining systemic inflammation (7 , 16, 17). These events are so critical that the loss or maintenance of Th17 cells can discriminate between pathogenic and non-pathogenic lentiviral infections (13, 17). Nevertheless, a proportion of infected Th17 cells

are long-lived and constitute a component of the viral reservoir in the gut (8, 18-22), underscoring their importance in maintaining viral persistence during ART.

Despite these major insights, we do not fully understand why Th17 cells are preferentially targeted by HIV-1 for infection and how they are lost during the early acute phases of the disease (10, 11). Also, the mechanisms that govern HIV-1 reactivation from latency in long-lived Th17 cells carrying viral reservoirs in ART-treated PLWH are still unclear.

The differentiation and effector functions of Th17 cells depend on the expression of the master regulator retinoic acid receptor-related orphan receptor 2, RORC2 (RORt in mice) (23-25). Here, we report that RORC2 is a bona fide host co-factor for HIV-1 gene expression in Th17 cells and identify small molecule RORC2 inhibitors that potently block HIV-1 replication/outgrowth without widespread cell toxicity. These findings establish a key molecular link between RORC2 expression and HIV-1 replication and suggest that RORC2 may be a novel Th17 cell-specific target to mitigate the loss of Th17 cells induced by their preferential HIV-1 infection.

RESULTS

RORC2 is a druggable host dependency factor for HIV-1.

In a previous compound screening experiment to find small molecules with anti-HIV-1 activity, we identified two hits (digoxin and digitoxin) that are known antagonists of both RORC2 and the Na+/K+ ATPase (26-28). Although we and others showed that digoxin affects HIV-1 gene expression mainly via the Na+/K+ ATPase (27-29), digoxin-mediated inhibition of HIV-1 infection was also observed in Jurkat cells expressing the mouse Na+/K+ ATPase (27), which does not bind to digoxin, suggesting that RORC2 might be a secondary target. To confirm and extend our initial observation, we tested several new well-characterized RORC2 inhibitors developed by GlaxoSmithKline (GSK). Compounds, GSK261805A, GSK2837270A, GSK2793955A and GSK283726 bound to the RORC2 ligand-binding domain (LBD) with nanomolar affinity and displaced steroid receptor co-activator-1 (SRC1) (30), whereas GSK2833332A and GSK2805956A had no measurable affinity up to 10 µM (SI Table 1). The compounds with high affinity for RORC2 LBD also inhibited expression of a reporter luciferase gene under the control of the human IL-17A enhancer/promoter with micromolar IC50s (SI Table 1), which goes in agreement with the observation that RORC2 induces IL-17 expression by binding at promoter and enhancer regions in the IL-17 locus (31). Initially, we sought to test the compounds' antiviral activity in Jurkat cells, which does not require stimulation, and whose susceptibility to HIV-1 infection is less variable than that of primary CD4+ T cells. To determine if Jurkat cells express RORC2 and hence may be sensitive to the compounds, we performed RT-qPCR using two sets of primers that specifically amplify either RORC2 or RORC1, an alternative spliced isoform that differs from RORC2 at its N-terminal region and is not normally expressed in CD4+ T cells (32). This analysis showed that Jurkat cells and memory CD4+ T cells express RORC2, but not RORC1 transcripts (SI Figure 1a-b); RORC2 protein expression was also confirmed in Jurkat cells by Western blot (SI Figure 1c). A single cycle HIV-1 LAI□env virus expressing GFP and pseudotyped with VSV/G (hereafter called HIV-1 LAIGFP) (33) was used to infect Jurkat cells in the presence of increasing concentrations of RORC2 inhibitors or DMSO. Infected cells were analyzed by flow cytometry 48 hours post-infection and assessed for cell toxicity by alamarBlue™ or LIVE/DEAD staining. Compound GSK2837269A was the most potent, followed by GSK2837270A and GSK2793955A, whereas

GSK2805956A and GSK2833332A showed lower potency (Figure 1), which is consistent with their low affinity for RORC2 (SI Table 1). None of the compounds showed detectable cytotoxicity.

Next, we tested the activity of the RORC2 inhibitors on HIV-1 replication in primary CD4+ T cells. Memory CD4+ T-cells were isolated from five HIV-uninfected healthy donors (HIV-), stimulated with CD3/CD28 antibodies (Abs) and infected with HIV-1THRO, a transmitted/founder virus (8), in the presence of GSK2691805A or DMSO (34, 35) (Figure 2a). We observed that GSK2691805A potently reduced HIV-1 replication (Figure 2b-d). The other RORC2 inhibitors GSK2837269A, GSK2793955A and GSK2833332A also reduced HIV-1 replication at a concentration of 5 uM, although differences did not reach statistical significance (Figure 2e-f). We observed some differences in the compounds' potency to inhibit HIV replication between Jurkat and primary CD4+ T cells at the 5 □M concentration: GSK2837270A was active in Jurkat cells, but not in primary CD4+ and conversely, GSK283332A was inactive in Jurkat cells, but showed an antiviral activity in primary CD4+ T cells. GSK2837270A may be less potent in primary cells due to its different susceptibility to drug transporters and efflux pumps (36), or faster degradation in these cells. All compounds, except for GSK2833332A, reduced IL-17A but not IFN-g production at 5µM (SI Figure 2a-b). This result suggested that GSK2833332A might inhibit HIV-1 infection in primary cells by a mechanism independent of RORC2. No cytotoxicity or changes in cell proliferation for any of the compounds was observed at the tested concentrations (SI Figure 3). Using the same experimental design, GSK2691805A also reduced replication of the primary isolate HIVNL4.3BaL, which is well-adapted to grow in primary CD4+ T cells (SI Figure 4d-e). Therefore, inhibition of RORC2 impairs the replication of different HIV strains in Jurkat and primary CD4+ T- cell models.

The pool of memory CD4+ T cells includes a Th17-polarized fraction, for which the expression of Th17 effector functions requires T cell receptor (TCR) triggering (10, 37). To determine if RORC2 expression in this Th17-polarized population promoted HIV-1 infection in vitro, memory CD4+ T cells from healthy donors were stimulated for 3 days and infected with HIV-1THRO, (SI Figure 5a). Six days post-infection, cells were analysed by flow cytometry to detect RORC2 and HIV-p24 expression. The percentage of HIV-p24+ cells was higher in RORC2+ than RORC2- cells in all five

donors, indicating better HIV-1 infection rates in cells expressing RORC2 (SI Figure 5b-c). In parallel, CD4+ T cells from these same donors were analysed by flow cytometry to detect HIV-p24 expression in cells producing IL-17A and/or IFN-g. IL-17A and IFN-g are markers of Th17 cells and Th1 cells, respectively) upon simulation with PMA/Ionomycin (SI Figure 5d-f). Notably, in these experimental settings, we confirmed that Th17 and Th17/Th1-polarized cells had a significantly higher proportion of HIV-p24+ cells than Th1 cells or unpolarised Th0 cells, which is consistent with their reported increased permissiveness to infection (10, 11).

To validate RORC2 as a host co-factor for HIV-1 infection, we generated three different hairpins targeting RORC2 mRNA and transduced them using lentiviral vectors into reporter Jurkat 1G5 cells, which express luciferase from the stably transfected HIV-1 LTR (38). We confirmed RORC2 knock-down (KD) by Western blot (Figure 3a) and infected the cells with HIV-1NL4.3, which is well-adapted to replicate in Jurkat cells. We found a reduced HIV-1 replication in KD cells relative to control cells and this phenotype was proportional to the degree of RORC2 depletion, indicating specificity (Figure 3b). To test if RORC2 was sufficient to promote HIV-1 infection, we expressed RORC2 cDNA in 293T cells, which do not normally express endogenous RORC2 (39) (Figure 3c), and infected them with VSV-G pseudotyped, single cycle HIV-1 LAIGFP in the presence of DMSO or the RORC2 inhibitor GSK2837269A, which had the best activity/toxicity profile in Jurkat cells (Figure 1). Exogenous expression of RORC2 in 293T cells enhanced HIV-1 LAIGFP infection above the already high basal levels and this phenotype was abrogated by GSK2837269A (Figure 3d). No inhibitory effect on infection was observed with GSK2837269A in control 293T cells at 12uM, consistent with the fact that they do not express the target. We also stably expressed a myc-tagged RORC2 cDNA in Jurkat cells using a retroviral vector construct and infected the cells with HIV-1 LAIGFP virus. We found that Jurkat cells expressing exogenous RORC2 sustained better infection relative to control cells (Figure 3e-f), in agreement with the 293T cells data. To confirm the specificity of the RORC2 drug action, HIV-1THRO-infected primary CD4+ T cells and ACH2 cells (a cell line carrying integrated HIV-DNA (40, 41)) were cultured in parallel in the presence or the absence of GSK261805A. As shown in SI Figure 1a-b, ACH2 cells express insignificant levels of RORC2 and RORC1 mRNA compared to memory CD4+ T cells and should therefore be insensitive

to the RORC2 inhibitors. As expected, GSK261805A inhibited HIV-1 infection in CD4+ T cells, but not in ACH2 cells, confirming RORC2 as the target mediating the phenotype (Figure 3g-h).

We next sought to further validate RORC2 as a co-factor for HIV-1 infection by mRNA depletion in primary CD4+ T cells. Memory CD4+ T cells were CD3/CD28-stimulated for 2 days, then nucleofected with a siRNA targeting RORC2 mRNA or a non-targeting control siRNA. One day after nucleofection, cells were infected with replication competent HIV-1NL4.3BaL. At day 3 post-infection, we observed partial inhibition of the RORC2 mRNA expression in all the tested donors, which resulted in a lower IL-17A production from the treated cells relative to controls (SI Figure 6), and a small but significant reduction of HIV-1 infection as measured by the quantity of proviral DNA (Figure 3i). Finally, because RORC2 is preferentially expressed by CCR6+ Th17 cells (42), RORC2 siRNA experiments were similarly performed on flow cytometry-sorted CCR6+ (Th17) and CCR6- (non-Th17) CD4+ T cells. The highest levels of HIV replication were detected in CCR6+ T cells, which agrees with previous reports by our group and others (8, 18, 19, 21, 22). It is noteworthy that the depletion of RORC2 mRNA resulted in a significant reduction in proviral DNA and HIV-p24 levels in CCR6+ subset, with no effects observed in CCR6- T cells, indicative of decreased HIV-1 replication upon RORC2 depletion (Figure 3j-k). Taken together, these pharmacological and genetic approaches demonstrated that RORC2 promote HIV-1 replication.

RORC2 regulates HIV-1 gene expression.

To determine the step of the viral replication cycle that was impaired by the anti-RORC2 compounds, we infected Jurkat cells with single cycle HIV-1 LAIGFP at an MOI of 0.1 in the presence of GSK2837269A, GSK2837270A, nevirapine (a non-nucleoside inhibitor of reverse transcriptase), raltegravir (a strand transfer inhibitor of integration) or DMSO. Total DNA was extracted from the cells at 24 hours post-infection and Taqman qPCR was used to measure the amount of GFP DNA (as a surrogate marker of negative strand viral DNA) and 2LTRs circular DNA, a hallmark of nuclear entry (43). To measure the amount of integrated viral DNA, we performed Alu-LTR quantitative PCR from DNA extracted 8 days post-infection (27). RORC2 inhibition did not significantly impair reverse transcription, nuclear entry or integration, in contrast to nevirapine,

which reduced both viral DNA and 2LTRs, or raltegravir, which suppressed integration (Figure 4a). We also infected primary CD4+ T cells with a single-round VSV-G-pseudotyped HIV (NL4.3 backbone, env-) in the presence of GSK2691805A and measured HIV-p24 levels by ELISA and integrated HIV-DNA levels by nested real-time PCR 72 h post-infection. While HIV-p24 levels were reduced (Figure 4b left panel), the number of integrated HIV-DNA copies was not statistically different (Figure 4b right panel), indicating that RORC2 acts at a step post-integration, such as gene expression.

To examine this aspect further, GSK2837269A was added on to Jurkat cells chronically infected with single cycle HIV-1 LAIGFP and the percentage of GFP+ cells was analysed by flow cytometry 48 hours later. A dose-dependent reduction of the percentage of GFP+ cells was observed (Figure 4c). To examine viral gene expression directly, Jurkat cells were infected with single cycle HIV-1 LAIGFP in the absence of compounds and analysed by flow cytometry 48 hours later (Figure 4d left panel). Next, GSK2837269A was added onto these infected cells, which were re-analysed by flow cytometry 24 hours later (Figure 4d middle panel). In parallel, RNA was extracted from the GSK2837269A-treated cells and used for RT-qPCR to detect GFP mRNA, which is transcribed from the viral LTR. At 5 uM, GSK2837269A reduced GFP mRNA 5-fold (Figure 4d right panel). The modest discrepancy between GFP mRNA and percentage of GFP+ cells is most likely explained by the longer half-life of the GFP protein relative to its mRNA. To confirm that RORC2 is important for HIV-1 gene expression, we transduced J-Lat cells (clone A2) (44), which harbour a latent HIV-1 provirus expressing GFP, with the same RORC2-targeting shRNAs shown in Figure 3b. Upon stimulation with 12-O-Tetradecanoylphorbol-13-Acetate (TPA), >60% J-Lat cells expressed GFP, while cells treated with the shRNAs showed lower GFP expression in terms of frequency and MFI, indicating that RORC2 silencing inhibited HIV-1 gene expression (Figure 4e-g).

RORC2 binds to specific DNA consensus elements, recruiting chromatin-activating co-factors via its ligand-binding domain to regulate transcription (23, 45). We therefore hypothesized that RORC2 might bind to such DNA elements on the HIV-1 provirus. The HIV-1 LTR contains a well characterized nuclear receptor responsive element (NRRE) that binds several retinoic acid

receptors (46, 47). In addition, we have detected in silico a consensus RORC DNA binding motif (23, 34) in the pol region of the HIV-1 genome (SI Figure 7). It has previously been reported that this region in Pol may have enhancer activity (48). To test the hypothesis, we performed chromatin immunoprecipitation followed by real-time PCR (ChIP-qPCR) for both the NRRE in the LTR and the RORC2 consensus sequence in Pol (CS Pol). There is no available ChIP-grade antibody against human RORC2. Hence we performed the experiments in Jurkat cells stably transduced with a retroviral vector expressing C-terminally myc-tagged RORC2. However, Jurkat cells do not express IL-17A, raising the possibility that the IL-17A locus might be defective thus making these cells unsuitable to detect RORC2 binding to the IL-17A regulatory elements by ChIP. To circumvent this problem, we transduced with the RORC2-myc expressing retroviral vector Jurkat cells stably transfected with a plasmid expressing luciferase driven by the IL-17A promoter and CNS-5 enhancer, which contains one RORC2 DNA consensus element each (Jurkat-Luc cells) (31, 49) (Figure 5a-b). Jurkat-Luc cells transduced with an empty retroviral vector (Jurkat Luc-EV) were generated to control for specificity. The resulting Jurkat Luc-RORC2 cells expressed luciferase at higher levels than Jurkat Luc-EV both at baseline and after stimulation, confirming their functionality (Figure 5c). Jurkat Luc-RORC2 and Luc-EV cells were infected in parallel experiments with HIV-1 LAIGFP at low MOI. Twenty-four hours after infection, cells were processed for ChIP using an anti-myc antibody. We also employed an antibody against H3K9me3, which is a histone marker of inactive but poised enhancer/promoters (50) and an antibody against NF-kB, a transcription factor known to bind both the HIV-1 LTR (51) and the IL-17 enhancer/promoter regions (52, 53). A specific ChIP signal for RORC2-myc was detected on the IL-17 enhancer and promoter regions of Jurkat Luc-RORC2 cells but not Jurkat Luc-EV cells (Figure 5d). Notably, a specific ChIP-qPCR signal was observed on the HIV LTR NRRE element whereas the signal for the CS Pol element was less convincing due to high background, which may be related to greater "stickiness" of the specific DNA sequence under study due to charge and/or secondary structure (Figure 5d).

These results showed that RORC2 binds to the HIV-1 LTR with similar or even greater strength than the IL-17 promoter/enhancer regions. Notably, a specific ChIP-qPCR signal was also detected for H3K9me3 in Jurkat-Luc cells, particularly on the IL-17A enhancer/promoter region. This

H3K9me3 signal was stronger in RORC2-expressing cells, suggesting that RORC2 might either associate with, or increase the proportion of poised enhancers. It should be noted that Jurkat cells were not stimulated before ChIP, hence some critical RORC2 co-factors important for chromatin remodelling might be present at low levels (23, 31, 53), reducing the rate of enhancer conversion from poised into active. Furthermore, the RORC2-specific ChIP-qPCR signal is likely to be an underestimation due to competition with the endogenous RORC2, which is expressed in Jurkat cells (SI Figure 1) but cannot be precipitated by the anti-myc Ab. Consistent with previous reports, we did not detect NF-kB binding to the IL-17 enhance/promoter in the absence of stimulation (52, 53), however expression of RORC2 appeared to stimulate recruitment of NF-kB on both the IL-17 enhancer/promoter region and the HIV-1 LTR, which is in line with the observed co-operativity of RORC2 and some transcription factors (23, 31, 52). Taken together, these results support the possibility that RORC2 regulates viral gene expression by binding to the HIV-1 LTR.

RORC2 is critical for HIV-1 outgrowth from patients' cells.

Since RORC2 promotes HIV-1 gene expression in acutely infected cells, we tested if its inhibition also prevented HIV-1 outgrowth from cells of PLWH. RORC2-mediated effector functions are not constitutive in Th17 cells but depend on TCR triggering (10, 37), thus we initially evaluated if the activation of memory CD4+ T cells with CD3/CD28 Abs was capable of inducing the expression of RORC2 at the transcriptional and protein levels. Memory CD4+ T cells were isolated from HIV-uninfected individuals and stimulated for 5 or 24h hours. The relative RORC2 mRNA and protein levels were assessed 5 or 24 hours later, respectively. We observed that cell activation induced the expression of RORC2, but not RORC1 mRNA (SI Figure 8c-d). At the mRNA and protein level, RORC2 expression was up-regulated by TCR activation and cells expressing RORC2 also expressed CCR6 (SI Figure 8c-d), which is a well-established marker of human Th17 cells (9) and HIV-reservoir enrichment (8, 10, 18, 22, 54). Next, to determine if cells expressing RORC2 harbour HIV-1, memory CD4+ T cells were isolated from ART+PLWH and stimulated in vitro with CD3/CD28 Abs for 24 hours in the presence of ARVs to prevent HIV cell-to-cell transmission (Figure 6a). This stimulation is required for optimal expression of RORC2, which, similar to all lineage-specific cytokines, is not constitutively expressed in Th17-committed CCR6+ T cells (37). Cells were then sorted based on CCR6 and/or RORC2 expression (Figure 6b) and analysed by

nested real-time PCR for HIV proviral DNA (Figure 6c). In all five ART+PLWH, proviral DNA was significantly more abundant in CCR6+RORC2+ cells compared to CCR6-RORC2- cells or CCR6+RORC2- cells (Figure 6c). To explore whether CCR6+RORC2+ cells carry translational-competent HIV-DNA, we performed a modified HIV Flow Assay to quantify HIV-p24 expression in RORC2+ cells (55). To this end, memory CD4+ T cells isolated from ART- PLWH were stimulated for 72 hours in the presence of ARVs, and RORC2 expression was measured in total T cells and productively infected CD4lowHIV-p24+ T cells. As expected, we found that cell stimulation induced the expression of both RORC2 and HIV-p24 (SI Figure 9). Notably, CD4lowHIV-p24+ T cells were enriched in RORC2 expression compared to total memory T cells (SI Figure 10a-c). Additionally, CD4lowHIV-p24+ T cells expressing RORC2 showed a higher HIV-p24 GMFI (geometric mean fluorescence intensity) compared to their RORC2- counterparts (SI Figure 10d), which supports the notion that RORC2 stimulated HIV-1 gene expression.

Based on these observations, we sought to test if RORC2 inhibition prevented HIV-1 reactivation from latency and/or viral outgrowth ex vivo. To this end, we performed a simplified viral outgrowth assay (VOA) we have previously described (56) using memory CD4+ T cells from both ART+ and ART- PLWH. Cells were stimulated with CD3/CD28 Abs for 3 days and maintained in culture for another 9 days by splitting each well into two new wells every 3 days, in the presence of DMSO or 5 uM GSK2691805A (Figure 7a). Cells were analysed to detect CD4lowHIV-p24+ cells by flow cytometry at day 12 post TCR triggering and the level of HIV-p24 in cell-culture supernatants was evaluated at days 9 and 12 post-activation by ELISA. In parallel, cell proliferation was evaluated by CFSE-based proliferation assay as described previously (57) on the cells from ART+ PLWH. No difference on cell proliferation was observed between the DMSO and GSK2691805A conditions (Figure 7 b-c). In the presence of GSK2691805A, there was a consistent reduction in the frequency of CD4lowHIV-p24+ cells compared to DMSO on T cells from both ART+ and ART- PLWH (Figure 7d-f). Similarly, HIV-p24 levels in the culture supernatants were significantly reduced by GSK2691805A treatment (Figure 7g-h). These results are consistent with the notion that RORC2 is critical for HIV-1 reactivation/outgrowth in infected Th17 cells from PLWH.

DISCUSSION

In this study, we have identified the Th17 cell master transcription regulator RORC2 as a host co-factor for HIV-1 infection and showed that it regulates viral gene expression upon infection in vitro and possibly reactivation from latency, as reflected by viral outgrowth ex vivo. These findings provide a new explanation as to why HIV-1 replicates more efficiently in Th17 cells, which express higher levels of RORC2 relative to other CD4+ T cell types.

The ChIP-qPCR results indicate that RORC2 binds to the NRRE in the HIV-1 LTR. This DNA element is well conserved among different HIV-1 subtypes and was previously shown to be recognized by several nuclear hormone receptors, including retinoic acid receptors that positively regulate viral gene expression in CCR6+ Th17 cells (8, 11, 18). The NRRE in the HIV-1 LTR might be a critical element providing transcriptional plasticity to the virus resulting in better adaptation to different cell types (58). It is noteworthy that oestrogen inhibits HIV-1 reactivation from latency in Th17 cells (59). This effect is mediated by binding of the nuclear hormone receptor oestrogen receptor-1 (ESR-1) to the LTR, which may explain why women appear to have a lower inducible reservoir than men (59). Oestrogen also inhibits Th17 cell differentiation and IL-17 secretion (60, 61), suggesting a broader interconnection between oestrogen and RORC2. It would be interesting to test if RORC2 and ESR-1 compete for binding to the LTR and exert antagonistic effects on viral transcription.

We therefore propose that RORC2, rather than being essential for HIV-1 gene expression, acts more like a positive modulator of HIV-1 transcription, perhaps by helping establish a suitable chromatin environment. This may explain why HIV-1 replicates better in Th17 cells. Higher HIV-1 gene and protein expression in Th17 cells may also make these cells better targets for being killed by CD8+ T cells that, in part, explains long-term depletion of such cells from the GALT. In addition to a direct role in the modulation of HIV-1 gene expression, RORC2 may have other indirect effects that promote viral replication via the transcriptional regulation of other HIV-1 permissiveness factors (62).

Furthermore, our results demonstrate that the pharmacological inhibition of RORC2 potently suppressed HIV-1 outgrowth ex vivo in cells from ART-treated or untreated PLWH. This is consistent with the idea that RORC2 stimulates HIV-1 gene expression and may be important in regulating the dynamics of the viral reservoir (63). Fluctuations in RORC2 expression levels correlate with the activation status of Th17 cells, which is dependent, among other things, on priming via the TCR by specific antigens from pathogens such as Candida albicans and Staphylococcus aureus (9). Thus, specific stimuli from the microbiota may upregulate RORC2 expression selectively in latently infected Th17 cells promoting HIV-1 reactivation from latency and viral rebound.

Pharmacological inhibition of RORC2 in vivo may help elucidate the contribution of Th17 cells on the latent viral reservoir. The GSK RORC2 inhibitors have been employed in animal studies for non-HIV indications and GSK2691805A has been used to study the effect of RORC2 inhibition on Th17 and ILC-3 cells in mice (64). It will therefore be possible to conduct experiments in pre-clinical animal models of HIV-1 infection to determine if RORC2 inhibition delays or prevents viral rebound after ART interruption and/or whether RORC2 inhibition reduces inflammation and immune activation. A limitation of this approach is that Th17 cell differentiation and their effector functions might also be affected by administration of the RORC2 inhibitors. Nevertheless, the effects of RORC2 drugs may be reversible, as indicated by the capacity of cells to produce IL-17 again after drug withdrawal in vitro. Optimal dosing regimens and regular monitoring of Th17 cell populations in various tissues may allow the safe testing of RORC2 inhibitors in pre-clinical and clinical interventions. Other potential limitations are linked to the fact that other cells also express RORC2, including ILC-3 and thymocytes (65, 66), although ILC-3 seem tolerant to prolonged inhibition of RORC2 (64). In consequence, these RORC2+ cells will need to be carefully monitored too. Despite these potential obstacles, which may be mitigated, targeting RORC2 in vivo may be a conceptually new approach to attenuate the loss of Th17 cells, which occurs during acute HIV-1 infection (10, 11)

MATERIAL AND METHODS

Ethics statement

The collection of leukapheresis from HIV-uninfected individuals, ART- PLWH and ART+ PLWH was conducted in compliance with the principles included in the Declaration of Helsinki. This study received approval from the Institutional Review Board of the McGill University Health Centre and the CHUM-Research Centre, Montreal, Quebec, Canada. Written informed consents were obtained from all study participants.

Human subjects

The human biological samples were sourced ethically and their research use was in accord with the terms of the informed consents under an IRB/EC approved protocol. HIV-uninfected individuals [HIV-; n= 18; 17 males and 1 female; median age of 57.5 years (range: 25-70), median CD4 counts 752 cells/ul (range: 511-1,115)], as well as virally suppressed ART-treated PLWH [ART+ PLWH; n=9; 9 males and 0 female; median age of 44 years (range: 30-57), median CD4 counts 514 cells/ul (range: 318-598)] and untreated PLWH (ART- PLWH; n=6; 6 males and 0 female; median age of 41 years (range: 24-50), median CD4 counts 459 cells/ul (range: 221-1,068)]) (Supporting information Table 2), were recruited at the Montreal Chest Institute, McGill University Health Centre and Centre Hospitalier de l'Université de Montréal (CHUM, Montreal, QC, Canada). Peripheral blood mononuclear cells (PBMC) (109–1010 cells) were collected by leukapheresis and frozen until use, as previously described (8, 18, 20). Plasma viral load in ART-treated PLWH was measured using the Amplicor HIV-1 monitor ultrasensitive method (Roche).

HIV infection in vitro of Primary memory CD4+ T cell

The molecular clones of CCR5-tropic HIV-1 strain used in this study were: transmitted Founder (T/F) THRO and NL4.3BAL HIV-1. The T/F THRO HIV plasmid was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: pTHRO.c/2626 (cat# 11745) from Dr. John Kappes and Dr. Christina Ochsenbauer. The NL4.3BaL HIV plasmid was provided by Dr. Michel Tremblay (Univerité Laval, Québec, Canada), originating from Dr Roger J Pomerantz (Thomas Jefferson

University, Philadelphia, Pennsylvania, USA). HIV-1 plasmid was amplified by MiniPrep and MaxiPrep and viral stocks were produced and titrated, as we described it previously (8, 20). Memory CD4+ T cells were isolated from PBMCs of HIV-uninfected individuals by negative selection using magnetic beads (Miltenyi Biotec, Auburn, CA, USA), as we previously described (8, 20). Then, memory CD4+ T cells (1x106 cells/ml per 48 well-plate well) were stimulated with immobilized CD3 and soluble CD28 Abs (1 μg/mL) for 3 days prior to infection. T cells were cultured with RPMI1640 media (Gibco, Grand Island, NY, USA) containing 10% FBS and 1% P/S. Memory CD4+ T cells were infected with HIV-1 (20-50 ng HIV-p24/106 cells) and then cultured in the presence of IL-2 (5 ng/ml; R&D Systems, Minneapolis, MN, USA) for up to 9 days, with 50% of the media being refreshed every 3 days. Viral replication was measured by flow cytometry analysis upon HIV-p24 and CD4 staining (HIV infected cells were identified as HIV-p24+ and CD4low) as well as by HIV-p24 ELISA in cell-culture supernatant, as previously described (18, 27).

Cell lines and viruses

293T cells (European Collection of Authenticated Cell Culture [ECACC] Public Health England, UK) were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco Labs, Paisley, UK) supplemented with 10% fetal calf serum (FCS) (Helena Bioscience, Newcastle, UK) and 2 mM glutamine (Gibco Labs) at 370C in 5% CO2. Jurkat E6.1 (ECACC) and Jurkat indicator line 1G5 containing the firefly luciferase gene driven by the HIV LTR (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: from Dr. Estuardo Aguilar-Cordova and Dr. John Belmont) were grown in RPMI medium (Gibco Labs) supplemented with 10% FCS at 370C in 10% CO2. Jurkat cell line IL-17CNS luciferase clone 539 (GSK BIOCAT128253) (here called Jurkat Luc) were generated by transfection of Jurkat E6.1 cells with plasmid pGL4-huIL-17 3-K CNS promoter containing the luciferase gene driven by the 1 Kb IL-17 CNS-5 enhancer fused to the 2 Kb promoter regions and were grown in RPMI medium with 10% FCS and 200ug/ml hygromycin at 370C in 10% CO2. Viral stocks were prepared by Fugene transfection of 293T cells as described previously (43) using pHIV LAIΔenv (gift of Michael Emerman, Fred Hutchinson Cancer Research Centre, Seattle, USA) and pMD.G expressing VSV-G or using HIV isolate NL4.3 (Centre for AIDS Reagents, Health protection Agency, UK). Supernatant containing viral particles was collected 48h and 72h post-transfection as described (43, 67). For infections, 13ml of 1G5 indicator cells

(~0.9x106/ml) were mixed with 2ml NL4-3 supernatant. The mix was dispensed robotically, 45 µl/well onto 384-plates preloaded with drug dilutions. Samples were analysed 48 h post-infection using the BrightGlo assay according to the manufacturer's instructions in a Pherastar plate reader. To generate Jurkat and 293T cells stably expressing human RORC2, the myc-DDK-tagged-RORC2 cDNA was PCR-amplified from plasmid RC212239 (Origene) and cloned into the MLV-based retroviral vector pMIG Blasti (gift of Jeremy Luban, UMASS, USA). Virus was produced in 293T cells as described and used to transduce Jurkat or 293T cells, which were selected in the presence of 5 µM blasticidin for 10 days. ACH2 cells were obtained through the NIH HIV Reagent Program, Division of AIDS, NIAID, NIH: ACH-2 Cells, ARP-349, contributed by Dr. Thomas Folks. ACH2 cells were kept in culture with RPMI, 10% FBS, 1% Penicillin/Streptomycin.

Flow cytometry staining and Fluorescence activated cell sorting (FACS)

The following fluorochrome-conjugated Abs were used for flow cytometry analysis: HIV-p24 FITC (KC57) (Beckman Coulter, Fullerton, CA, USA), HIV-p24 PE (KC57) (Beckman Coulter, Fullerton, CA, USA), CD3 Pacific blue (UCHT1), CD4 PerCP/Cy5.5 (RPA-T4) (Biolegend, San Diego, CA, USA), CD4 Alexa Fluor 700 (RPA-T4), CCR6 PE (11A9), CD45RA Alexa eFluor 780 (HI100), RORC2 Alexa Fluor 647 (Q31-378), Ki-67 BUV395 (B56), IL-17A PE (eBio64DEC17) and IFN-γ Alexa Fluor 700 (B27). Live/Dead Fixable Aqua Dead Cell Stain Kit (Vivid, Life Technologies, Burlington, Ontario, CA) was used to exclude dead cells. Intracellular staining was performed using the BD Cytofix/Cytoperm kit (BD Biosciences) and intranuclear staining was performed using the eBioscience Foxp3/Transcription Factor Staining Buffer Set. Cells were analysed with the BD-LSRII cytometer, BD LSRFortessa and BD-Diva (BD Biosciences) and FlowJo version 10 (Tree Star, Inc., Ashland Oregon, USA). The positivity gates were placed using fluorescence minus one (FMO) strategy (8, 20). For FACS, memory CD4 T cells from PBMCs of ART+ PLWH were isolated by negative selection using magnetic beads. CCR6+RORC2+, CCR6+RORC2- and CCR6-RORC- T cells were sorted by FACS (BDAria II; BD Biosciences) using the Abs CD3 Pacific blue (UCHT1), CCR6 PE (11A9), CD45RA Alexa eFluor 780 (HI100), and RORC2 Alexa Fluor 647 (Q31-378).

Compounds

GSK2793955A, GSK2805956A, GSK2833332A, GSK2837269A and GSK2837270A were provided by GlaxoSmithKline at 100 mM stock in DMSO or freeze-dried and reconstituted in DMSO. GSK2691805A was synthesized in house according to (35) (see also Supporting Materials and Methods).

ChIP-qPCR

ChIP assays were performed as described previously (67) with some modifications. Briefly, 5 × 107 Jurkat cells expressing myc-tagged RORC2 or the pMI-blasti "empty" vector (EV) were infected with the VSV-G pseudotyped LAIΔenv-GFP at an m.o.i. of 0.3. Twenty four hours later, cells were collected in 50-ml tubes and chemically cross-linked by the addition of 1/10th volume of fresh 11% formaldehyde solution added directly to cell culture media and incubated for 20 min at room temperature with gentle rotation followed by the addition of 1/20th volume of cold 2.5 M glycine and incubated at 4 °C for 5 min. Cells were then collected by centrifugation at 4 °C, and the pellet was rinsed twice with PBS and flash-frozen in liquid N2. Cells were resuspended and lysed in 1 mL lysis buffer 1 (50 mM Hepes-KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% IGEPAL, 0.25% Triton X-100) for 10 min at 4 °C with slow rotation. Nuclei were pelleted by centrifugation and gently resuspended in 1 mL Nuclei Wash buffer (200 mM NaCl, 1 mM EDTA) for 10 mins at 4 °C with gentle rotation. Nuclei were pelleted by centrifugation and 1 mL of Lysis Buffer 3 (LB3) (10 mM Tris, pH 8, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% sodium deoxycholate, 0.5% N-laurylsarcosine) was added without disturbing the pellet followed by incubation for 10 mins at 4 °C with gentle rotation. This step was repeated once then nuclei were pelleted by centrifugation, resuspended in 300 µL LB3 and kept on ice for 10 mins. Samples were sonicated using a Diagenode Bioruptor device (30s pulse/90 sec pause x 10 cycles). Samples were centrifuged at 13,000 rpm for 6 mins at 4 °C and supernatant was collected in pre-cooled 1.5 mL tubes. The resulting whole-cell extract (WCE) was incubated overnight at 4 °C with 100 µL of protein-G magnetic Dynabeads preincubated with 10 µg of the appropriate antibody for 3 h on rotating platform (9 rpm) in a cold room. Antibodies used were: rabbit polyclonal anti-H3K9me3 (abcam, ab8898), normal rabbit IgG (Merk Millipore, 12-370), mouse mAb anti-c-Myc (ThermoFisher, 9E10) and rabbit polyclonal anti-NF-κB p65 acetyl K310 (Abcam ab19870). The next day beads were washed (5 mins with slow rotation) two times with low salt buffer (10mM

Tris HCl pH 8, 150 mM NaCl, 1mM EDTA, 1% Triton X100, 0.1 % SDS, PMSF) then once with high salt buffer (10mM Tris HCl pH 8, 500 mM NaCl, 1mM EDTA, 1% Triton X100, 0.1 % SDS, PMSF) then once with LiCl buffer (10mM Tris HCl pH 8, 1mM EDTA, 0.5 mM EGTA, 250 mM LiCl, 1% IGEPAL, 1% NaDOC, PMSF) then once with TE buffer and finally with elution buffer (TE + 1% SDS). Bound complexes were eluted from the beads by heating at 65 °C with occasional vortexing and cross-linking in the immunoprecipitation and WCE samples was reversed by incubating at 65 °C for 6–7 h. Immunoprecipitation and WCE DNA was then purified by treatment with RNase A, proteinase K and extracted with phenol/chloroform/isoamyl alcohol extractions. ChIP products were quantified by Real Time qPCR. Primer sequences were as follows: IL-17 enhancer forward: 5' TGATAGCCCAACCACAATGTG – 3' (IL-17 gene nt 1051 - 1072). IL-17 enhancer reverse: 5'-ACCTATACGTTAGCAGGCACA – 3' (IL-17 gene nt 1220 - 1241). IL-17 promoter forward: 5'-TCTGCCCTTCCCATTTTCCT-3' (IL-17 gene nt 2886 – 2906)

IL-17 promoter reverse: 5'- ATGGATGAGTTTGTGCCTGC-3' nt 3064 – 3084. Nuclear hormone receptor responsive element (NRRE-1) in the HIV-1 LTR Forward 5'-TCTACCACACACAAGGCTACT-3', Reverse 5'-ACAAGCTGGTGTTCTCTCCT-3'; RORC2 consensus sequence (CS) in HIV-1 Pol forward: 5'-GGGAAAGCTAGGGGATGGTT-3' (HIV-1 nt 5137 - 5157), RORC2 CS reverse: 5'-TCAGGGTCTACTTGTGTGCT -3 (HIV-1 nt 5322- 5342). Real time PCR was carried out in an Eppendorf Realplex in a final volume of 20 µL containing 1x SYBR green master mix (Applied Biosystems), 0.4 µM each primer and 2 µL DNA (pre-diluted 1:10). Cycles parameters were: 95 °C for 2 minutes for 1 cycle, followed by 95 °C for 1 minute, 55 °C for 55 seconds and 65 °C for 1min 30 seconds for 45 cycles. ChIP signal was calculated using the percent input method.

Viral outgrowth assay

A simplified VOA was performed, as we previously described (56). Briefly, memory CD4+ T cells were cultured at 1×106 cells/well in 1 ml of media (RPMI, 10% FBS, 1% Penicillin/Streptomycin) in a 48-well plate (Costar) coated with CD3 Abs (1 µg/ml; BD Biosciences, Clone UCHT1) and in the presence of soluble CD28 Abs (1 µg/ml; BD Biosciences, Clone CD28.2). At day 3 post-stimulation, cells from each original replicate were individually washed with media, split into two new CD3 Abs-uncoated 48-well plate wells, and cultured in media containing IL-2 (5 ng/ml; R&D

Systems), in the presence or in the absence of GGSK2691805A (5 uM). Cells from each well were further split into two new wells (without washing) at day 6 and 9 post-stimulation, with 50% of the media being refreshed with IL-2 with/without GSK2691805A. Cells were kept in culture for a total of 12 days.

Statistical Analysis

Statistical analyses were performed with GraphPad Prism 7 software (GraphPad Software, Inc., La Joya, CA, USA). One-way ANOVA and Friedman along with Dunnett's and Dunn's multiple comparisons test respectively evaluated the statistical differences. The use of non-parametric tests is justified by the fact that data sets did not pass the normal distribution test Kolmogorov-Smirnov. P-values of ≤0.05 were arbitrarily considered statistically significant.

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Competing interest statement

AF and PA received funding from GSK for this study. HA-M and DI are GSK employees. The other co-authors declare no financial or non-financial competing interests to disclose.

Authors' contributions

TRWS, YZ DS, AZ, LRM, DP, AFert, ML, DC, KK, and SO designed and performed experiments, analysed data and prepared figures. JPR provided access to clinical samples/information, set up clinical research protocols, and contributed to manuscript revision. HA-M and DI provided critical reagents and expertise. PA and AF conceived the research study, designed research, analysed data and wrote the manuscript. All co-authors revised and approved the manuscript.

Data Availability

Source numerical data files and images for all Figures have been deposited with UCL Data Repository. (https://doi.org/10.5522/04/14892822.v1)

Materials availability

GSK compounds GSK2837270A, GSK2793955A, GSK283726, GSK2833332A and GSK2805956A are available upon reasonable request. GSK2691805 is commercially available.

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MAIN FIGURE LEGENDS

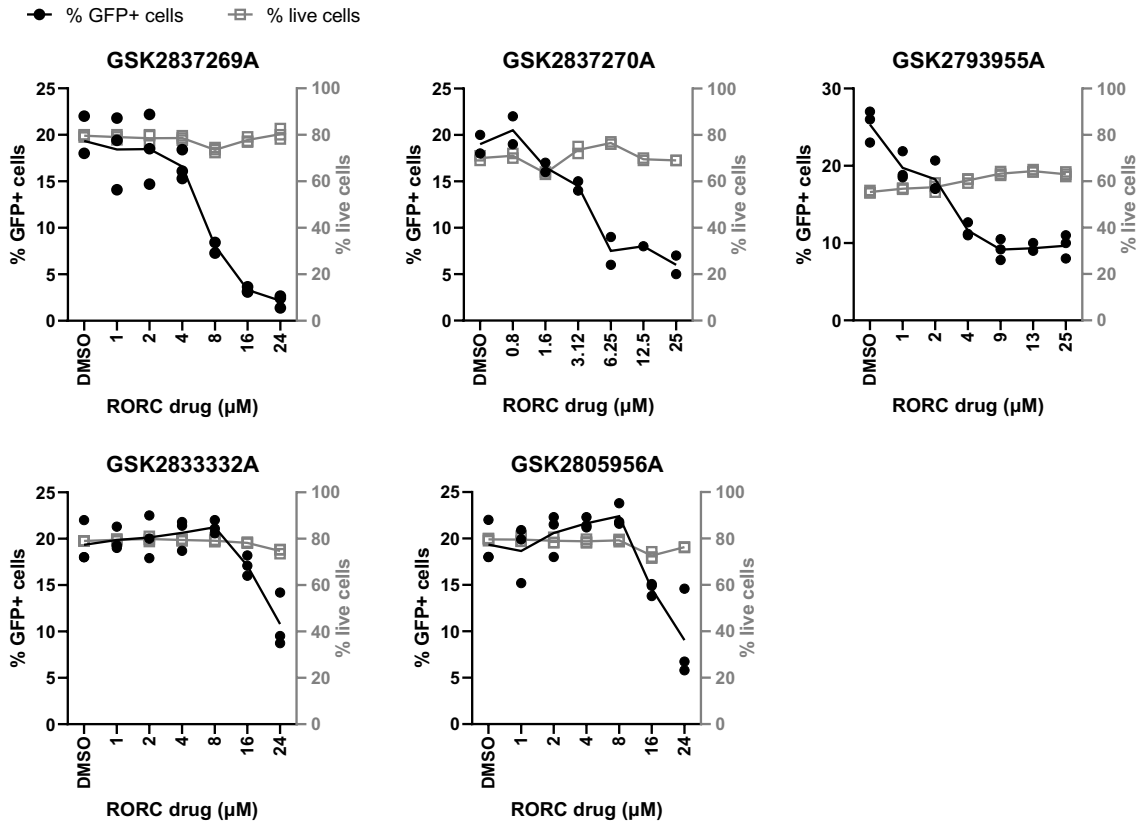


Figure 1. RORC inhibitors inhibit HIV-1 infection. Jurkat cells were infected with single cycle, VSVG-pseudotyped HIV-1 LAIGFP at an MOI of 0.2 in the presence of the indicated concentrations of RORC2 inhibitors. Forty-eight hours post-infection, cells were analysed by flow cytometry to measure the percentage of GFP+ cells. The proportion of live cells was simultaneously assessed by AlamaR blue, n=3.

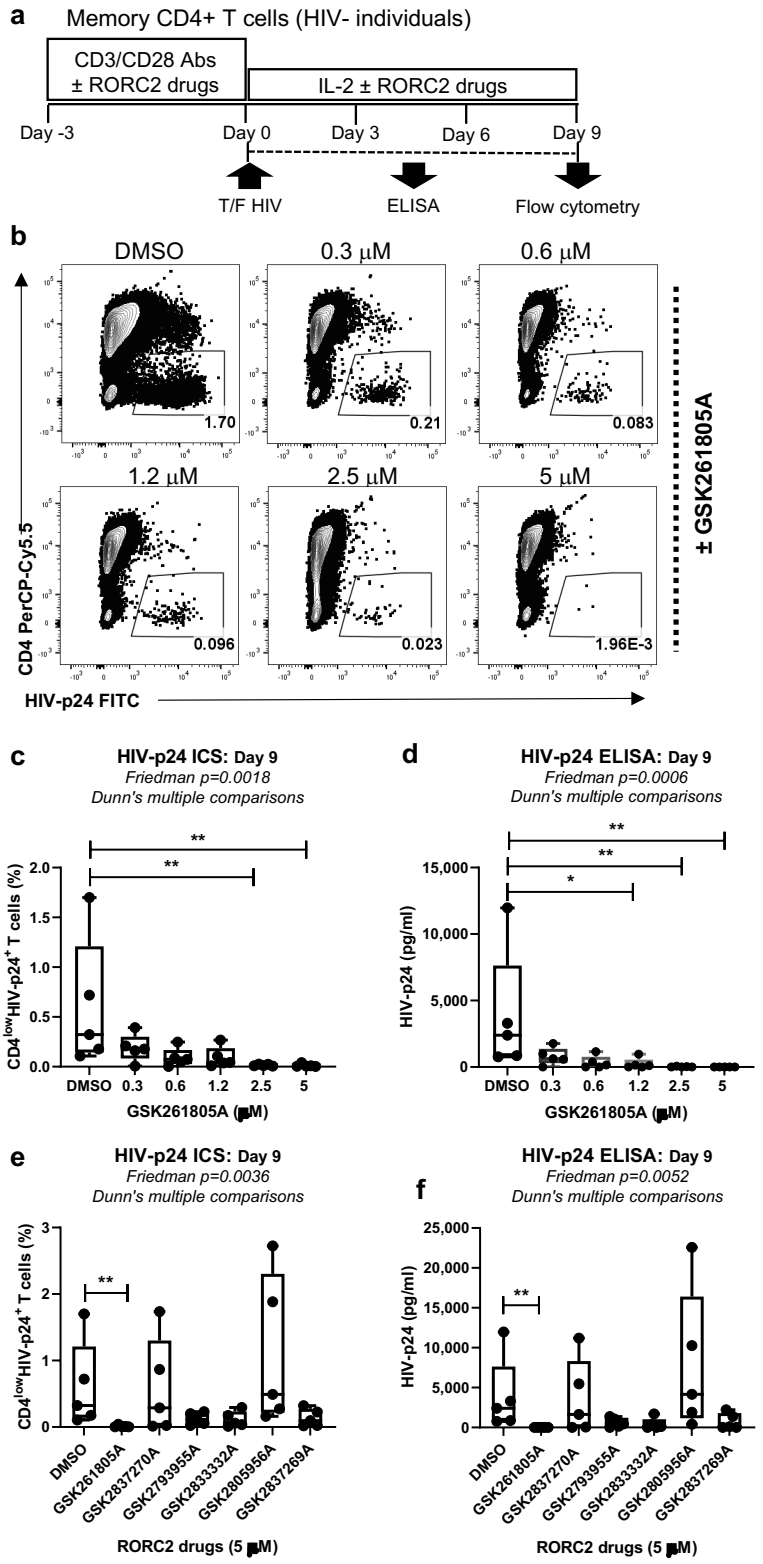


Figure 2. RORC inhibitors reduce HIV-1 replication in primary CD4+ T cells in vitro. Shown is the experimental flow chart (a). Memory CD4+ T cells isolated from n=5 healthy donors were stimulated with CD3/CD28 antibodies in the presence or absence of the indicated concentrations

of RORC2 inhibitors for 3 days. Then, cells were exposed to HIVTHRO for 3 hours. HIV-infected CD4+ memory T cells were cultured with IL-2 in the presence of the indicated concentrations of RORC2 inhibitors for 9 days. Media was refreshed with IL-2 and RORC2 inhibitors every 3 days. (b) The intracellular expression of HIV-p24 in CD4+ memory T cells was quantified by flow cytometry after staining with fluorochrome-conjugated CD4 and HIV-p24 Abs at day 9 post-infection. Shown are contour plots of the frequency of CD4lowHIV-p24+ T cells in one representative donor. (c) Statistical analysis of the frequency of CD4lowHIV-p24+ T cells within the total memory CD4+ T cells exposed to the indicated concentration of GSK261805A from n=5 donors 9 days post-infection (d) HIV-p24 levels measured in cell culture supernatants by ELISA at Day 9 post-infection. (e) Statistical analysis of the frequency of CD4lowHIV-p24+ T cells within the memory subset and (f) HIV-p24 levels in the cell culture supernatant in the presence of the indicated RORC2 inhibitors (5 uM). Friedman p-values, with Dunn's multiple comparison significance, are indicated on the graphs.

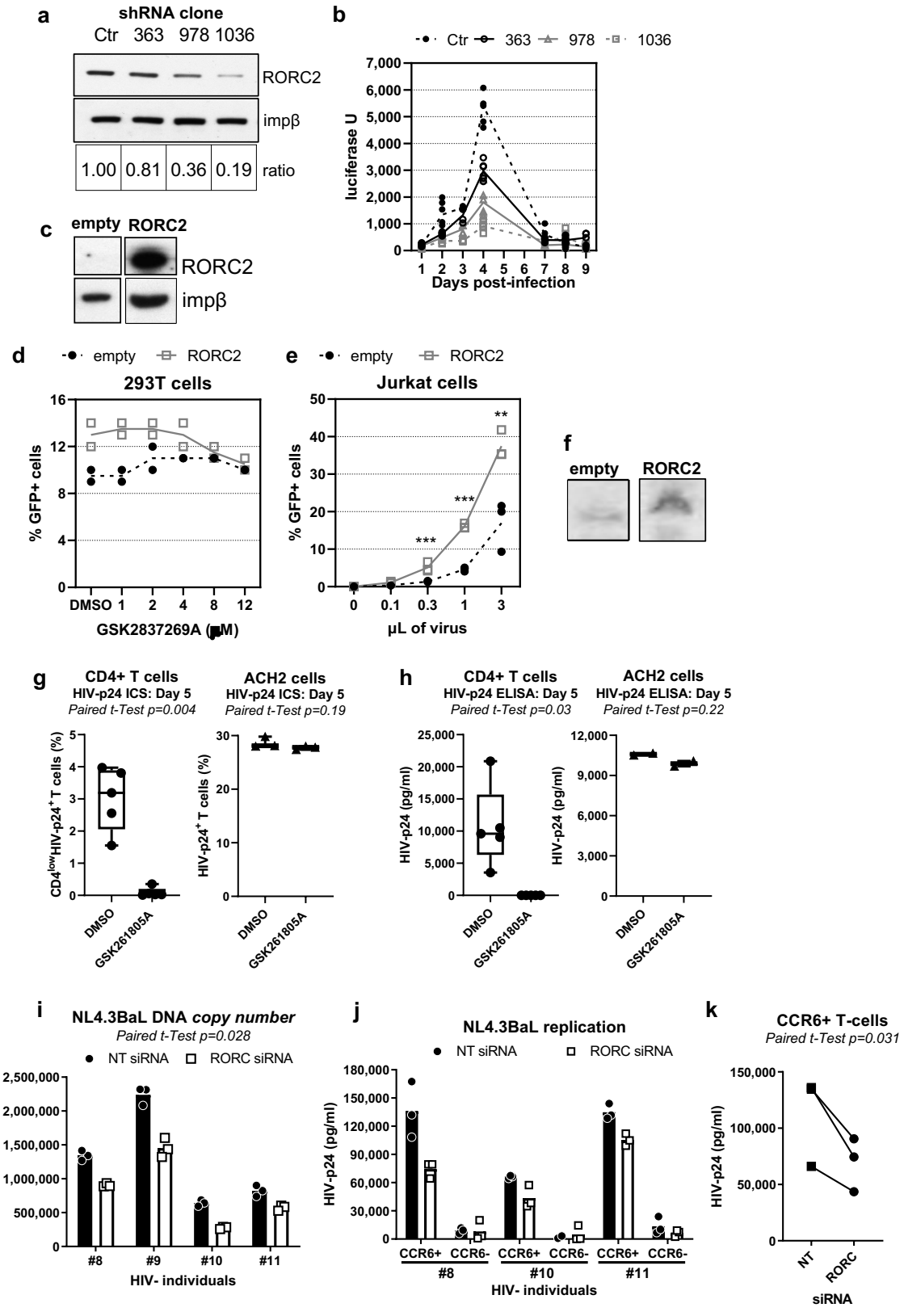


Figure 3. RORC2 is a host co-factor for HIV-1 infection of CD4+ T cells. (a) Luciferase Jurkat 1G5 indicator cells were transduced with three different shRNAs that target RORC2 or a non-targeting

shRNA (Ctr) and selected in puromycin. RORC2 protein levels were analysed by Western blot and impB was used as a loading control. The signal was quantified by ImageJ and the ratio of RORC2 versus impB is indicated in the lower panel. (b) 1G5 cells transduced with the same shRNAs were infected with HIV-1NL4.3 and the luciferase signal was measured at the indicated time points. (c) An empty vector plasmid or the myc-RORC2 cDNA was transfected into 293T cells. After selection in puromycin, cells were analysed by Western blot as above. (d) The same 293T cells were infected with single cycle HIV-1 LAIGFP at an MOI of 0.1 in the presence of the indicated concentrations of GSK2837269A and 40 hours later cells were analysed by flow cytometry to measure the percentage of GFP+ cells (n=2). (e) Jurkat cells were transduced with a retroviral vector expressing myc-RORC2 or an empty vector, selected in media containing blasticidin for 7 days and infected with the indicated volumes of single cycle HIV-1 LAIGFP (107 i.u./mL). Forty hours after infection cells were analysed by flow cytometry to measure the percentage of GFP+ cells. *** $p<0.005$, ** $p = 0.008$ two tailed Student's T-test, n=3. (f) Expression of RORC2 in these cells was confirmed by Western blot. (g-h) Memory CD4+ T cells isolated from n=5 HIV- participants and ACH2 cells were cultured in parallel using the experimental design depicted in Figure 2A. CD3/CD28-activated CD4+ T cells exposed to HIVTHRO for 3 hours and ACH2 cells were cultured with IL-2 in the presence or the absence of GSK261805A (5 µM) for 5 days. (g) The intracellular expression of HIV-p24 was quantified by flow cytometry upon staining with fluorochrome-conjugated CD4 and HIV-p24 Abs. (h) HIV-p24 levels in cell culture supernatants were measured by ELISA. Paired t-Test p-values are indicated on the graphs. (i-k) CD3/CD28-activated total memory CD4+ T cells from n=4 HIV- donors (i) or FACS-sorted memory CCR6+ and CCR6- CD4+ T cells from n=3 HIV- donors (j-k) were nucleofected with Dharmacon On target smart siRNA pools specific for RORC2 or a non-targeting (NT) siRNA using the Amaxa technology.. Cells were then exposed to HIVNL4.3BaL (50 ng per 106 cells) and HIV-DNA integration were quantified by nested real-time PCRat day 3 post-infection. Shown is HIV-DNA integration in memory CD4+ T cells (i) and HIV replication in sorted CCR6+/CCR6- T cells (j), as well as statistical analysis of HIV-p24 levels in sorted CCR6+ T cells (k).

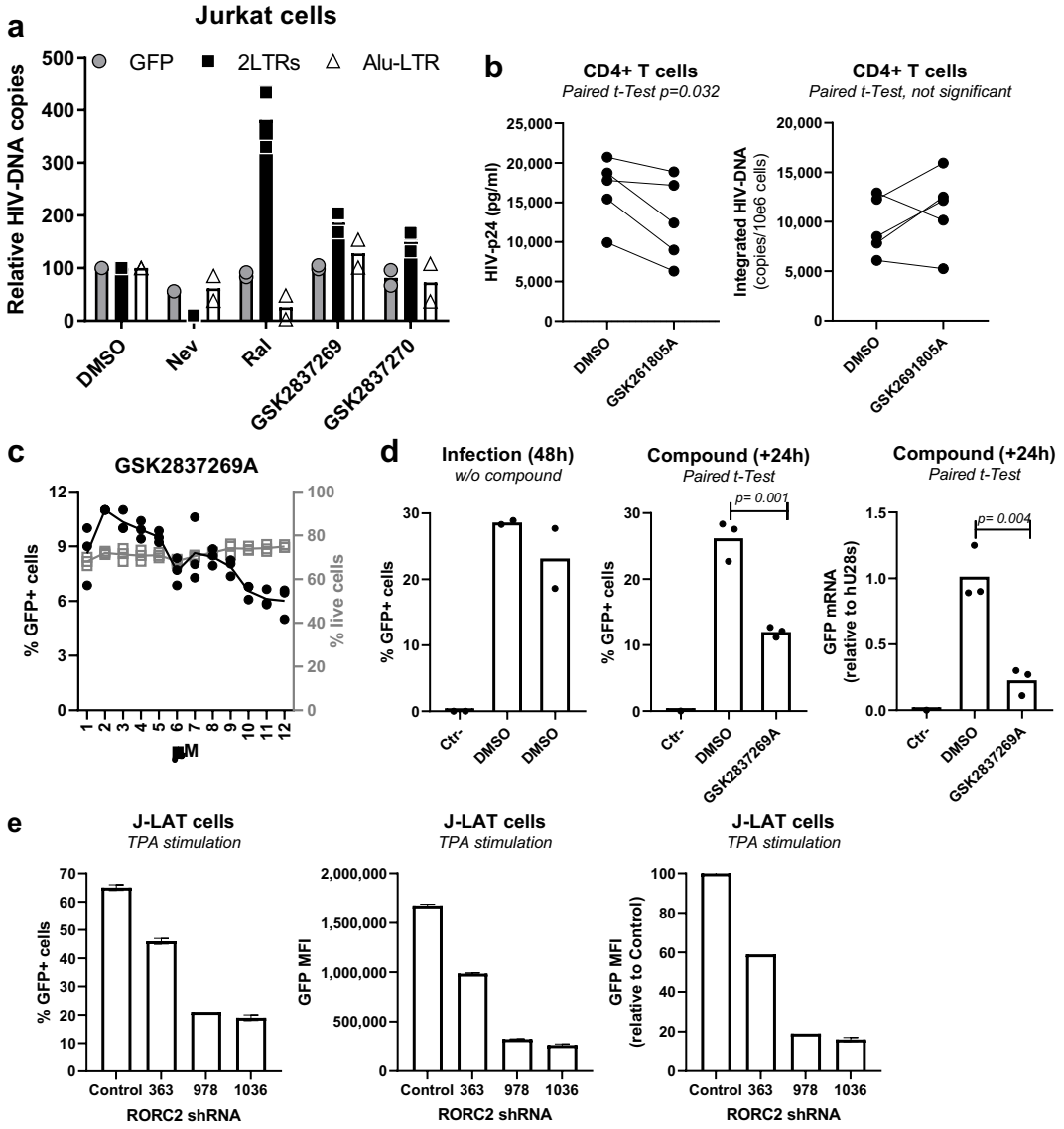


Figure 4. RORC2 promotes HIV-1 gene expression and binds to the HIV-1 LTR. (a) Jurkat cells were infected with VSV-G pseudotyped single cycle HIV-1 LAIGFP in the presence of the reverse transcriptase inhibitor nevirapine (500 nM), or the integrase inhibitor raltegravir (100 nM) or the indicated RORC inhibitors (5 uM). The amount of negative strand viral DNA (GFP) and 2-LTR circular DNA was quantified 24h post-infection by Taqman qPCR. Integrated viral DNA was quantified by Alu-LTR qPCR on DNA extracted 8 days after infection, n=2. (b) Memory CD4+ T cells from n=5 HIV- donors were stimulated with CD3/CD28 Abs in the presence or the absence of GSK261805A (5 μM) for 3 days and then infected with single round VSV-G pseudotyped NL4.3HIV-1. Cell were cultured with IL-2 in the presence or the absence of GSK261805A (5μM) for additional 3 days. Shown are HIV-p24 levels in cell culture supernatants measured by ELISA

(left panel) and integrated HIV-DNA levels (right panel) quantified by nested real-time PCR at day 3 post-infection. (c) Jurkat cells were infected with VSV-G pseudotyped single cycle HIV-1 LAIGFP and 24h post-infection they were treated with the indicated concentrations of GSK2837269A. Cells were analysed 24h later by flow cytometry to determine the percentage of infected (GFP+) cells, n=3. (d) Jurkat cells were infected with single cycle HIV-1 LAIGFP. Forty-hours after infection, cells were analysed by flow cytometry to determine the percentage of infected (GFP+) cells (left panel). These cells were then treated with DMSO or GSK2837269A (5 uM) for 24h before being re-analysed by flow cytometry (middle panel). RNA was extracted from the cells and the amount of GFP mRNA relative to 28S ribosomal RNA was quantified by RT-qPCR. Average values are shown (n=3) (left panel). (e) J-Lat (clone A2) cells were transduced with the same RORC2-targeting shRNAs shown in Fig. 3a, selected with puromycin for 7 days and stimulated with TPA for 24h before analysis by flow cytometry.

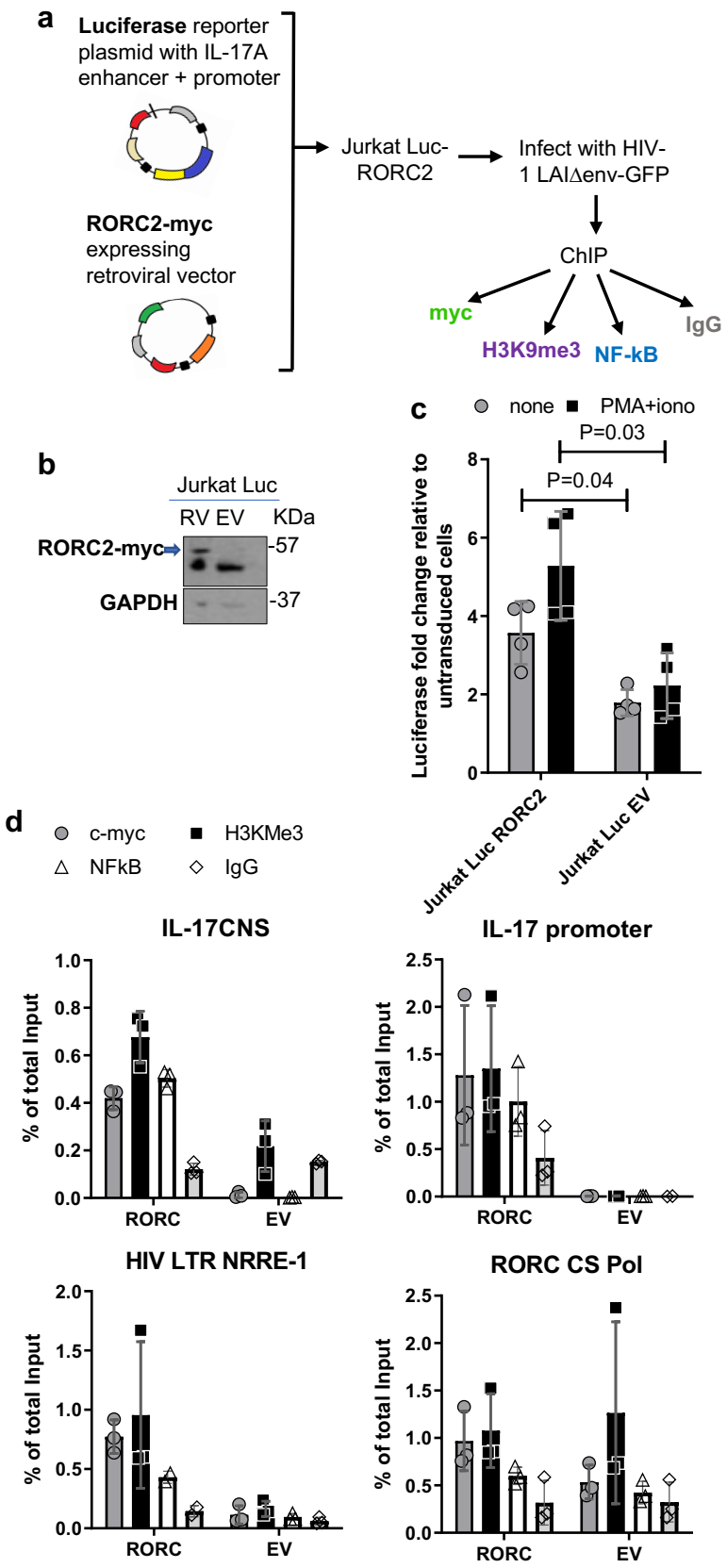


Figure 5. RORC2 binds to the HIV promoter. (a) Schematic diagram explaining the experimental steps for ChIP. Jurkat cells expressing luciferase driven by the IL-17A enhancer/promoter region

were transduced with a retroviral vector expressing RORC2-myc (Luc -RORC2) or an empty vector control (Luc-EV). Luc-RORC2 and Luc-EV cells were infected in parallel with VSV-G pseudotyped HIV-1 LAI deltaenvGFP (HIV-1GFP) and 24 hours later processed for ChIP with the four indicated Abs. (b) Western blot with anti-myc antibody to detect RORC-myc following ChIP (the lower band is IgG heavy chains); GAPDH in the total lysate samples was used as a loading control. (c) Fold change in Luciferase expression relative to untransduced Jurkat Luc cells with or without stimulation with PMA and ionomycin. Shown is average ± SD, n=3, P value based on two tailed t-test. (d) Real time PCR signal following ChIP for the indicated DNA regions and antibody. Top left, IL-17A enhancer region; top right, IL-17A promoter region; bottom left, NRRE region in the HIV-1 LTR; bottom right, HIV-1 pol region containing the putative RORC2 consensus element. Shown are replicate values, as well as average ± SD, n=3.

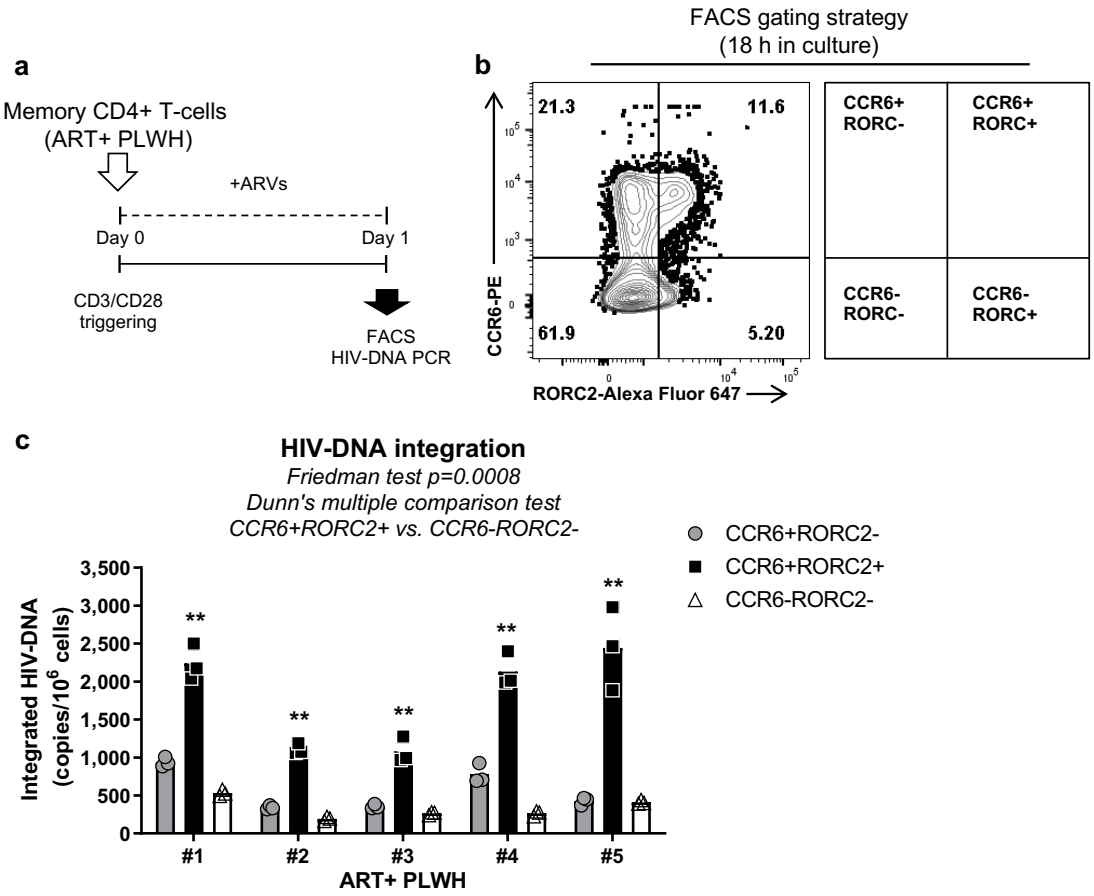


Figure 6. Memory CD4+ T cells expressing the Th17 markers CCR6 and RORC2 are enriched in integrated HIV-DNA in ART-treated PLWH. (a) Flow chart of experimental approach; memory CD4+ Tcells isolated from ART+ PLWH were stimulated with CD3/CD28 Abs for 18 hrs in the presence of antiretroviral drugs (AZT 180 nM, Efavirens 100nM, Raltegravir 200nM) to prevent cell-to-cell HIV transmission in vitro. Highly pure CCR6+RORC2-, CCR6+RORC2+ and CCR6-RORC2- cell subsets were sorted by FACS and Integrated HIV-DNA levels were quantified by nested real-time PCR. (b) Gating strategy used to sort the three cell populations mentioned above and (c) the statistical analysis of Integrated HIV-DNA in sorted CD4+ T cell subsets from n=5 ART-treated PLWH. Shown are individual replicates, with the bars representing median values.

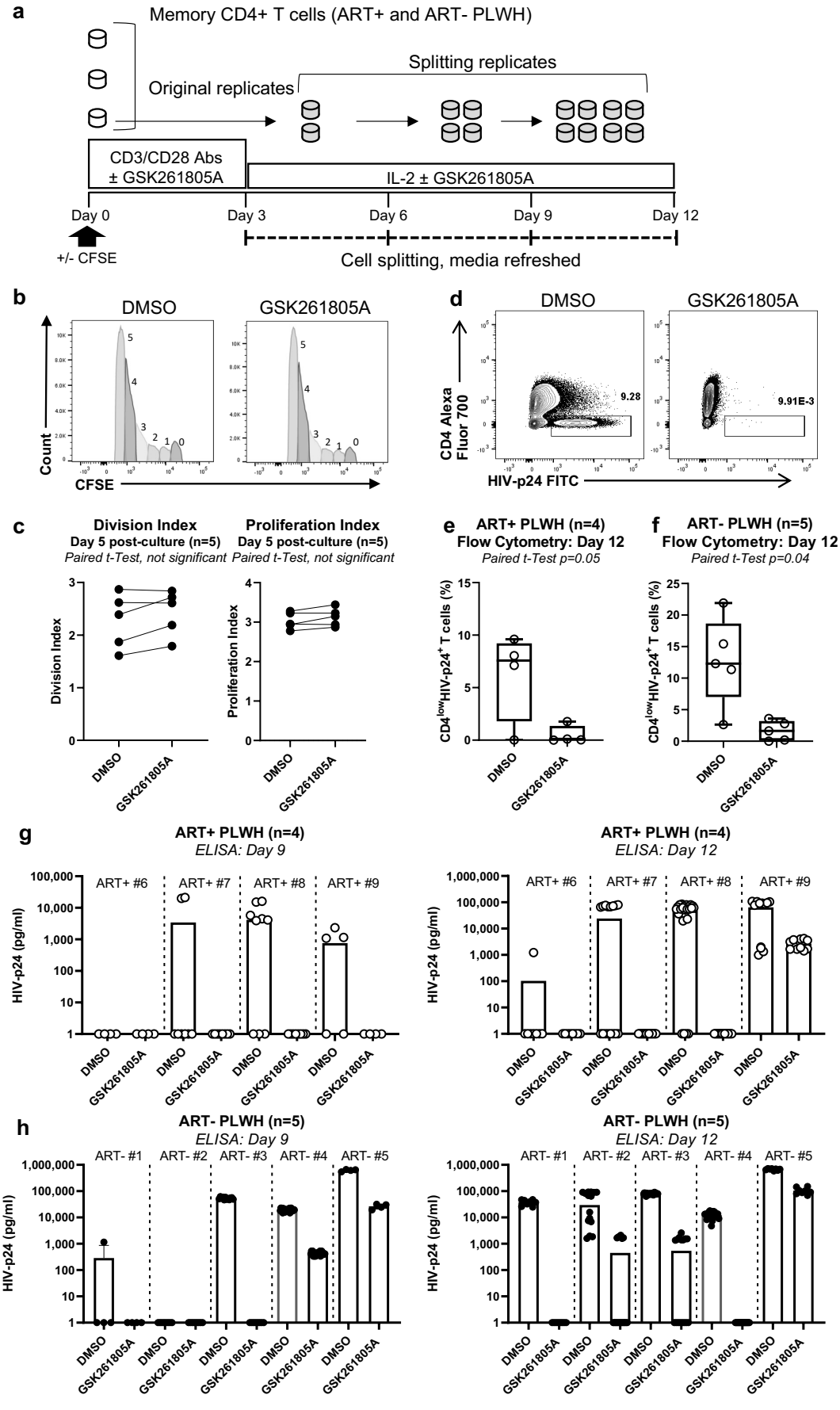

a
Memory CD4+ T cells (ART+ and ART- PLWH)
Splitting replicates
Original replicates
CD3/CD28 Abs
± GSK261805A
IL-2 ± GSK261805A
Day 0
Day 3
Day 6
Day 9
Day 12
+/- CFSE
Cell splitting, media refreshed
b
DMSO
GSK261805A
Count
CFSE
d
DMSO
GSK261805A
CD4 Alexa Fluor 700
HIV-p24 FITC
9.28
9.91E-3
c
Division Index
Day 5 post-culture (n=5)
Paired t-Test, not significant
Proliferation Index
Day 5 post-culture (n=5)
Paired t-Test, not significant
e
ART+ PLWH (n=4)
Flow Cytometry: Day 12
Paired t-Test p=0.05
f
ART- PLWH (n=5)
Flow Cytometry: Day 12
Paired t-Test p=0.04
g
ART+ PLWH (n=4)
ELISA: Day 9
ART+ PLWH (n=4)
ELISA: Day 12
HIV-p24 (pg/ml)
ART+ #6
ART+ #7
ART+ #8
ART+ #9
h
ART- PLWH (n=5)
ELISA: Day 9
ART- PLWH (n=5)
ELISA: Day 12
ART- #1
ART- #2
ART- #3
ART- #4
ART- #5

Figure 7. RORC antagonism inhibits HIV-1 outgrowth in memory CD4+ T cells of ART-treated and untreated PLWH. (a) Experimental flow chart of the viral outgrowth assay; memory CD4+ T cells isolated from PLWH receiving ART (ART+PLWH) or not (ART- PLWH) were activated with CD3/CD28 Abs in the presence of DMSO or GSK2691805A (5 uM) at a cell concentration of 1 million cells/ml in triplicates for 3 days. Then, cells were washed and split at day 3 post-stimulation and cultured in medium containing IL-2 (5 ng/ml) up to 12 days (split every three days) in the presence of DMSO or GSK2691805A (5 uM). At day 12, HIV-infected cells were identified as CD4lowHIV-p24+ by flow cytometry. HIV-p24 levels in cell-culture supernatants were measured by ELISA. (b-c) In parallel, using the same experimental set up, a VOA was performed with CFSE-loaded memory CD4+ T cells obtained from ART+ PLWH. CFSE dilution was evaluated by flow cytometry at day 5 post-activation. Shown is an histogram of CFSE dilution showing cell divisions (division 0 to 5) of one representative individual (b) and a statistical analysis of the division and proliferation index obtained with cells of n=5 ART+PLWH (c). (d) Frequency of CD4lowHIV-p24+ cells in one representative individual. (e-f) Statistical analysis of results obtained with cells of n=4 ART+PLWH (e) and n=5 ART-PLWH (f). (g-h) Effect of GSK2691805A on HIV-p24 levels in cell-culture supernatants of n=4 ART+PLWH (g) and n=5 ART-PLWH (h) at day 9 and 12 post CD3/CD28 activation. Paired t-Test p-values are indicated on the graphs. Shown are box and whiskers plots with individual values and maximum and minimum (e-f), and individual replicate values, with bars indicating median values (g-h).

SUPPORTING INFORMATION

Supporting Information Table 1: RORC2 compounds inhibit HTRF-ligand binding and Jurkat IL-17 luciferase reporter expression.

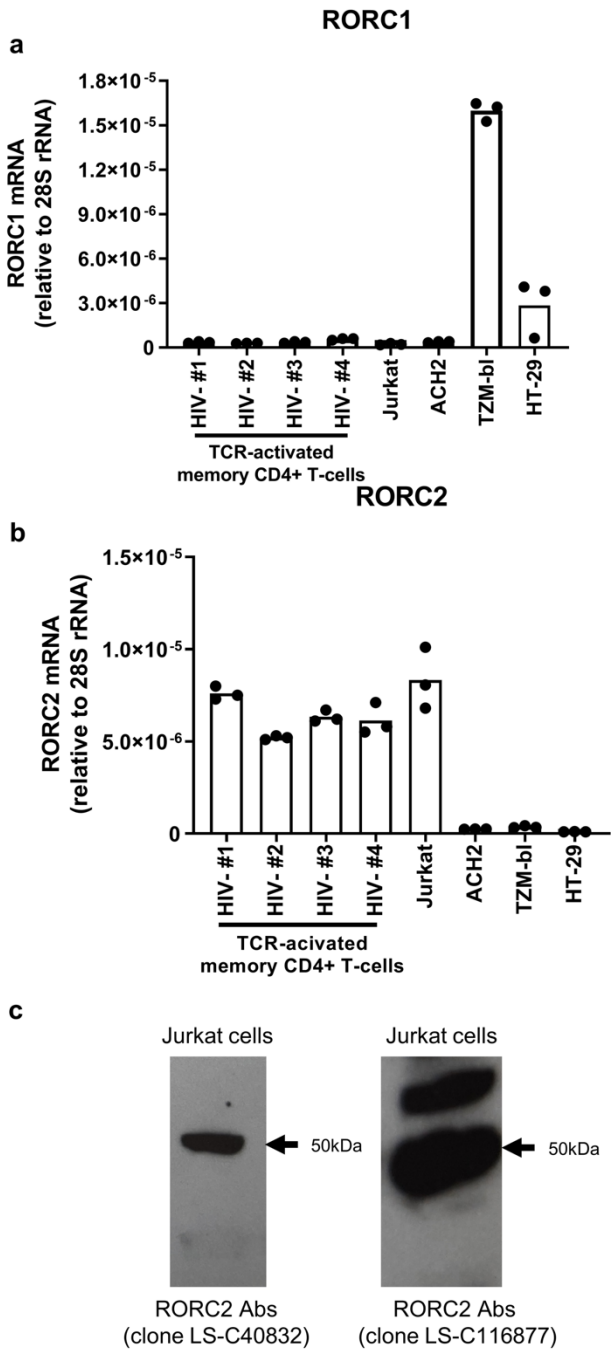
	HTRF-Ligand Binding		Jurkat IL-17 Luciferase	
Compound	**IC50 Mean µM (+/- SD), n**	**Vmax Mean (+/- SD), n**	**IC50 Mean µM (+/- SD), n**	**Vmax Mean (+/- SD), n**
GSK2691805A	0.039 (0.001), 3	38 (10), 3	0.040 (0.002), 3	110 (0), 3
GSK2837270A	0.016 (<0.001), 2	94 (3), 2	0.501 (0.025), 2	110 (0), 2
GSK2793955A	0.025 (0), 1	86 (0), 1	0.794 (0.010), 2	110 (0), 2
GSK2833332A	>10 (0), 1	-	-	-
GSK2805956A	>10 (0), 1	-	-	-
GSK2837269A	0.016 (<0.001), 2	94 (4), 2	0.079 (0.001), 4	110 (1), 4

Supporting information Table 2: Clinical parameters of HIV-infected untreated and ART-treated study participants.

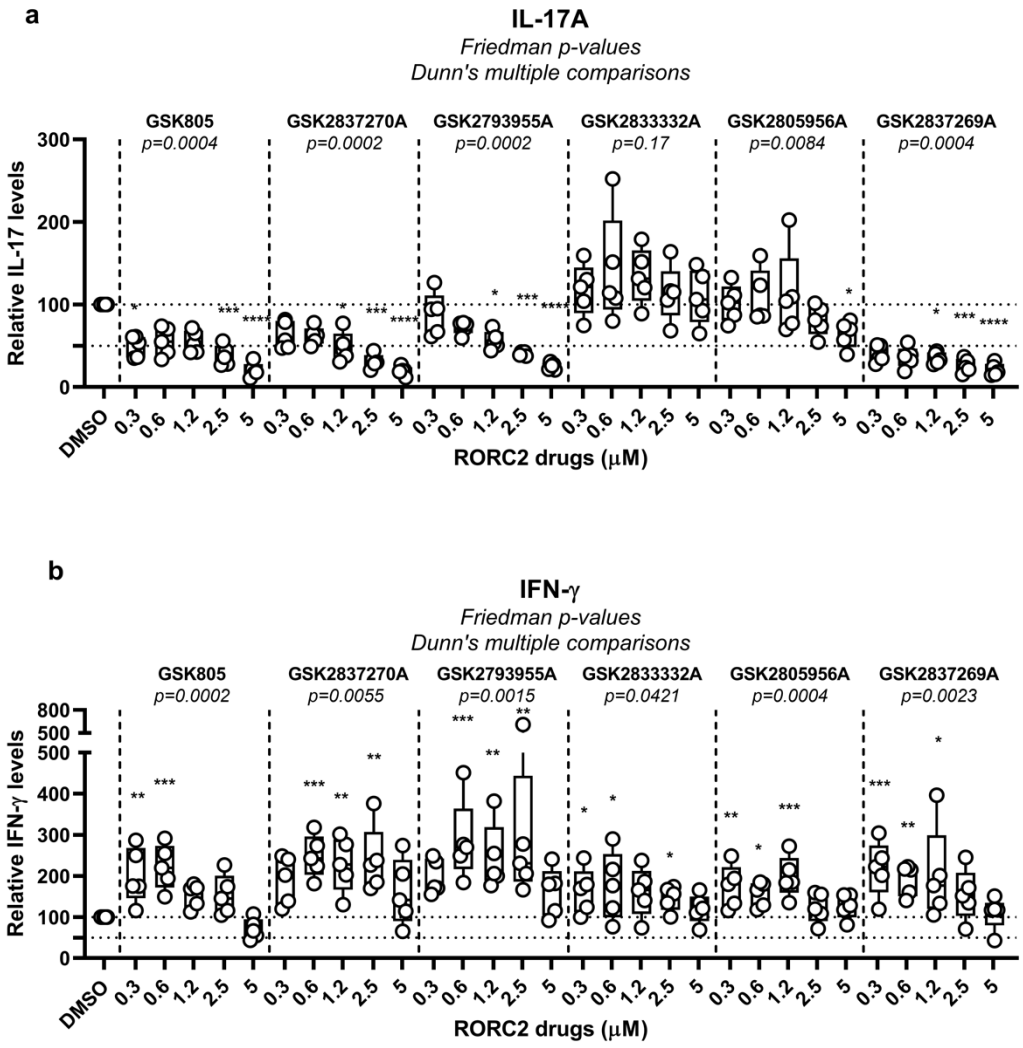
ID	Sex	Age″	CD4 count#	CD4:CD8 Ratios	Viral load&	Time since infection*	ART	Time on ART*
ART+ #1	M	45	318	0,7	<40	150	Delaviridine Kivexa	50
ART+ #2	M	44	459	0,8	<40	189	Truvada Raltegravir	-
ART+ #3	M	46	581	0,5	<40	99	-	94
ART+ #4	M	32	523	1,0	<40	52	Truvada Reyataz	48
ART+ #5	M	57	514	0,9	<40	16	Tivicay Truvada	11
ART+ #6	M	44	398	0,5	<40	154	Complera	25
ART+ #7	M	36	542	0,7	<40	13	Stribild	12
ART+ #8	M	49	458	0,5	<40	227	Truvada Viramune	201
ART+ #9	M	30	598	1,0	<40	80	Stribild	77
ART- #1	M	24	316	0,5	9,496	55	None	N.A
ART- #2	M	47	529	1,2	3,189	110	None	N.A
ART- #3	M	42	221	0,5	41,774	89	None	N.A
ART- #4	M	50	389	0,3	97,552	150	None	N.A
ART- #5	M	40	1,068	1,2	22,812	2	None	N.A

ART- #6	M	24	897	1,3	18,621	-	None	N.A

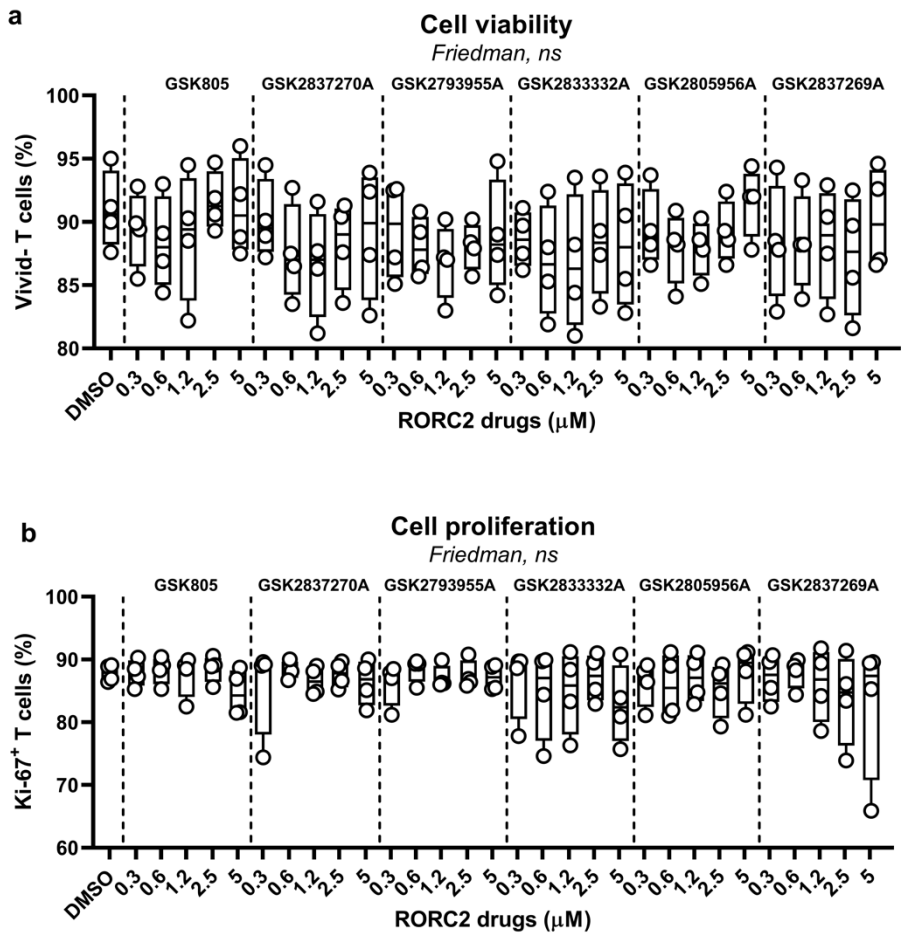
M, male; F, female; ART-, ART-untreated PLWH; ART+, ART-treated PLWH; ″ , years; #, cells/µl; &, HIV-RNA copies/ml plasma; *, months on ART; -, information not available; N.A, not applicable



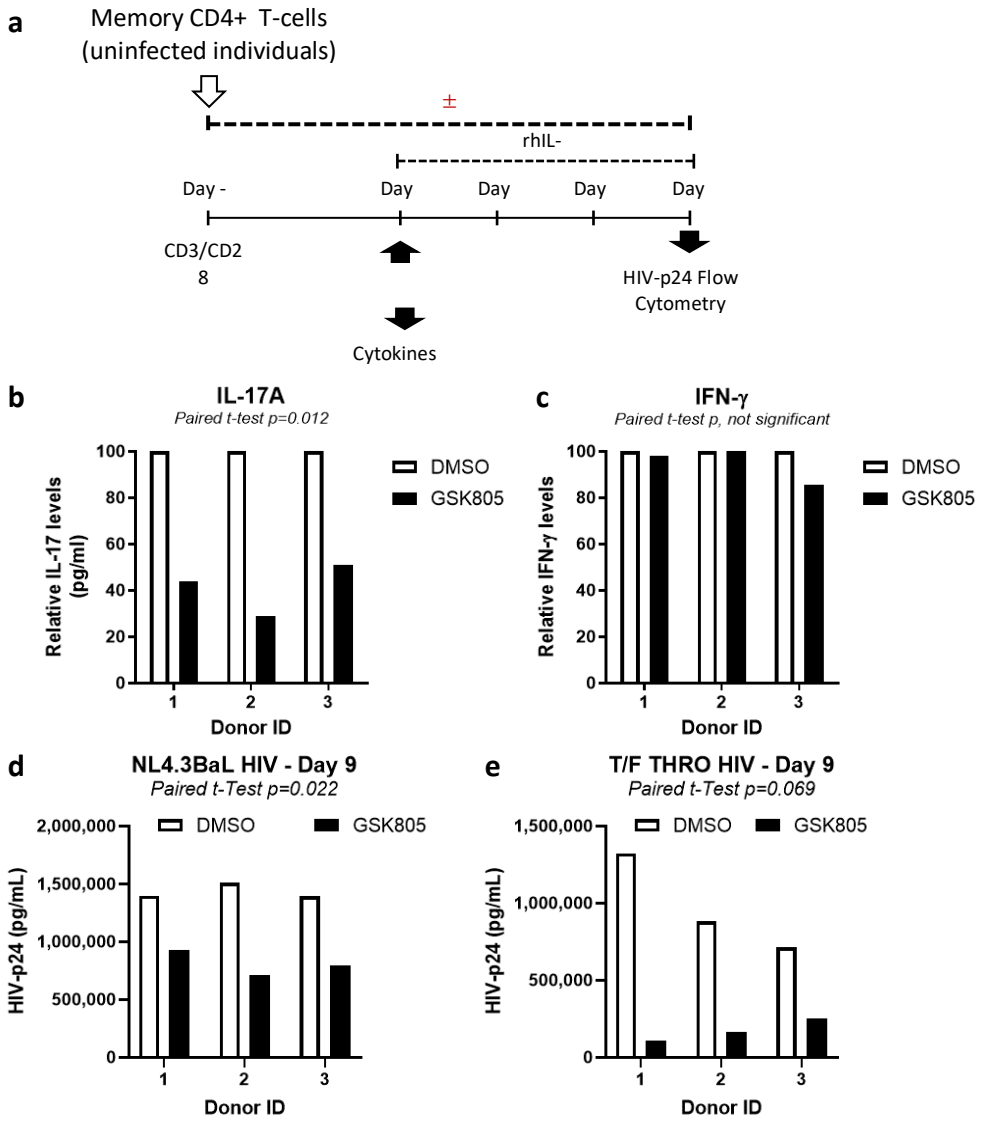
Supporting information Figure 1. **RORC2, but not RORC1, is expressed in primary CD4+ T cells and Jurkat cells**. **(a-b)** The relative gene expression of RORC1 mRNA **(a)** and RORC2 mRNA **(b)** was evaluated by real-time RT-qPCR in CD3/CD28-activated memory $CD4^+$ T cells isolated from n=4 HIV- uninfected individuals as well as in the Jurkat, ACH2, TZM-bl and HT-29 cell lines. **(c)** Western blot of Jurkat cell lysates to detect RORC2 expression. The antibody used is indicated below each panel.



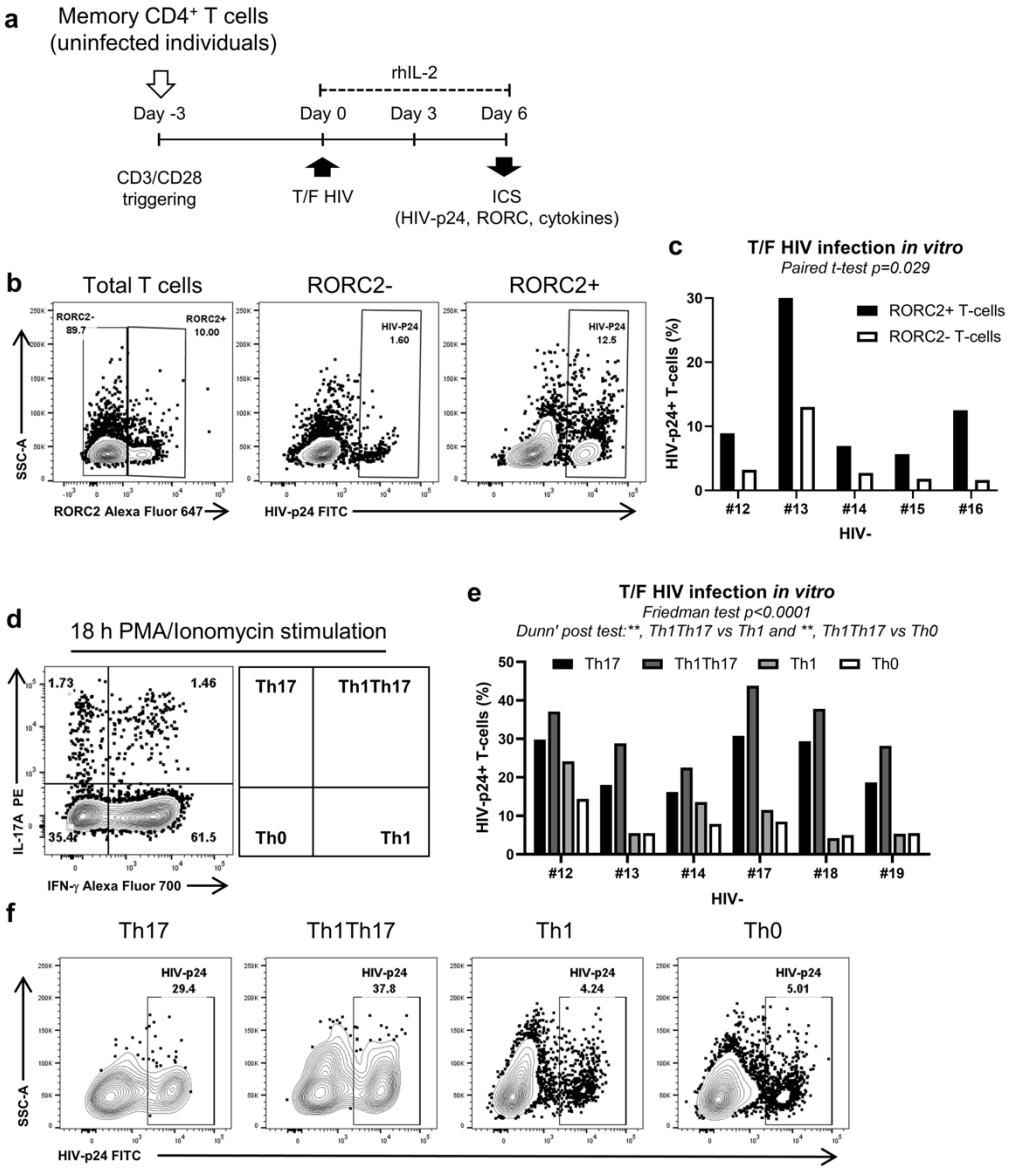
Supporting information Figure 2. RORC2 inhibitors reduce IL-17A, but not IFN-γ production. Memory CD4+ T cells were isolated and stimulated with CD3/CD28 Abs in the presence or absence ofthe indicated RORC2 inhibitors, as described in Figure 2. Levels of IL-17A and IFN-γ were measured by ELISA at day 3 post-stimulation. Statistical analysis of IL-17A **(a)** and IFN-γ **(b)** levels in cell culture supernatants of experiments performed with cells from n=5 HIV-uninfected individuals. Friedman p- values, with Dunn's multiple comparison significance, are indicated on the graphs (*, p<0.05; **, p<0.01;***, p<0.001; ****, p<0.0001).



Supporting information Figure 3. No effect of RORC2 inhibitors on cell viability and proliferation. A fraction of memory CD4$^+$ T cells from experiments depicted in **Figure 2** were harvested 3 days after CD3/CD28 stimulation and stained with Live/dead Fixable Aqua dead stain kit and fluorochrome-conjugated Ki-67 Abs for flow cytometry analysis. Shown are the statistical analysis of the frequency of Live (Vivid-) **(a)** and Ki-67+ cells **(b)** in experiments performed with cells from n=5 HIV-uninfected individuals.

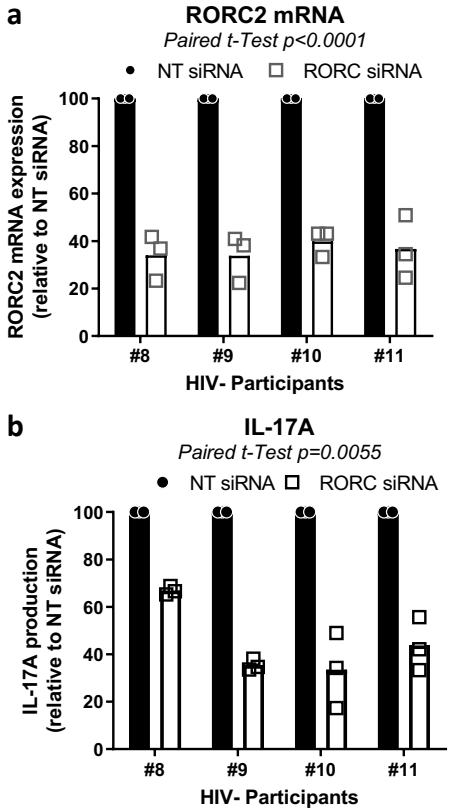


Supporting information Figure 4. The RORC inhibitor GSK261805 decreases $HIV_{NL4.3BaL}$ replication in memory $CD4^+$ T cells. Shown is the effect of GSK261805 on IL-17A **(b)** and IFN-γ **(c)** production in memory $CD4^+$ T cells of n=3 HIV- participants at D3 post-TCR riggering (prior to infection), as well as replication of $HIV_{NL4.3BaL}$ **(d)** and HIV_{THRO} **(e)** strains at 9 days post-infection.



Supporting information Figure 5. **Preferential HIV-1 replication in Th17-polarized RORC2$^+$ cells *in vitro*.** Experimental flow chart; memory CD4$^+$ T cells isolated from n=5 HIV-uninfected individuals were stimulated with CD3/CD28 Abs for 3 days and exposed to HIVTHRO. After infection, cells were cultured in the presence of IL-2 for 6 days. The intracellular expression of HIV-p24, RORC2 and cytokines (IL-17A and IFN-γ) was analysed by flow cytometry **(a)**. Shown are contour plots of RORC2 expression **(b, left panel)**, HIV-p24 expression in RORC2$^-$ and RORC2$^+$ cells of one representativeindividual **(b, middle/right panels respectively)** and statistical analysis of results obtained with cells form n=5 individuals **(c)**. Gating strategy used to identify Th17 (IL-17A+IFN-γ-), Th1Th17 (IL-17A+IFN-γ+), Th1 (IL-17A-IFN-γ+), Th0 (IL-17A-IFN-γ-) **(d).** Shown are the contour

plots of HIV-p24 expression in Th subsets of one representative individual **(e)** and statistical analysis of experiments performed with cells from n=6 individuals **(f)**.

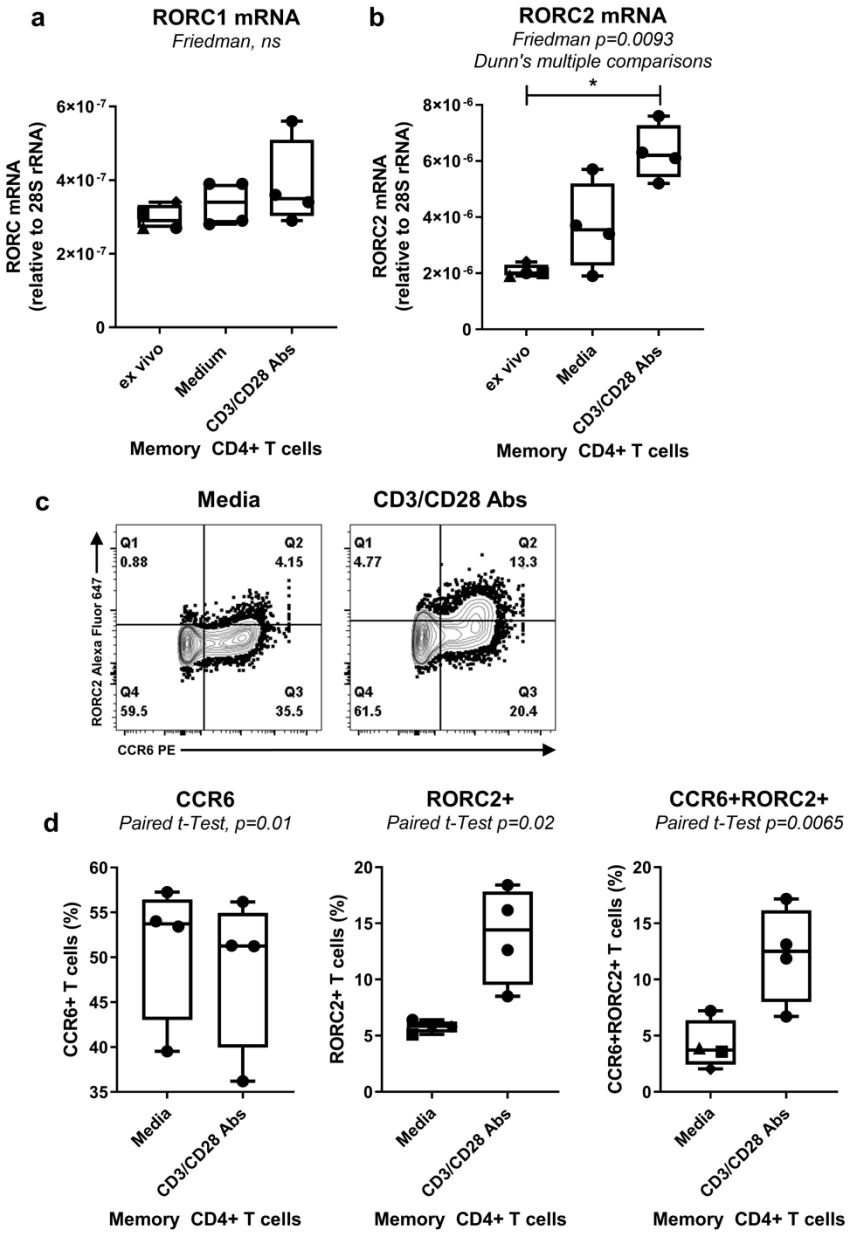


Supporting information Figure 6. Effect of RORC2 RNA interference on RORC2 and IL-17A expression. Memory $CD4^+$ T cells from n=4 HIV- donors were activated via CD3/CD28 for 3 days and nucleofected with Dharmacon On target smart siRNA pools specific for RORC2 or a non-targeting (NT) siRNA using the Amaxa technology. RORC2 mRNA expression was quantified by nested real-time PCR (upper panel), while IL-17A production was measured by ELISA (bottom panel; relative production *versus* the NT siRNA control)), at day 3 post-infection.

```
IIIB              CAGGAGAAAGAGACTGGCATTTGGGTCAGGGAGTCTCCATAGAATGGAGGAAAAAGAGAT 4866
NL4-3             CAGGAGAAAGAGACTGGCATTTGGGTCAGGGAGTCTCCATAGAATGGAGGAAAAAGAGAT 5320
C.96BW06.H51      CAGGAGAAAGAGAGTGGCATTTGGGTCATGGAGTCTCCATAGAATGGAGATTGAGAAAAT 5310
RORC              ------------------AWNTAGGTCA------------------------------- 10
                                     **********
```

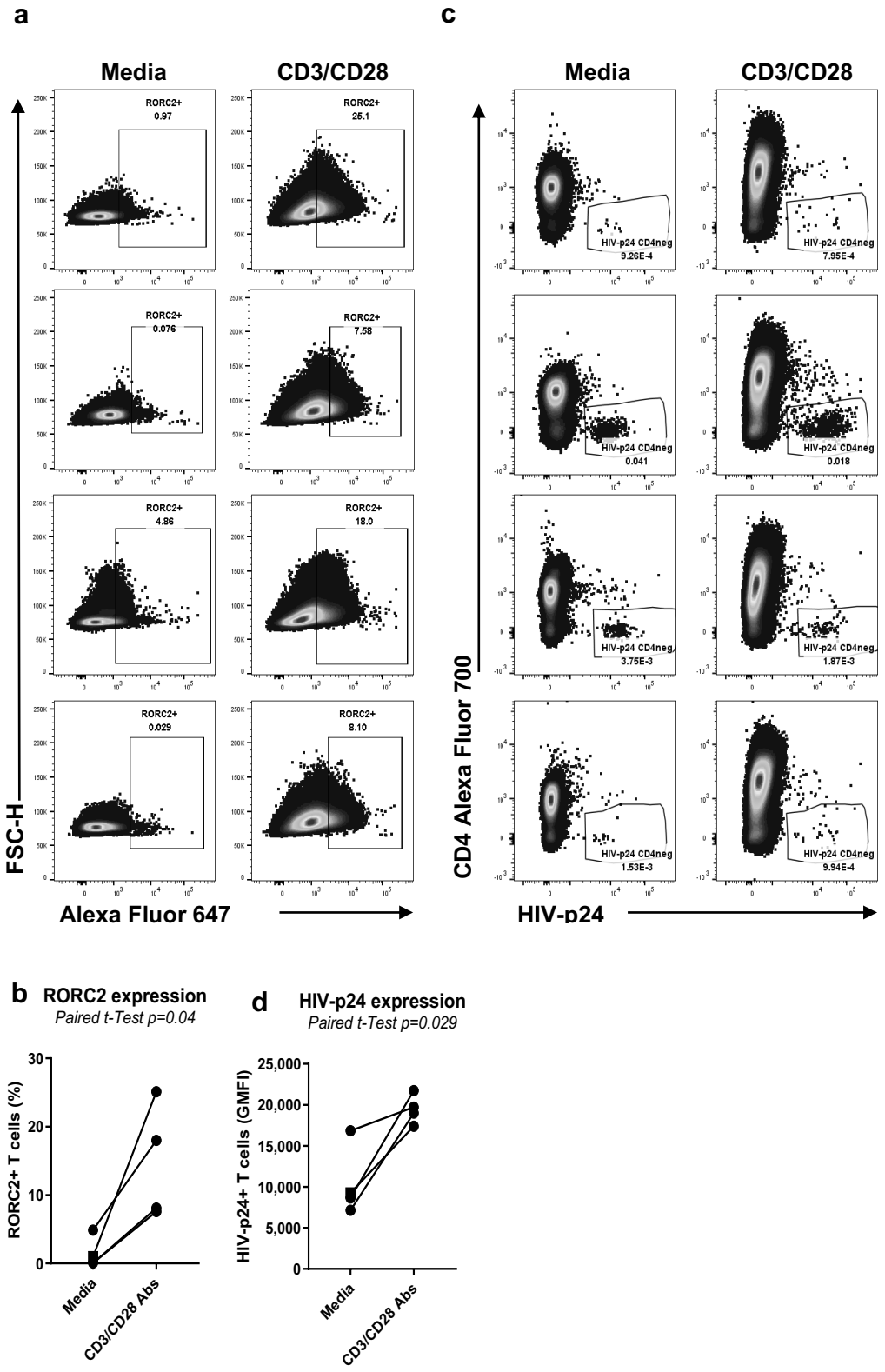
RORC2 binding motif 

Supporting information Figure 7. RORC2 DNA consensus sequence in HIV-1 Pol. The consensus sequence (Ciofani et al. 2012, Xiao et al. 2014) was aligned using ClustalW2 to the proviral genome sequence of HIV-1 IIIB, NL4.3 and subtype C 96BW06.H51.

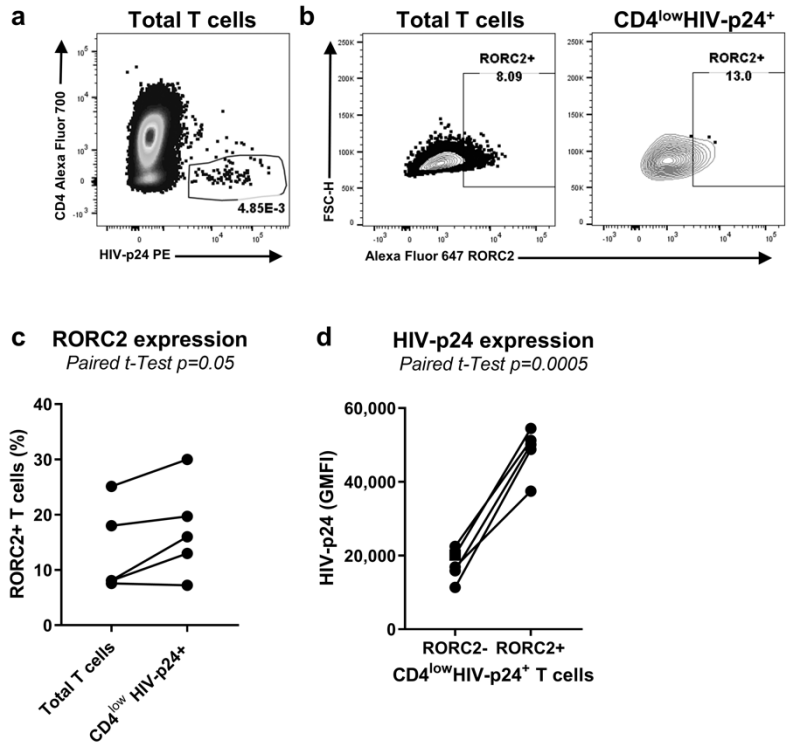


Supporting information Figure 8. TCR triggering promotes RORC2 expression in primary CD4⁺ T cells without interfering with CCR6 expression. Memory CD4⁺ T cells isolated from n=4 HIV-uninfected individuals were stimulated with CD3/CD28 Abs for 5 hours to evaluate RORC1 and RORC2 mRNA expression by real-time RT-PCR and for 24 hours to evaluate RORC2 and CCR6 expression by flow cytometry. Shown are the statistical analysis of RORC1 (a) and RORC2 mRNA (b) expression *ex vivo*, and in CD3/CD28-activated and non-activated (medium) T cells. Gating strategyand frequency of cells expressing RORC2 and/or CCR6 in one representative individual

(c) and statistical analysis of the frequency of CCR6+, RORC2+ and CCR6+RORC2+ cells in n=4 individuals (d).


a
Media
CD3/CD28
RORC2+
0.97
RORC2+
25.1
RORC2+
0.076
RORC2+
7.58
RORC2+
4.86
RORC2+
18.0
RORC2+
0.029
RORC2+
8.10
FSC-H
Alexa Fluor 647
c
Media
CD3/CD28
HIV-p24 CD4neg
9.26E-4
HIV-p24 CD4neg
7.95E-4
HIV-p24 CD4neg
0.041
HIV-p24 CD4neg
0.018
HIV-p24 CD4neg
3.75E-3
HIV-p24 CD4neg
1.87E-3
HIV-p24 CD4neg
1.53E-3
HIV-p24 CD4neg
9.94E-4
CD4 Alexa Fluor 700
HIV-p24
b RORC2 expression
Paired t-Test p=0.04
RORC2+ T cells (%)
30
20
10
0
Media
CD3/CD28 Abs
d HIV-p24 expression
Paired t-Test p=0.029
HIV-p24+ T cells (GMFI)
25,000
20,000
15,000
10,000
5,000
0
Media
CD3/CD28 Abs

Supporting information Figure 9. Inducible expression of RORC2 and HIV-p24 after TCR triggering. Memory CD4+ T cells isolated from n=4 ART- PLWH individuals were stimulated or not with CD3/CD28 Abs for 3 days. Intracellular expression of RORC2 and HIV-p24 by flow cytometry upon staining with fluorochrome-conjugated CD3, CD4, RORC2 and HIV-p24 Abs. **(a)** Contour plots for RORC2 expression on cells stimulated (right panels) or not (left panel) with CD3/ CD28 Abs for 3 days. **(b)** Statistical analysis of the frequency of RORC2+ T cells in n=4 individuals. **(c)** Contour plots for $CD4^{low}$HIV-$p24^{+}$ T cells after stimulation with (right panels) or without (left panel) CD3/ CD28 Abs for 3 days. **(d)** The geometric meanfluorescence intensity (GMFI) of HIV-p24 expression in within $CD4^{low}$HIV-$p24^{+}$ T cells. Paired t-Test values are indicated on the graphs.



Supporting information Figure 10. High RORC2 expression in memory CD4$^+$ T cells of ART- PLWH carrying translationally-competent HIV reservoirs. Memory CD4+ T cells isolated from n=5ART-PLWH individuals were stimulated with CD3/CD28 Abs for 3 days in the presence of antiretroviral drugs (raltegravir 0.2 µM and BMS806 1 µM) to prevent HIV cell-to-cell transmission *in vitro*. Intracellular expression of HIV-p24 and RORC2 was quantified by flow cytometry after staining with fluorochrome-conjugated CD3, CD4, RORC2 and HIV-p24 Abs. Contour plots for CD4 and HIV-p24 expression, with CD4^{low}HIV-p24$^+$ T cells identified as productively infected cells (a). RORC2 expression in total T cells (CD3+) and CD4^{low}HIV-p24$^+$ T cells in one representative individual (b). Statistical analysis of the frequency of RORC2$^+$ T cells n=5 individuals (c) and the geometric meanfluorescence intensity (GMFI) of HIV-p24 expression in RORC2- and RORC2+ within CD4^{low}HIV-p24$^+$ T cells (d). Paired t-Test values are indicated on the graphs.

SUPPLEMENTARY MATERIALS AND METHODS

Synthesis of GSK GSK2691805A

1H NMR spectral data were recorded on a Bruker 400 NMR spectrometer operating at 400 MHz. CDCl3 is deuterochloroform, DMSO-d6 is hexadeuterodimethyl sulfoxide. Chemical shifts are given in parts per million downfield from the NMR solvent. Abbreviations for NMR data are as follows: s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet, dd = doublet of doublets, dt = doublet of triplets, app = apparent, br = broad. Step 1: To a solution of ethyl 2-(4-ethylsulfanylphenyl) acetate (1g, 4.7mmol) in dichloromethane (50mL) at 0oC was added 3-chloroperbenzoic acid (2.4g, 14.0mmol) and the reaction was stirred for 16 h at room temperature. The reaction was then quenched with saturated aqueous sodium carbonate solution (50mL) and extracted into dichloromethane (2 x 30mL). The organics were separated, dried (MgSO4) and reduced in vacuo. Purification on the Biotage gave ethyl 2-(4-ethylsulfonylphenyl) acetate (980mg, 81% yield) as a gum. 1H NMR (CDCl3): δH 1.25-1.30 (m, 6H), 3.11 (q, 2H), 3.71 (s, 2H), 4.18 (q, 2H), 7.49 (d, 2H) and 7.86 (d, 2H).

Step 2: To a solution of ethyl 2-(4-ethylsulfonylphenyl) acetate (900mg, 3.5mmol) in ethanol (10mL) was added a solution of sodium hydroxide (562mg, 14mmol) in water (10mL) and the reaction stirred at room temperature for 16h. The ethanol was then reduced in vacuo and the remaining aqueous solution was extracted with dichloromethane (2 x 30mL) and then acidified with 2M aqueous HCl (to pH 1). This solution was then extracted with ethyl acetate (2 x 30mL) and the combined organics were dried (MgSO4) and reduced in vacuo to give 2-(4-ethylsulfonylphenyl) acetic acid (710mg, 88% yield). 1H NMR (CDCl3): δH 1.28 (t, 3H), 3.12 (q, 2H), 3.77 (s, 2H), 7.51 (s, 2H) and 7.88 (s, 2H).

Step 3: To a solution of 2-(4-ethylsulfonylphenyl)acetic acid (700mg, 3.1mmol) in dichloromethane (20mL) was added N-(3-dimethylaminopropyl)-N-ethylcarbodiimide hydrochloride (705mg, 3.7mmol), 1-hydroxybenzotriazole hydrate (497mg, 3.7mmol) and 4-bromo-3,5-dichloroaniline (733mg, 3.1mmol) and the reaction was stirred at room temperature for 16h. The solution was then diluted with water (20mL) and extracted into dichloromethane (2 x 20mL). The organics were washed with 2M aqueous HCl solution (30mL), then saturated aqueous NaHCO3 solution (30mL) and brine (30mL), dried (MgSO4), reduced in vacuo and

purified on the Biotage Isolera to give N-(4-bromo-3,5-dichloro-phenyl)-2-(4-ethylsulfonylphenyl)acetamide (520mg, 37% yield) as a light brown solid. 1H NMR (DMSO): δH 1.10 (t, 3H), 3.27 (q, 2H), 3.84 (s, 2H), 7.60 (d, 2H), 7.84-7.86 (m, 4H) and 10.64 (s, 1H). Step 4: A mixture of N-(4-bromo-3,5-dichloro-phenyl)-2-(4-ethylsulfonylphenyl)acetamide (100mg, 0.22mmol) , 2-(trifluoromethoxy)benzeneboronic acid (91mg, 0.4mmol), tetrakis(triphenylphosphine)palladium(0) (26mg, 0.02mmol) and potassium carbonate (61mg, 0.44mmol) in DMF (2mL) were reacted in the microwave at 100oC for 20min. After cooling to room temperature, the mixture was diluted with ethyl acetate (10mL) and washed with water (10mL). The organics were separated, dried, reduced in vacuo and purified on the Biotage Isolera to give N-[3,5-dichloro-4-[2-(trifluoromethoxy)phenyl]phenyl]-2-(4-ethylsulfonylphenyl)acetamide GSK2691805A (25mg, 21% yield) as an off-white solid. 1H NMR (CDCl3): δH 1.32 (t, 3H), 3.15 (q, 2H), 3.84 (s, 2H), 7.24-7.27 (m, 1H), 7.36-7.42 (m, 3H), 7.46-7.50 (m, 1H), 7.55 (d, 2H), 7.66 (s, 2H) and 7.91 (d, 2H).

Homogenous Time Resolved Fluorescence (HTRF) RORC2 Ligand Binding Assay

The Homogenous Time Resolved Fluorescence (HTRF) RORC2 Ligand Binding assay measures the interaction of co-factor SRC1 peptide with a purified bacterial-expressed RORγ ligand-binding domain (LBD). This assay is based on the knowledge that nuclear hormone receptors interact with cofactors in a ligand dependent manner. RORγ has a basal level of interaction with the co-activator SRC1 in the absence of ligand, thus it is possible to find ligands that enhance or inhibit the RORγ/SRC1 peptide interaction. The sites of interaction have been mapped to LXXLL Leucine Charge Domain 2 motifs in the co-factor sequence, and to the AF2 domain of the nuclear receptor. Short peptide sequences containing the LXXLL motif mimic the behaviour of full-length co-factors. A biotinylated SRC1 peptide residues 676-700 (CPSSHSSLTERHKILHRLLQEGSPS-CONH2) was used as an inhibitor (i.e. 'cold peptide') to compete-off the biotinylated peptide from the RORγ LBD. The biotinylated peptide can be displaced by the unbiotinylated form. For this assay, an equal volume of biotinylated SRC1 peptide/Europium-labeled streptavidin (Perkin Elmer) was added to biotynlated RORγ /APC-labeled strepavidin (Perkin Elmer), each in 10 mM DL-dithiothreotol (DTT, JT Baker) with 400 nM D-biotin (Pierce), to give a final solution of 20 nM biotinylated-SRC1 peptide, 1 nM Europium-streptavidin, 20 nM biotinylated-RORγ, 10 nM APC-

streptavidin in 10 mM DTT with 400 nM biotin. After a 5 minute incubation at room temperature, 25 µL of the peptide/RORγ solution was added to 384-well assay plates containing 1 µL of titrations of compounds in 100% DMSO. Plates were incubated for one hour and read on ViewLux ultra HTS Microplate Imager (Perkin Elmer) in Lance mode for EU/APC. For dose response curves the APC counts (@671) were divided by the europium (@618) counts to compensate for quenching effects and account for well to well variation due to liquid handling errors. Data were normalized using the following equation: (unknown- ave basal)/ (ave basal - ave background) * 100 = % activation or inhibition. A response of 0% would be inactive, >0% indicates enhancement of the protein peptide interaction and < 0% (negative values) indicates inhibition of the protein peptide interaction. Results were analyzed with ActivityBase (IDBS) using a 4-parameter fit equation.

Jurkat KD RORC2

To generate 1G5 Jurkat indicator cells with a stable KD of RORC2, four shRNA hairpins were obtained from Sigma mission catalogue 3-1245h1C1; 4-1036h21C1; 4-363h21C1 and 3-978h1C1 and were cloned into lentiviral vector pLKO.1-puro (Addgene, catalogue #8543). Lentiviral vectors were produced in 293T cells by Fugene transfection and the supernatant was used to infect Jurkat cells. Forty-eight hours post-infection, cells were selected with 5 µM puromycin for 4 days.

Jurkat RORC2 IL-17F Promoter Luciferase Reporter Assay

The Jurkat RORC2 IL-17F Promoter Luciferase Reporter Assay measures RORC2-specific (human IL-17 conserved non-coding sequence (CNS)) promoter driven luciferase-reporter activity to indirectly assess RORC2 activity. Both RORC gene and reporter construct were sequentially transfected into Jurkat cells and stably integrated. The Jurkat double stable cell line expressing the human RORC2 and IL-17 luciferase-reporter construct was adjusted to a concentration of 0.5 x 106 cells/mL in medium (RPMI-1640, 10% FBS, 2 mM Glutamax) and mixed with 166 ng/mL of anti-CD3 antibody (GlaxoSmithKline). 50 µL of cells/antibody was added to each well of 384-well plates containing compound titrations in 100% DMSO and plates were incubated at 37 °C for 18

h. 20 µL of Steady-Glo Luciferase Assay reagent (Promega) was added to each well and plates were incubated for 30 minutes at room temperature. Luminescence was measured on a ViewLux ultra HTS Microplate Imager (Perkin Elmer) and data were analyzed with ActivityBase (IDBS) fitting a 4-parameter equation.

CFSE-based proliferation assay

A modified short-term VOA was performed,as described in Figure 7A. Briefly, memory CD4+ T cells of ART+PLWH were loaded with Carboxy Fluoroscein Succinimidyl Ester (CFSE) (0.5 µM CFSE; Sigma) for 8 minutes at room temperature. The CFSE reaction was then rapidly stopped with human serum for 1-2 minutes. Cells were subsequently washed once with PBS and once with RPMI1640 before being cultured at 1×106 cells/well in 1 ml of proliferation media (RPMI, 10% human serum, 1% HEPES, 1% L-glutamine, 1% Penicillin/Streptomycin). The VOA was stopped at day 5 post-activation at which point cells were harvested, stained with Live/Dead Fixable Aqua Dead Cell Stain Kit (Vivid, Life Technologies, Burlington, Ontario, CA) to exclude dead cells and analysed with the BD LSRFortessa and FlowJo version 10 (Tree Star, Inc., Ashland Oregon, USA). Division and proliferation index were calculated using the proliferation tool of Flow Jo. The Division index represents the average number of cell divisions that a cell in the original population has undergone and the Proliferation index represents the total number of divisions divided by the number of cells of cells that went into division.

Western blot

2x106 cells were centrifugated (100g, 5minutes), washed with ice cold phosphate buffered saline (PBS) once and lysed with 150µl of SDS sample buffer (140mM Tris pH8, 2% SDS, 50 mM DTT, 0.5M sucrose, 2mM MgCl2, Bromophenol-blue). Lysates were incubated at 100°C for 5 minutes. Samples were loaded to a NuPAGETM Bis-Tris Protein Gel. Western blotting was performed following wet electrophoretic transfer (1h, 100mV) to a PVDF membrane. After probing with the primary antibody at 40C overnight, HRP conjugated secondary antibodies were used for detection by luminescence. Primary antibodies were: rabbit polyclonal anti-RORC/RORC2 cat. LS-

C40832, rabbit polyclonal anti-RORC/RORC2 L-C116877 both from LSBio (Seattle, WA) and mouse mAb anti-c-Myc (ThermoFisher, 9E10).

qPCR

For Taqman qPCR, approximately 1x106 Jurkat cells were washed twice in PBS and total DNA was extracted with the Qiamp® DNA Minikit (Qiagen, Manchester, UK). Quantitative PCR reactions were carried out as previously described (1) using 0.3pmol each primer and 0.15 pmol of the probe in 25 uL volume containing 100-300ng total DNA using an ABI Prism® 7000 Sequence Detection System (SDS). For amplification of (-) DNA strand (GFP), primers used were forward: CAACAGCCACAACGTCTATATCAT, reverse ATGTTGTGGCGG ATCTTGAAG and probe 5'-FAM-CCG ACA AGC AGA AGA ACG GCA TCA A-3'TAMRA. For amplification of 2LTR circular DNA, the same conditions were used with primers 2LTRqPCR-F: 5'-AACTAGAGATCCCTCAGACCCTTTT-3' and 2LTRqPCR-RC: 5'-CTTGTCTTCGTTGGGAGTGAATT-3' and 2LTR probe 5'-FAM-CTAGAGTTTTCCACACTGAC-0-TAMRA-3'. Standards were prepared by PCR amplification of DNA from acutely infected cells with primers 2LTRF 5'-GCCTCAATAAAGCTTGCCTGG-3' and 2LTRRC 5'-TCCCAGGCTCAGATCTGGTCTAAC-3'. The amplification product was cloned into TOPO vector, amplified and confirmed by sequencing (1). Alu-LTR Taqman qPCR was carried out as previously described (1) using primers ALU-forward, AAC TAG GGA ACC CAC TGC TTA AG and LTR1-reverse, TGC TGG GAT TAC AGG CGT GAG (for first round amplification) and ALU-forward AAC TAG GGA ACC CAC TGC TTA AG, LTR2-reverse, TGC TAG AGA TTT TCC ACA CTG ACT, ALU-probe, FAMRA – TAG TGT GTG CCC GTC TGT TGT GTG AC – TAM (for second round Taqman qPCR). Integrated HIV-DNA in human primary memory CD4+ T-cells was quantified by nested real-time PCR using specific primers and amplification conditions as previously described (2, 3).

RT-qPCR

Jurkat cells were grown in RPMI + 10% FCS. 1.5 x 106 cells/ml were seeded onto 12-well plates. Cells were infected with HIV-1 LAIGFP and 24 hours cells an aliquot was analyzed by FACS and another aliquot was treated with GSK2837269A (5 μM) or DMSO. After 24 hours, cells were

collected, and RNA was isolated using RNAesy mini kit (Qiagen) and reverse transcribed using SuperScriptTM III Reverse Transcriptase (Invitrogen) after digestion with RNAse-free DNAse I (Promega). Quantitative TaqMan qPCR was performed using GFP forward primer (5'-CAA CAG CCA CAA CGT CTA TAT CAT-3'), GFP reverse primer (5'-ATG TTG TGG CGG ATC TTG AAG-3') and GFP probe (5'-FAM-CCG ACAAGC AGA AGA ACG GCA TCA A- TAM-3') in an Eppendorf MasterCycler Realplex. HIV-GFP mRNA expression was normalized to that of hu28S rRNA expression using hu28S rRNA forward primer (5'-TTG AAA ATC CGG GGG AGA G-3') and hu28S rRNA reverse primer (5'-ACA TTG TTC CAA CAT GCC AG-3'). For GFP mRNA, the TaqMan PCR assay was set in a final volume of 20µl containing 250ng cDNA, 0.5µM of each primer, 0.15µM of GFP probe and TaqMan universal master mix II (2X) (Qiagen). For hu28S rRNA, the SYBR Green qPCR reaction was carried out in a final volume of 20µl using 250 ng cDNA, 0.4 µM hu28S rRNA forward and reverse primers and PowerUpTM SYBRTM Green Master Mix. For primary memory CD4+ T-cells, Jurkat, ACH2, TZM-bl and HT-29 cell lines shown in Supporting information Figure 1, total RNA was extracted using the RNeasy kit (Qiagen) and quantified by Pearl nanophotometer (Implen, Germany). RORC1 and RORC2 gene expression was evaluated by One step SYBR green real-rime RT-PCR (Qiagen) using a Light-Cycler 480 II as follows; reverse transcription at 50°C for 30 min, 15 min at 95°C and then 45 cycles each at 94°C for 10 s, 61°C for 10 s, and 72°C for 10 s. The sequence of primers used for RORC isoforms were: RORC2 rRNA forward primer, 5'--CTGCTGAGAAGGACAGGGAG-3'; RORC1 rRNA forward primer, 5'-CACAGAGACAGCACCGAGC-3'; RORC2/RORC1 rRNA reverse primer (same for both isoforms) 5'-AGTTCTGCTGACGGGTGC-3'. The relative expression of RORC was normalized relative to the internal control 28S. The sequence of primers used for 28S were 28s rRNA forward primer 5′ -CGAGATTCCTGTCCCCACTA-3′ and 28s rRNA reverse primer, 5′ GGGGCCACCTCCTTATTCTA-3′. Primers were obtained from IDT. Each reaction was performed in triplicates. After real time amplification, melting curve analysis was used to determine the uniformity of the thermal dissociation profile for each amplification product.

RNA interference in primary CD4+ T cells

RNA interference was performed as previously described by our group (4). Briefly, PBMCs were thawed and rested overnight at 37 °C. Memory CD4+ T-cells were isolated by negative selection using magnetic beads (Miltenyi Biotec), as described previously (2, 3). Cells were stimulated by CD3/CD28 Abs for 2 days and nuclofected with 100 µM RORC or non-targeting (NT1) siRNA (ON-TARGETplus SMART pool, Dharmacon) using the Amaxa Human T cell Nucleofector Kit (Amaxa, Lonza), according to the manufacturer's protocol. Cells were suspended in the NF solution (100 µl/2×106 cells) and nucleofected using the Amaxa Nucleofector II Device and the human activated T-cell protocol (T-20). Cells (2×106) were transferred into 48-well plates containing 1 ml of RPMI1640 (10 % FBS, 5 ng/ml IL-2, w/o antibiotics) and cultured for another 24 hours at 37 °C before HIV exposure.

ELISA

HIV-p24 levels in cell culture supernatant were quantified using a homemade sandwich ELISA, as described previously (3, 5). Briefly, virions in cell supernatants were lysed using a homemade buffer solution (PBS 1X, Tween 20 0.05%, Triton X-100 2.5%, Trypan Blue 1% and Thimerosal 0.02% in deionized water) for 1h at 37°C. Levels of IL-17A and IFN-γ were measured in the cell culture supernatant, according to the manufacturer's protocols (Thermofisher).

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Chapter 5

Manuscript #3: A BLOOD IMMUNOLOGICAL SIGNATURE OF SUBCLINICAL CORONARY ARTERY ATHEROSCLEROSIS IN PEOPLE LIVING WITH HIV-1 RECEIVING ANTIRETROVIRAL THERAPY

Manuscript submitted to The Lancet EBioMed journal

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Authors' contributions

TWS contributed to the study design, performed most of the experiments, analyzed results, prepared figures, and wrote the manuscript.

YZ contributed to the study design and performed experiments.

AG and NFDR contributed to experiments. MEF was instrumental in the study design and provided protocols.

AF performed all multivariate statistical analyses. JPR contributed to participant recruitment.

CCL performed CT-SCAN angiographies and interpreted the results in all participants.

ALL, MD, and CLT contributed to patient recruitment, the establishment of the biobank of plasma and PBMC, and provided access and valuable guidance in the analysis of clinical information and statistical analysis.

PA designed the study, analyzed results, contributed to figure preparation, and wrote the manuscript. All authors reviewed and approved the manuscript.

A Blood Immunological Signature of Subclinical Coronary Artery Atherosclerosis in People Living with HIV-1 Receiving Antiretroviral Therapy

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ABSTRACT

Cardiovascular disease (CVD) remains an important co-morbidity in people living with HIV-1 (PLH) receiving antiretroviral therapy (ART). Our previous studies performed on ART-treated PLH (HIV+) versus uninfected controls (HIV-) included in the Canadian HIV/Aging Cohort Study (CHACS) (>40 years-old; Framingham Score (FS) >5%), revealed a 2-3-fold increase in non-calcified coronary artery atherosclerosis (CAA) plaque burden, measured by Computed tomography angiography scan (CTAScan) as total (TPV) and low attenuated plaque volume (LAPV). Here, we sought to identify biomarkers of subclinical CAA in HIV+ individuals. Markers of intestinal damage (sCD14, LBP, FABP2); cell trafficking/inflammation (CCL20, CX3CL1, MIF, CCL25); subsets of Th17-polarized and regulatory (Tregs) CD4+ T-cells, monocytes, and myeloid/plasmacytoid dendritic cells, were studied in relationship with TPV/LAPV and traditional CVD markers (D-dimer, fibrinogen, LDL, HDL, triglycerides). The TPV detection/values were higher in TPV+HIV+ (32/61; median TPV: 608.7 mm3) compared to TPV+HIV- (7/21; median TPV: 192.8 mm3), and coincided with higher plasma sCD14, FABP2, CCL20, MIF, CX3CL1 and triglyceride levels, increased CD4/CD8 ratios and Treg frequencies, and lower CD4 counts and Th17/Treg ratios. Among HIV+, TPV+ versus TPV- exhibited decreased Th17 frequencies, reduced Th17/Treg ratios, and higher non-classical CCR9lowHLADRhigh monocyte frequencies, together with longer time on ART and increased plasma fibrinogen. Finally, Th17/Treg ratios and non-classical CCR9lowHLADRhigh monocyte frequencies remained associated with TPV/LAPV after adjusting for FS, age, smoking and/or LDL in a logistic regression model. These findings point to Th17 cell paucity and the expansion of non-classical monocytes as novel putative markers of subclinical CAA that may fuel the CVD risk in ART-treated PLH.

Keywords (4-6): HIV-1, antiretroviral therapy (ART), cardiovascular disease (CVD), Th17/Treg cells, non-classical monocytes, myeloid/plasmacytoid dendritic cells

INTRODUCTION

AIDS-related mortality in people living with HIV (PLH) has considerably diminished after the implementation of antiretroviral therapy (ART). Nevertheless, non-AIDS co-morbidities, such cardiovascular disease (CVD), remain highly prevalent in ART-treated PLH.[1-6] A modeling study performed in the Netherlands estimated an increase in the life expectancy of ART-treated PLH from 43.9 years in 2010 to 56.6 years in 2030, and predicted that 78% of PLH anticipated will be diagnosed with CVD.[7, 8] Indeed, PLH tend to present clinical signs of CVD approximately 10 years earlier compared to general population.[9] In addition to traditional CVD risk factors (e.g., dyslipidemia, insulin resistance, diabetes, hypertension, smoking, and/or sedentary life-style),[10] HIV-specific mechanism are linked to HIV infection per se and metabolic alterations due to long-term administration of ART.[8, 11-13] In ART-treated PLH, the persistence of HIV reservoirs is associated with impaired intestinal mucosal barrier functions, microbial translocation, and immune dysfunction, which together with CMV and other co-infections contribute to an increased CVD risk in this group.[14, 15] Thus, CVD represents a major non-AIDS co-morbidity in ART-treated PLH and novel intelligently designed therapeutic interventions are needed to reduce this risk.

Cells of the innate and adaptive immune system are key players in CVD pathogenesis.[10, 16, 17] T lymphocytes with yet unclear antigenic specificities infiltrate atherosclerotic plaques and are recruited into the heart via mechanisms involving the hepatocyte growth factor receptor c-Met, CCR4, CXCR3, and CCR5.[18] There is evidence that ART-treated PLH with carotid atherosclerotic plaque, increased carotid intima media thickness, or arterial stiffness presented with increased circulating CD8+ T-cell activation (CD38+HLA-DR).[19, 20] Also, various CD4+ T-cell subsets are documented to participate to CVD pathogenesis.[21] In uninfected individuals, T helper 1 (Th1) cells have pro-atherogenic features, whereas regulatory T cells (Tregs) can act as both anti- and pro-atherogenic.[22] Th17 cells appear to play dual roles in the development of atherosclerosis, either protective or pathogenic.[21] In HIV-uninfected individuals with unstable angina or acute myocardial infarction, the frequency of Th17 cells was higher compared to those in participants with stable angina or healthy individuals.[23] The role of CD4+ T-cells and their associated cytokines

in atherosclerosis or coronary artery disease in ART-treated PLH remains poorly documented, with some recent studies documenting the expansion Tregs [24] and surprisingly Th17 cells.[25]

In addition to T cells, innate immune cells such as monocyte activation and dendritic cell (DC) also contribute to atherosclerotic plaque formation/rupture.[26, 27] Arterial inflammation is higher in PLH than in uninfected individuals and correlates with higher circulating levels of the pro-inflammatory cytokine IL-6 and activated monocytes.[28, 29] Mice deficient in CCR2 and CX3CR1, two chemokine receptors important for monocyte migration, exhibit decreased severity of atherosclerotic plaque thus, pointing to the deleterious role of monocytes in CVD.[30] In HIV-individuals, an elevated frequency of circulating non-classical (CD14dimCD16++) monocytes was associated with an amplified carotid intima-media thickness (IMT) over 10 years in a prospective cohort study.[31] Similarly, in PLH, the intermediate (CD14+CD16+) monocyte counts were associated to subclinical atherosclerosis[32] and the expression of CX3CR1 on CD16+ monocytes independently predicted carotid artery thickness.[33] Of note, a decreased expression of CXCR4 was observed on non-classical monocytes in women with subclinical atherosclerosis.[34] Moreover, ex vivo experiments with monocytes of virologically suppressed PLH showed an increased potential to form atherosclerosis promoting foam cells compared to uninfected individuals.[35] Furthermore, atherosclerosis plaque burden was associated with increased levels of the monocyte chemoattractant protein-1 (MCP-1/CCL2) in ART-treated PLH.[36, 37]

Although it is documented that plasmacytoid DC (pDC) infiltrate atherosclerotic lesions,[38, 39] their contribution to atherosclerosis remains controversial. Some mice studies have shown that pDCs promote atherogenesis through their capacity to produce interferon (IFN)-γ while others support their role in atheroprotection.[40-41] pDCs contribute to peripheral and central tolerance role by the induction of Tregs; or, the anti-atherogenic role of pDCs was associated with indoleamine 2,3-dioxygenase 1 (IDO1)-dependent induction of aortic Treg cells.[42] During HIV infection, there is a decline in pDC counts and alterations in their functions, which are not restored with ART.[43, 44] Whether interventions to restore pDC may limit the CVD risk in ART-treated PLH requires investigations.

The objective of this study was to identify an immunological signature associated with subclinical coronary artery atherosclerosis (CAA) in ART-treated PLH. To reach this objective, we had access to plasma and PBMC samples collected at baseline from ART-treated PLH (HIV+; n=61) and HIV-uninfected participants (HIV-; n=21) included in the cardiovascular imaging sub-study of the longitudinal Canadian HIV/Aging Cohort Study (CHACS). Our results reveal alterations in Th17/Treg ratios and non-classical CCR9lowHLA-DRhigh monocyte frequencies, together with plasma fibrinogen levels, that correlated with the magnitude of subclinical CAA in ART-treated PLH, independently of other traditional risk factors. While the predictive CVD risk value of the identified markers remains to be validated in larger cohorts and longitudinal studies, our findings emphasize the need for novel therapies aimed at restoring Th17, Treg, and monocyte counts/functions in ART-treated PLH.

METHODS

Ethics statement

This study was approved by the Institutional Review Boards (IRB) of the Centre de recherche du Centre hospitalier de l'Université de Montréal (CRCHUM) (Ethical approval #CE.11.063) and the IRBs of all participating sites. This study was conducted with the ethical principles for medical research involving human subjects established by the Declaration of Helsinki in 1975. All study participants provided written informed consent for the collection of blood and the use of plasma and peripheral blood mononucleated cells (PBMC) for the current research investigation.

Study Participants

The design of the Canadian HIV and Aging Cohort Study (CHACS) has been previously reported.[45] The first eligible HIV- (n=21) and HIV+ (n=61) participants recruited in the CHACS were included in this sub-study. Briefly, inclusion criteria for the cardiovascular imaging sub-study of the CHACS cohort were subjects older than 40 years old, without clinical manifestations or diagnosis of CAA at recruitment and a 10 year risk of cardiovascular disease according to the Framingham risk score ranging from 5-20% (Table 1). Exclusion criteria were renal impairment and hypersensitivity to contrast agents. Plasma and PBMC samples collected at baseline from ART-treated PLH (HIV+) and uninfected (HIV-) participants were available for this study. PBMCs were thawed, washed, and stained for flow cytometry analysis.

Computed Tomography Angiography Scan

All study participants underwent Computed tomography angiography scan (CTA-Scan) to determine total plaque volume (TPV) and low attenuated plaque volume (LAPV, high-risk atherosclerotic plaque rupture), as we previously described.[5, 46, 47] Briefly, TPV represents the total volume (calculated from 3D reconstructions) of all coronary artery plaque present in an individual's coronary arteries. Coronary arteries that are normal (free of CVD) will have a TPV and LAPV of zero, while any volume greater than zero indicates the presence of CVD. For this sub-study, HIV+ and HIV- participants were stratified based on TPV and LAPV values of zero or greater than zero, indicative of the absence or the presence of CVD, respectively.

ELISA

Markers of microbial translocation (LPS binding protein, LBP), mucosal damage (Intestinal-type Fatty Acid Biding Protein, I-FABP), immune activation (sCD14), and chemokines (CCL20, CX3CL1, CCL25, MIF) were quantified in plasma by ELISA, according with the manufacturer's protocol (R&D Systems, Minneapolis, MN).

Flow Cytometry

Fluorescence-activated cell sorting (FACS) was used to identify subsets of CD3+CD4+ T-cells [Th17 (CCR6+CD26+CD161+), Tregs (CD127-CD25+Foxp3+), central memory (CM, CD45RA-CCR7+), effector memory (EM, CD45RA-CCR7-), effector memory RA+ (TEMRA, CD45RA+CCR7-), and naive (CD45RA+CCR7+) cells]; subsets of monocytes (CD3-CD4lowHLA-DR+CD1c-) [classical (CD14++CD16-), intermediate (CD14++CD16+), non-classical (CD14-CD16++), and Slan/M-DC8+]; as well as myeloid (mDC; HLA-DR+CD1c+) and plasmacytoid dendritic cells (pDC, BDCA2+CD123+); and measure their expression of chemokine receptors (CCR2, CCR6, CCR9, CX3CR1). Cells were analyzed by FACS using BD-LSRII cytometer, BD-Diva (BD Biosciences), and FlowJo version 10 (Tree Star, Inc., Ashland, Oregon, USA) software. The antibodies used in this study are presented in Supplemental Table 1. All Abs were titrated for an optimal noise/ratio. The gating strategy used to identify the different immune cell subsets is described in Supplemental Figures 1, 5 and 8. A combination of gates using the Boolean tool gating from Flow Jo was used to determine the expression of chemokines.

Statistical Analysis

The Shapiro-Wilk test was used to determine the normal distribution of the continuous variables. Variables with normal distribution were presented as means with standard deviation, and variables with non-normal distribution were presented as median with interquartile range (IQR). Comparison of two groups for the same variable with normal distribution was analyzed using t-Test, and non-normal distributed variables were analyzed with Mann Whitney. Categorical variables were represented as proportions, and comparisons for the same variable between groups were analyzed using Fisher's Exact Test. A logistic regression model was used to determine the association between covariates and the presence or absence of CAA plaque (TPV and LAPV). The following covariates were included in a basic multivariate model: age, LDL and

smoking. In a second analysis, triglycerides, the use of statins and smoking were included. P-values lower or equal to 0.05 were considered statistically significant.

RESULTS

Laboratory and imaging markers of subclinical atherosclerosis in ART-treated PLH

In previous studies performed by our group, CTA-Scan was used as a noninvasive tool to visualize and quantify CAA plaque as total (TPV) and low attenuated plaque volume (LAPV) in ART-treated PLH (HIV+) and uninfected controls (HIV-) included in our CHACS cohort.[5, 46, 47] In the current cardiovascular imaging sub-study, a total of n=61 HIV+ and n=21 HIV- were included, with demographic and clinical characteristics depicted in Table 1. Briefly, differences between HIV+ and HIV- groups in terms of age (mean 55.38 versus 56.94 years), body mass index (BMI; median 24.43 versus 25.72 Kg/m2), and Framingham score (median 10 versus 8; p=0.08) did not reach statistical significance; also, similar proportions of participants received treatment with statins (23% versus 23.8%). The HIV+ group included 61/61 males (100%), while the HIV- group included 17/21 males (81%) (Table 1). The measurement of CAA plaque volume using CT-Scan angiograms was performed in all sub-study participants, as we previously reported.[5, 46, 47] Among the HIV+ group, 39/61 (64%) participants were identified with detectable plaque (TPV+), while 22/61 (36%) participants had undetectable plaque (TPV-) measured as TPV and LAPV (arbitrary identified as 1) (Supplemental Figure 1A-B; Table 1). Similarly, the HIV- group included 13/21 (62%) with detectable plaque, while 8/21 (38%) participants had undetectable plaque (Supplemental Figure 1A-B; Table 1). Differences in the prevalence of CAA [TPV (median 108.3 versus 49.7) and LAPV values (median 34.4 versus 10.53)] between HIV+ versus HIV- groups did not reach statistical significance (p=0.32 and 0.39, respectively). However, in participants with detectable CAA (TPV>10; LAPV>1), both TPV and LAPV levels tended to be higher in HIV+ [TPV (median: 608.7; IQR: 142.6- 881.8); and LAPV (median: 164.8; IQR: 36-296)] compared to HIV- participants [TPV (median: 192.8; range: 34-2,253), and LAPV (median: 71.3; range: 5-632)] (Figure 1, Table 1, and data not shown) (5). Also, the number of participants with TPV values >100 mm3 was higher in HIV+ (32/61; 52.4%; median TPV: 608.7 mm3) compared to HIV- (7/21; 33.3%; median TPV: 192.8 mm3) (data not shown).

In terms of laboratory parameters, the HIV+ and HIV- groups expressed similar white blood counts, lymphocyte counts, and LDL levels; however, HIV+ compared to HIV- groups had

significantly lower levels of HDL (median: 1.23 versus 1.44) and higher levels of triglyceride (median: 1.73 versus 1.21) (Table 1). Since low HDL and high triglycerides are two well-documented CVD risk factors,[48] such differences may accelerate the occurrence of subclinical CVD events in ART-treated PLH, consistent with our recently reported findings.[5]

We further sought to identify clinical and laboratory markers associated with subclinical CAA imaging among HIV+ participants (Table 2). Considering the fact that TPV and LAPV values were strongly positively correlated in HIV+ and HIV- participants (Supplemental Figure 1C; $p<0.0001$, $r=0.9913$), all subsequent statistical analysis were based on the TPV values equal or higher than zero. TPV+ and TPV- HIV+ participants were similar in age (mean: 56.24 versus 53.85 years), BMI (median: 24.05 versus 25.29 Kg/m2) and the frequency of PLH with undetectable plasma viral loads (94.8 versus 86.36%). However, TPV+ compared to TPV- HIV+ participants were on ART for a longer time (mean 16.14 versus 10.69; $p=0.003$) and the time since infection tended to be superior (median: 19.6 versus 14.7; $p=0.06$) (Table 2). Also, the Framingham score (median 11 versus 8; $p=0.084$) and the number of HIV+ participants under statin treatment [(12/39; 30.8%) versus (2/22; 9.1%); $p=0.064$] tented to be superior among TPV+ compared to TPV- participants (Table 2). Regarding laboratory parameters, TPV+ and TPV- HIV+ participants showed similar white blood counts, lymphocyte counts, LDL, HDL, and triglycerides levels; however, fibrinogen levels, indicative of coagulopathy,[49] were significantly increased in the TPV+ compared to TPV- HIV+ (Table 2; $p=0.005$). Thus, subclinical signs of CVD reflected by CAA were strongly associated with a longer time on ART, as well as increased plasma fibrinogen levels.

Plasma markers in relationship with HIV-1 status and subclinical atherosclerosis

To identify systemic biomarkers associated with subclinical atherosclerosis in ART-treated PLH, we quantified plasma levels of well-established markers of gut damage (sCD14, LBP, FABP2) and systemic inflammation (CCL20/MIP-3α, MIF, CX3CL1/FKN, CCL25/TECK) in the HIV- and HIV+ groups in relationship with subclinical CVD. While higher plasma levels of sCD14, FABP2, CCL20, MIF and CX3CL1 distinguished HIV+ from HIV- participants (Supplemental Figure 2A-B), only levels of CCL20, previously associated with HIV disease progression,[50] tended to be significantly

increased in TPV+ compared to TPV- among HIV+ participants (Supplemental Figure 2C-D; p=0.0514). Thus, limited subtle differences in plasma markers distinguish subclinical CVD in ART-treated PLH.

T-cell profile alterations in relationship with HIV-1 status and subclinical atherosclerosis

To study the relationship between key players in adaptive immunity and subclinical CVD status, polychromatic flow cytometry was used to identify specific T-cells subsets in the peripheral blood of HIV+ and HIV- participants with/without CAA (Supplemental Figure 3). Th17-polarized T-cells were identified by Boolean combination of gates between CCR6, CD26 and CD161 and defined as cells with a CCR6+CD26+CD161+ phenotype.

HIV+ compared to HIV- participants showed decreased frequencies of CD4+ T-cells within CD3+ T-cells, with increased frequencies of CD8+ T-cells and lower CD4/CD8 ratios (Supplemental Figure 4A), consistent with previous literature identifying the CD4/CD8 ratio as a marker of HIV disease progression [51] and viral reservoirs.[52, 53] No differences in the latter parameters were observed among TPV+ and TPV- HIV+ participants (Supplemental Figure 4B). The frequencies of total memory (CD45RA-) and naïve (CCR7+CD45RA+) CD4+ T-cells, as well as effector memory (EM, CD45RA-CCR7-), central memory (CD45RA-CCR7+; CM), and EM expressing CD45RA (EMRA) CD4+ T-cells, did not differ between HIV+ and HIV- participants (Supplemental Figure 5A-B) neither between TPV+ and TPV- HIV+ participants (Supplemental Figure 5C-D). Of notice, HIV+ compared to HIV- participants showed a statistically significant increase in the frequency of Tregs (CD127lowCD25highFoxP3+), similar frequencies of CD4+ T-cells with a Th17 phenotype (CCR6+CD26+CD161+) within total memory, EM and CM subsets, and reduced Th17/Treg ratios (Supplemental Figure 6A-C). Among HIV+ participants, TPV+ compared to TPV- individuals exhibited similar frequencies of Tregs, but decreased frequencies of total memory, EM and CM Th17 subsets, and decreased Th17/Treg ratios among memory and EM subsets (Figure 1A-C). The same trend was not observed in HIV- individuals, although the relatively low number of HIV- participants should be considered (Supplemental Figure 7A-C). Thus, ART-treated HIV-1 infection

is associated with an expansion of Tregs and an alteration of Th17/Treg ratios, with the paucity of Th17 cells being further exacerbated in HIV+ participants with subclinical CVD.

Monocyte alterations in relationship with HIV-1 status and subclinical atherosclerosis

Changes in monocyte heterogeneity have been associated with various pathological conditions, including HIV-1 infection.[54] Thus, we investigated changes in monocyte subset frequencies and phenotypes in our cohort. The flow cytometry gating strategy used to identify monocyte subsets is depicted in Supplemental Figure 8, as previously described.[30, 55, 56] An expansion of classical (CD14++CD16-) to the detriment of intermediate (CD14++CD16+) monocytes was observed in HIV+ compared to HIV-, whereas the frequencies of non-classical (CD14-CD16++) and M-DC8+ (Slan+) monocytes were similar (Supplemental Figure 9A). Classical, intermediate, nonclassical and Slan monocytes were similar in frequency regardless of the plaque status (Supplemental Figure 9B).

The phenotype of monocyte subset was further analyzed, including the expression of the chemokine receptors CCR2 (involved in monocyte trafficking from bone marrow into peripheral tissues, [57] CX3CR1 (involved in intermediate and non-classical monocyte patrolling onto vascular beds,[58] and CCR9 (involved in cell recruitment into multiple sites including the atherosclerotic plaque.[59] Consistent with previous reports, CCR2 and CCR9 were mainly expressed on classical monocytes, while CX3CR1 was predominantly expressed on non-classical and M-DC8+ monocytes (Figure 2; Supplemental Figure 10).[60-62] The expression of CCR2 and CX3CR1 on monocyte subsets did not differ between HIV+ and HIV- (Supplemental Figure 10A-D). In contrast, the expression of CCR9 on intermediate and non-classical monocytes was diminished in HIV+ compared to HIV- (Supplemental Figure 10B-C). The highest expression of HLA-DR was observed on non-classical and Slan/M-DC8+ monocytes of HIV+ compared to HIV- (Supplemental Figure 10C-D). Further, the stratification based on TPV in HIV+ participants demonstrated similar expression of CCR2 and CX3CR1 on all monocyte subsets, a decreased expression of CCR9 on non-classical monocytes, and an increased expression of HLA-DR on non-classical and Slan/M-DC8+ monocytes from TPV+ compared to TPV- HIV+ participants (Figure 2A-D). Thus, in the cohort used for this study, no

significant alterations were observed in terms of frequency of monocyte subsets in relationship with the HIV status; however, changes were observed in the expression of HLA-DR and CCR9, with the predominance of HLA-DRhighCCR9low non-classical and HLA-DRhigh M-DC8+ monocytes in TPV+ versus TPV- HIV+ participants.

mDC and pDC alterations in relationship with HIV-1 status and subclinical atherosclerosis

DC are important immune players acting at the interface between innate and adaptive immunity. While myeloid DC (mDC) are mainly involved in antigen-presentation and priming the T cell response,[63, 64] plasmacytoid DC (pDC) are specialized in sensing viruses and producing type I IFN.[63] The flow cytometry gating strategy to identify mDC (HLA-DR+CD1c+) and pDC (BDCA2+CD123+) is depicted in Supplemental Figure 11. Briefly, mDC and pDC were gated on CD3-CD4- Live cells and the expression of CCR6 and CCR9 was also evaluated in each subset. The frequency of mDC and their expression of CCR6 or CCR9 was similar in HIV+ and HIV- participants (Supplemental Figure 12A), with no differences between TPV+ and TPV- HIV+ participants (Supplemental Figure 12B). Similar results were obtained for the frequency and phenotype of pDC (Supplemental Figure 13A-B). Thus, in this cohort, the mDC and pDC frequency and CCR6/CCR9 phenotype did not distinguish between groups with different HIV and TPV statuses.

Multivariate analysis identifies an immunological signature associated with subclinical atherosclerosis in ART-treated PLH

A logistic regression model was used to determine the association between covariates [65] and the presence or the absence of subclinical CAA plaque (i.e., TPV). In crude analysis fibrinogen levels, the frequency of CCR9lowHLA-DRhigh non-classical monocytes, and the Th17/Treg ratios among Memory, EM, and CM CD4+ T-cell subsets were associate with the presence of CAA plaque, while the frequency of CCR9+HLA-DRlow non-classical monocytes were associated with the absence of CAA plaque (Table 3). When performing a first adjustment for LDL, smoking and age (Model 1), the later association remained statistically significant for all variables mentioned above, except for the Th17/Treg ratio in CM CD4+ T-cells. When a second adjustment for statins, smoking and

triglycerides was also performed (Model 2), only fibrinogen levels and the frequency of CCR9lowHLA-DRhigh non-classical monocytes remained significantly associated with the presence of plaque volume, while CCR9+HLA-DRlow non-classical monocytes were associated with the absence of CAA plaque. Interestingly, M-DC8+ monocytes were also associated with the presence of TPV after this second adjustment (Table 3). Lastly, after adjustment with the Framingham score, fibrinogen levels and the frequency of CCR9lowHLA-DRhigh and CCR9+HLA-DRlow non-classical monocytes remained associated with the presence and the absence of plaque, respectively (Table 3).

Our studies reveal alterations in Th17/Treg ratios and non-classical monocyte frequency/phenotype that coincide with subclinical CVD, alterations that may directly/indirectly fuel CAA plaque formation in ART-treated PLH.

DISCUSSION

In this study, we explored whether alterations documented to occur during HIV-1 infection, including gut barrier impairment, microbial translocation, Th17 cell paucity,[66-68] and monocyte subset expansion/activation,[54] were associated with subclinical CAA plaque in ART-treated PLH. To test this possibility, we had access to plasma and PBMC samples from ART-treated PLH and HIV- participants included in the cardiovascular imaging sub-study of the CHACS. Markers of gut dysfunction (i.e., sCD14, LBP, I-FABP) and chemokines involved in cell trafficking (i.e., CCL20, CX3CL1, CCL25) were quantified in the plasma, while the frequency and phenotype of CD4+ T-cell subsets (i.e., Th17, Tregs), CD8+ T-cells, monocyte (i.e., classical, intermediate, non-classical, M-DC8+) and DC (i.e., mDC, pDC) subsets were monitored in the peripheral blood. Variations in these immunological parameters, together with multiple clinical measurements, were studied in relationship with the presence of CAA plaque, visualized/monitored by CT Scan, as we previously reported.[5, 46, 47] Our results support a model in which the paucity of Th17 cells and the predominance of CCR9lowHLA-DRhigh non-classical monocytes favor a state of systemic immune activation that may fuel CAA in ART-treated PLH.

Studies previously performed by our group on a total of 181 ART-treated PLH (HIV+) and 84 HIV-uninfected controls (HIV-) included in the CHACS at baseline, revealed a two to three-fold increase in the coronary non-calcified plaque burden in ART-treated PLH compared to HIV-individuals.[5] The current immunological sub-study was performed on a fraction of HIV+ (n=61) and HIV- (n=21) participants, the first enrolled in the CHACS. In this sub-study, we did not have sufficient statistical power to observe differences between HIV+ and HIV- groups in the prevalence of CAA plaque detection (TPV >0). Nevertheless, the number of participants with TPV values >100 mm3 was higher in HIV+ (32/61; 52.4%; median TPV: 608.7 mm3) compared to HIV- (7/21; 33.3%; median TPV: 192.8 mm3). Consistently, we observed that levels of HDL were lower, while the levels of triglycerides were higher in ART-treated PLH compared to HIV- participants. In addition, plasma markers of intestinal damage (FABP2), systemic inflammation (sCD14), and chemokines involved in cell trafficking (CCL20, MIF, and CX3CL1) were significantly higher in HIV+ compared to HIV- participants. This is indicative that ART does not restore gut barrier functions

nor counteracts immune activation in PLH, thus explaining an increased CVD risk associated with HIV-1 infection relative to the general population.

The detection of subclinical CAA plaque in HIV+, as measured by TPV and LAPV, was associated with a longer time on ART and elevated plasma levels of fibrinogen, as well as a tendency for an increased time since infection, Framingham score, and the use of statins. No differences in nadir CD4 counts were observed. Among soluble factors monitored in plasma, only CCL20 levels tended to be higher in TPV+ compared to TPV- HIV+ ($p=0.0514$). CCL20 is a chemokine produced by inflamed epithelial cells, endothelial cells and monocytes, with the binding of CCL20 on its receptor CCR6 regulating the recruitment into effector sites of CCR6+ cells, such as Th17 cells and DC. The CCL20-CCR6 axis plays an important role in CVD by facilitating the recruitment of immune cells into the vascular beds, thus facilitating atherosclerotic plaque formation . Studies in CCR6-/-Apo-/- mice demonstrated the role of CCR6 in monocyte recruitment into atherosclerotic plaques, as well as monocyte emigration from bone marrow.[69] Of note, monocyte per se can produce CCL20 upon exposure to HIV.[70]

Th17-polarized CD4+ T-cells represent keystone players of immunity at mucosal barriers.[66-68] Multiple studies documented alterations in Th17 function and frequency during HIV/SIV infection and identified Th17 depletion as the major cause for gut barrier damage, which results in microbial translocation-induced systemic immune activation.[66-68] An important finding of our study is represented by the significant reduction in the frequency of Th17 cells with memory, EM and CM phenotypes in the peripheral blood of TPV+ compared to TPV- HIV+ participants. The preferential depletion of Th17 cells during HIV-1 infection was linked to their relatively high susceptibility to integrative HIV-1 infection.[66-68] The abundance of Th17 cells during acute infection correlated with reduced immune activation during progressive HIV disease.[71] In line with this evidence, the loss of Th17 cells during early acute HIV infection is correlated with systemic immune activation measured by the proportion of activated CD8+ T-cells.[72] Additionally, higher plasma lipopolysaccharide, which is a marker of microbial translocation and gut damage, correlated with lower frequencies of Th17 cells in chronic HIV infection.[73] ART initiation during

very early acute HIV infection restored Th17 cells. It was also reported that although long-term ART adherence normalizes the frequency of sigmoid Th17 cells, high levels of plasma lipopolysaccharide were still observed in those individuals.[73, 74] In our cohort, we did not observe differences in the frequencies of Th17 cells between HIV- and HIV+ participants. This similarity could be explained by the fact that the median ART duration of our participants was relatively high, exactly 15.67 years (IQR: 8.14-19.37), raising the possibility that long-term ART may have restored the Th17 compartment in the blood. Nevertheless, the detection of CAA plaque in ART-treated PLH was associated with a reduced frequency of Th17 cells, which may mirror alterations in mucosal barrier integrity.

The existence of a link between alterations occurring at the level of the mucosal intestinal barrier and the pathology of the cardiovascular system supports the existence of the gut-heart axis.[75-78]

The detection of IL-17A-producing CD4+ T-cells in the atherosclerotic plaque of mice [79] supports the possibility that subsets of Th17 cells may be recruited into the vascular beds and locally fuel atherosclerosis. This scenario is supported by the fact that in our study TPV+ compared to TPV- ART-treated PLH exhibit increased plasma levels of CCL20, a chemokine essential for CCR6+ Th17 cell trafficking.[66-68] Considering that T-cell functions depend on TCR triggering, future studies should determine the antigenic specificity of Th17 cells recruited into the atherosclerotic plaques. Indeed, studies reported that CD4+ T-cells infiltrating the atherosclerotic plaque recognize CMV and HIV peptides, as well as LDL and apolipopotein B peptides.[21] Th17 cell mainly recognize components of the microbiota.[80, 81] Or, specific components of the microbiota were reported to be present into atherosclerotic plaques,[82, 83] thus supporting a cognate contribution of Th17 cells to the process of atherosclerosis. Nevertheless, the role of Th17 cells may be dual, a positive one at the intestinal level, where they maintain the integrity of the mucosal barrier, and a deleterious one when recruited into the atherosclerotic plaque and exerting effector functions in response to translocated microbial components.

Another CD4+ T-cell subset altered during HIV-1 infection are Tregs, a subset reported to be expanded during HIV-1 infection despite the initiation of ART, with their suppressive functions

being detrimental for the proper development of anti-HIV immunity.[84] Due to alterations in the frequency of Th17 and Tregs during HIV-1 infection, the Th17/Tregs ratio is decreased during HIV infection.[71, 85] In primary HIV-1 infection, the Th17/Tregs ratio was negatively correlated with the proportion of activated CD8+ T-cells, plasma viral load, and markers of monocyte activation such as sCD14 and IL-1RA.[85] In line with these results, we observed increased frequencies of Tregs and decreased Th17/Tregs ratios in ART-treated PLH compared to HIV- participants. Although we did not observe statistically significant differences in the frequency of Tregs between TPV+ and TPV- in our group of n=61 ART-treated PLH, another sub-study performed by our group on n=84 participants from the same CHACS cohort found an increase in Treg frequency in TPV+ compared to HIV+ participants. This difference may be explained by differences in the number of participants included in the two studies. Nevertheless, we consistently report here that CAA is associated with a reduction in Th17/Treg ratios in ART-treated PLH.

A large body of evidence supports the contribution of specific monocyte subsets to the process of atherosclerosis,[30] with studies in mice demonstrating the recruitment of non-classical monocytes into the plaque via the chemokine receptor CX3CR1 and its ligand CX3CL1/FKN.[86] Non-classical monocytes express indeed preferentially CX3CR1 and patrol the vascular beds rich in membrane-associated CX3CL1.[54] Of note, an increased proportion of non-classical monocytes and monocytes expressing CX3CR1 were positively associated with coronary intima-media thickness.[33, 87] They also produce inflammatory cytokines (IL-6), chemokines (CCL2), and MMP-9 upon interaction with CX3CL1-expressing endothelial cells.[88] In addition, the CCL2 receptor CCR2 is expressed on classical and intermediate monocytes and is key in establishing atherosclerosis in mice models.[30] In our study, we did not observe an expansion of intermediate/non-classical CCR2-CX3CR1+ monocytes in ART-treated PLH with subclinical atherosclerosis. Nevertheless, the presence of subclinical CAA was associated with the predominance of CCR9lowHLA-DRhigh non-classical monocytes. The significance of this correlation was maintained after cofounder factor adjustment and multivariate analyses, thus raising new questions on the role of these monocytes in CVD in ART-treated PLH.

The expression of CCR9 on monocytes and the CCR9/CCL25 axis have been associated with autoimmune diseases such as inflammatory bowel disease, rheumatoid arthritis (RA) and ankylosing spondylitis.[61, 89] In relation to CVD, in a mouse model of atherosclerosis, the presence of monocytes and resident macrophages expressing CCR9 with characteristics of plaque-foaming cells was observed. In addition, CCR9 silencing by RNA interference decreased the size of atherosclerotic plaques.[59] CCR9 is also associated to cardiac hypertrophy in mice models.[90] Furthermore, in humans, CCR9+ cells were visualized infiltrating the atherosclerotic plaques.[59] In our cohort, we observed that a decreased expression of CCR9 on non-classical monocytes in ART-treated PLH with subclinical CAA. One of the possible explanations is the infiltration of CCR9+ non-classical monocytes into the atherosclerotic plaques, explaining why they are not observed in the periphery. At the opposite, CCR9+ pDCs have been reported to be tolerogenic and CCR9 loss to be deleterious in the development of atherosclerosis,[42] which suggests that CCR9 is not necessary a marker of inflammation. In our study, the predominance of CCR9+ monocytes was associated with the absence of CAA plaque. Therefore, one cannot exclude the possibility that CCR9+ monocytes play a protective role in atherosclerosis. Future mechanistic studies should address this possibility.

Our study has multiple limitations. First, we performed the immunological studies only on baseline samples included in the CHACS cohort. This pan-Canadian cohort was initiated in Montréal in 2013 and includes now 805 HIV+ and 244 HIV- controls for a 5-year longitudinal follow-up. Participants to the CHACS cardiovascular imaging sub-study (181 HIV+ and 84 HIV-) are presently undergoing repeat Cardiac CT angiogram, which will grant data to inform us on the validity of the identified markers of subclinical CAA in their ability to predict CVD progression in ART-treated PLH. Third, the CHACS participants are in majority males, although it is reported that differences exist between males and females in terms of HIV and CVD pathogenesis,[91] with an increased CVD risk observed in female versus male PLH.[92] Also, molecular mechanisms of CVD appear to be different in women versus men PLH, as reflected in part by our group findings on IL-32 in CVD pathology.[93-95] Second, we did not correlate alterations in the Th17/Tregs ratios with the size of HIV reservoirs. Indeed, studies performed by our group on the CHACS samples revealed an increased HIV-DNA reservoir in CD4+ T-cells of TPV+ versus TPV- HIV+.[96] Third, we

were not able to confidently conclude on immunological differences between HIV- participants with and without subclinical CAA due to reduced number of individuals enrolled at the time we performed the study. Nevertheless, despite the small sample size, there was no decrease in Th17 frequency nor Th17/Treg ratios between TPV+ and TPV- HIV- participants, indicative that the immunological signature associated with subclinical CAA is distinct in HIV+ versus HIV- groups.

In conclusion, we report results generated on peripheral blood cells collected from participants included in our CHACS cohort, a longitudinal cohort established to identify novel biomarkers predictors of subclinical CVD in ART-treated PLH. Our investigations revealed an immunological signature associated with subclinical CAA in ART-treated PLH, including i) a reduced ratio between Th17-polarized CD4+ T-cells (the guardians of mucosal barrier integrity) and regulatory CD4+ T-cells (the negative regulators of immune responses); together with ii) increased frequencies of non-classical CD16+ monocytes with a CCR9lowHLA-DRhigh phenotype and iii) increased plasma fibrinogen levels indicative of coagulopathy. Our results reveal immune cell subsets that may predict CAA ART-treated PLH and emphasize the need for new therapeutic strategies designed to restore immune competence, especially at mucosal barrier levels, in an effort to reduce the CVD risk especially in PLH under long-term ART.

Declaration of competing Interest

Contributors

TRWS contributed to study design, performed most of the experiments, analyzed results, prepared figures, and wrote the manuscript. YZ contributed to study design and performed experiments. AG and NFDR contributed to experiments. MEF was instrumental to the study design and provided protocols. AF performed all multivariate statistical analysis. JPR contributed to participant recruitment. CCL performed CT-SCAN angiographies and interpreted the results in all participants. ALL, MD and CLT contributed to patient recruitment, establishment of the biobank of plasma and PBMC, and provided access and valuable guidance in the analysis of clinical information, as well as statistical analysis. PA designed the study, analyzed results,

contributed to figure preparation, and wrote the manuscript. All authors reviewed and approved the manuscript.

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TABLES

Table 1: Description of study participants

	HIV$^+$ (n=61)	**HIV$^-$** (n=21)	**p-value**
Demographics and clinical information			
Age (years)*	55.38±6.99	56.94±7.99	0.39
Male&	61 (100%)	17 (81%)	***0.003***
BMI (kg/m2)#	24.43 (22.10-28.29)	25.72 (24.34-28.33)	0.2023
Framingham Score#	10 (6-14.25)	8 (8-12.5)	0.67
Current Statin Treatment&	14 (23%)	5 (23.8%)	1
Smocking&			
Never	18 (29.5%)	8 (38.1%)	
Current Smoker	23 (37.7%)	5 (23.8%)	
Former Smoker	20 (32.8%)	8 (38.1%)	
Coronary Artery Disease Parameters			
Total Plaque Volume (mm^3)#	108.30 (0-698)	49.70 (0-279.1)	0.32
Low attenuated plaque volume (mm^3)#	34.40 (0-194.5)	10.53 (0-118.9)	0.39
Laboratory Parameters			
White blood cells (xE9/L)#	5.6 (4.7-7.1)	6.15 (5.65-7)	0.2580
Lymphocytes (xE9/L)*	1.90± 0.66	1.87± 0.47	0.85
LDL (mmol/L)#	2.58 (2.13-3.40)	3.10 (2.30-3.82)	0.21
HDL (mmol/L)#	1.23 (1.07-1.3)	1.44 (1.30-1.6)	***0.002***
Triglycerides (mmol/L)#	1.73 (1.19-2.99)	1.21 (0.89-1.68)	***0.008***

NA, not applicable; *, for numeric parametric variables: mean±SD, and t-Test were used; #for numeric non-parametric variables: median, interquartile range (IQR), and Mann-Whitney test were used. &, Categorical variables were compared with Fisher's Exact test.

Table 2: Comparisons between ART-treated PLWH with and without atherosclerosis

	TPV+ (n=39)	**TPV-** (n=22)	**p-value**
Demographics and clinical information			
Age (years)*	56.24±6.35	53.85±7.93	0.1625
Male&	39 (100%)	22 (100%)	NA
BMI (kg/m2)#	24.05 (21.05- 28.38)	25.29 (22.91- 28.27)	0.23
Framingham Score#	11.00 (7- 18)	8.00 (5.5-13)	0.085
Current Statin Treatment&	12 (30.8%)	2 (9.1%)	0.064
Smocking& Never Current Smoker Former Smoker	 7 (17.9%) 20 (51.3%) 12 (30.8%)	 11 (50%) 3 (13.6%) 8 (36.4%)	NA
Coronary Artery Disease Parameters			
Total Plaque Volume (mm^3)#	608.70 (142.6- 881.8)	0	NA
Low attenuated plaque volume (mm^3)#	164.80 (36- 296)	0	NA
HIV disease parameters			
Undetectable Viral Load&	37 (94.8%)	19 (86.36%)	0.34
Duration of HIV (years)#	19.16 (14.29- 24.4)	14.71 (4.58- 24.14)	0.06
Duration of ART (years)*	16.14±5.68	10.69±7.82	***0.003***
Nadir CD4 (xE9/L)#	195 (100- 330)	170 (78- 255)	0.397
Laboratory Parameters			
White blood cells (xE9/L)#	5.90 (4.7- 7.8)	5.40 (4.3- 6.85)	0.27
Lymphocytes (xE9/L)*	1.91±0.60	1.89±0.78	0.924
D-Dimer (ug/L)#	300.00 (197.5- 410)	290.00 (175- 380)	0.52
Fibrinogen (G/L)#	3.23 (2.73- 3.78)	2.67 (2.27- 3)	***0.005***
LDL (mmol/L)#	2.40 (1.99- 3.48)	2.90 (2.23- 3.40)	0.35
HDL (mmol/L)#	1.21 (1.07- 1.37)	1.25 (0.98- 1.37)	0.96
Triglycerides (mmol/L)#	2.07 (1.26- 3.1)	1.38 (1.09- 2.03)	0.109
Serology			
Co-infection CMV#	36 (92.3%)	17 (77.3%)	0.39

NA, not applicable; *, for numeric parametric variables: mean±SD, and t-Test were used; #, for numeric non-parametric variables: median, IQR, and Mann-Whitney test were used. &, Categorical variables were compared with Fisher's Exact test.

Table 3. Association between cell subsets and coronary plaque volume.

	Crude Model	*p*	Model 1	*p*	Model 2	*p*	Framingham	*p*
Fibrinogen	2.51 (1.22-5.18)	***0.01***	2.23 (0.99-5.00)	***0.05***	3.91 (1.38-11.09)	***0.01***	2.31 (1.09-4.9)	***0.03***
$CCR9^{+}HLA\text{-}DR^{low}$ Non-classical monocytes	0.30 (0.15-0.63)	***0.01***	0.19 (0.07-053)	***0.01***	0.21 (0.08-0.59)	***0.02***	0.25 (0.11-0.59)	***0.01***
$CCR9^{low}HLA\text{-}DR^{high}$ Non-classical monocytes	3.56 (1.45-8.74)	***0.02***	3.74 (1.41-9.94)	***0.03***	4.9 (1.57-15.32)	***0.03***	4.06 (1.59-10.35)	***0.01***
EM Th17/Treg ratio	0.48 (0.27-0.84)	***0.03***	0.38 (0.18-0.83)	***0.05***	0.48 (0.22-1.05)	0.12	0.52 (0.28—0.95)	***0.05***
Memory Th17/Treg ratio	0.51 (0.29-0.89)	***0.03***	0.47 (0.23-0.94)	***0.05***	0.53 (0.26-1.07)	0.12	0.53 (0.30-0.95)	***0.05***
CM Th17/Treg ratio	0.58 (0.33-0.99)	***0.05***	0.59 (0.31-1.11)	0.11	0.61 (0.32-1.17)	0.15	0.58 (0.33-1.01	0.06
M-DC8/Slan	2.71 (1.24-5.91)	0.10	3.76 (1.32-10.71	0.07	5.54 (1.64-18.72	0.04	3.33 (1.36-8.14)	0.06

Model 1: LDL, smoking and age

Model 2: Statins, smoking and triglycerides

*shown are Odds ratio and the upper and lower limits of the confidence interval.

MAIN FIGURES

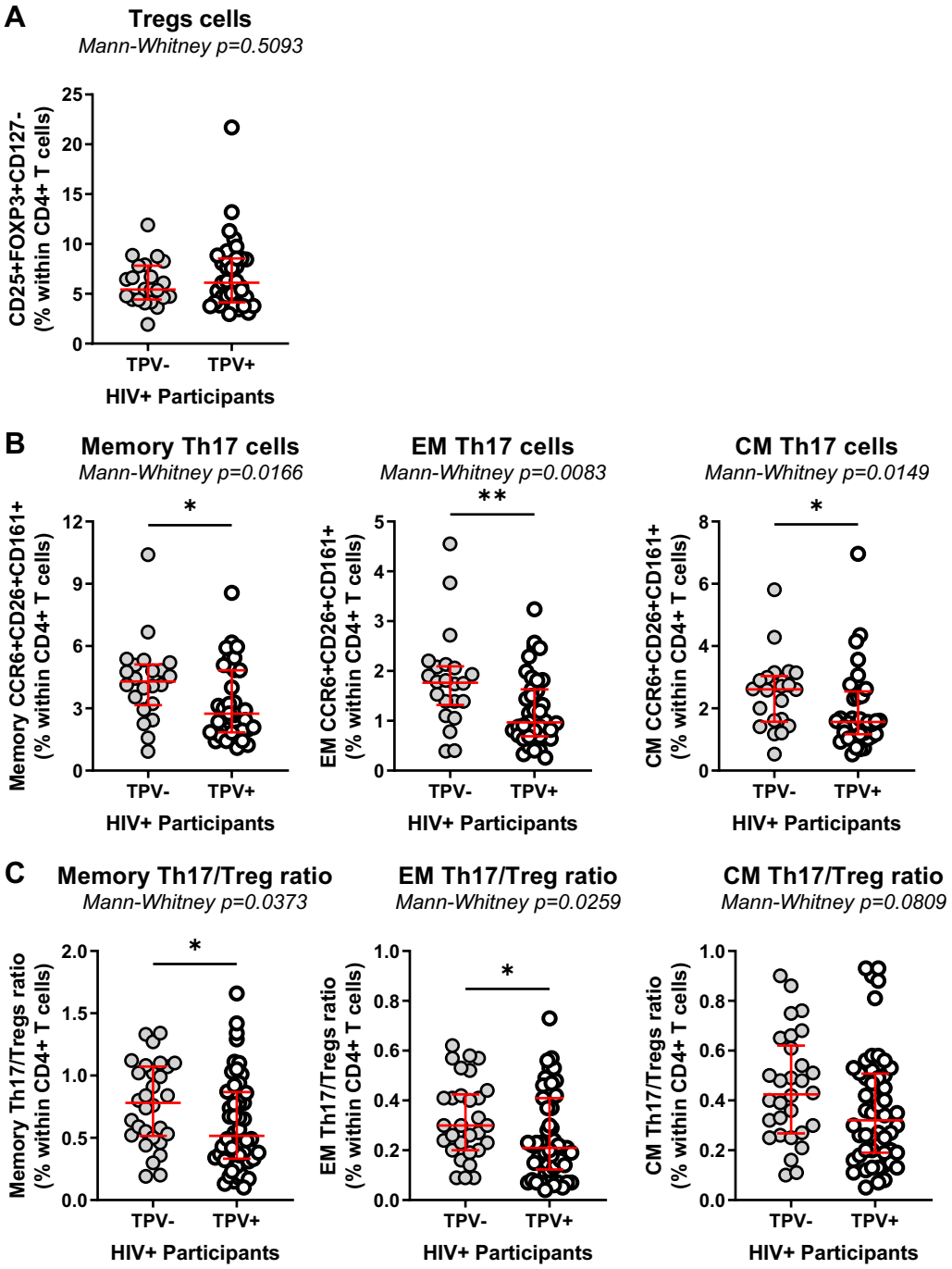


Figure 1: Analysis of Tregs and Th17 frequencies, and Th17/Treg ratios in TPV+ and TPV- HIV+ participants. The frequencies of regulatory CD4+ T-cells (Tregs; CD25+FOXP3+CD127-CD4+) (A), as well as Th17-polarized CD4+ T-cells (CCR6+CD26+CD161+) with memory (CD45RA-), effector memory (EM) (CD45RA-CCR7-) and central memory (CM) (CD45RA-CCR7+) phenotypes (B), were compared in PBMC between TPV+ (n=39) and TPV- (n=22) HIV+ participants. (C) Shown are the

Th17/Treg ratios within the memory, EM and CM Th17 subsets among TPV+ versus TPV- HIV+ participants. Median and IQ range are indicated in red as horizontal lines. Differences among study groups were determined by Mann-Whitney rank test. P-values and statistical significance are indicated in the figures (*, $p < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

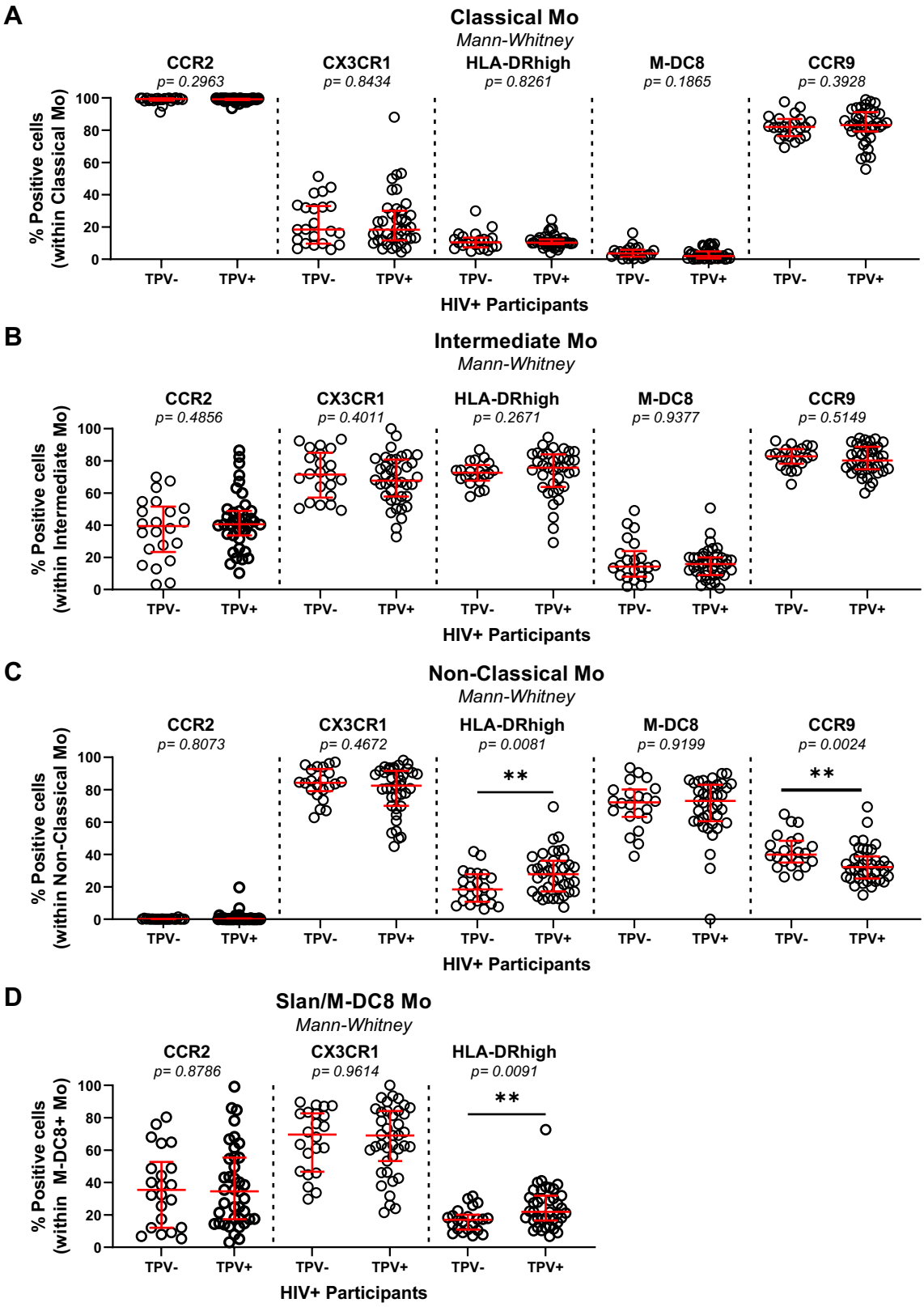


Figure 2. Phenotypic characterization of monocyte subsets in TPV+ and TPV- HIV+ individuals.

Total monocytes (Mo) were identified as cells lacking the T cell lineage markers CD3 and CD4 and

the DC marker CD1c, and expressing HLA-DR (Supplemental Figure 7). Shown is the expression of CCR2, CX3CR1, CCR9, HLA-DR and M-DC8 on classical (CD14++CD16-) (A) intermediate (CD14++CD16+) (B), and non-classical (CD14+CD16++) Mo (C) , as well as the expression of CCR2, CX3CR1 and HLA-DR on M-DC8+ Mo (D), in PBMC of TPV+ (n=39) versus TPV- (n=22) HIV+ participants. Median and IQ range are indicated in red as horizontal lines. Differences among study groups were determined by Mann-Whitney rank test. P-values and statistical significance are indicated in the figures (*, $p < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

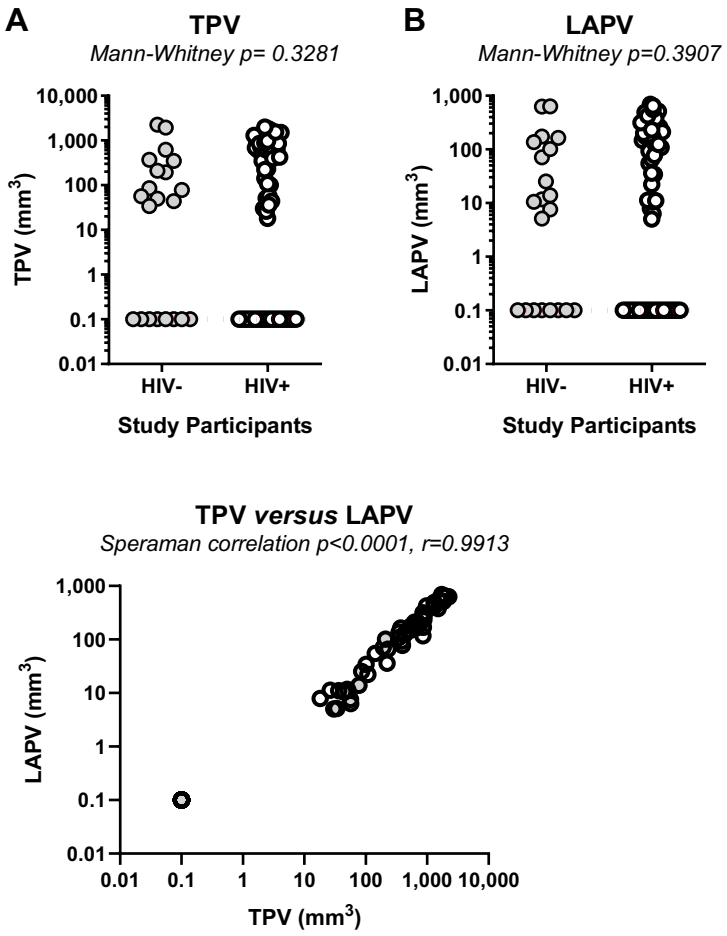
SUPPLEMENTAL INFORMATION

Supplemental Table 1: Antibodies and dyes used for polychromatic flow cytometry

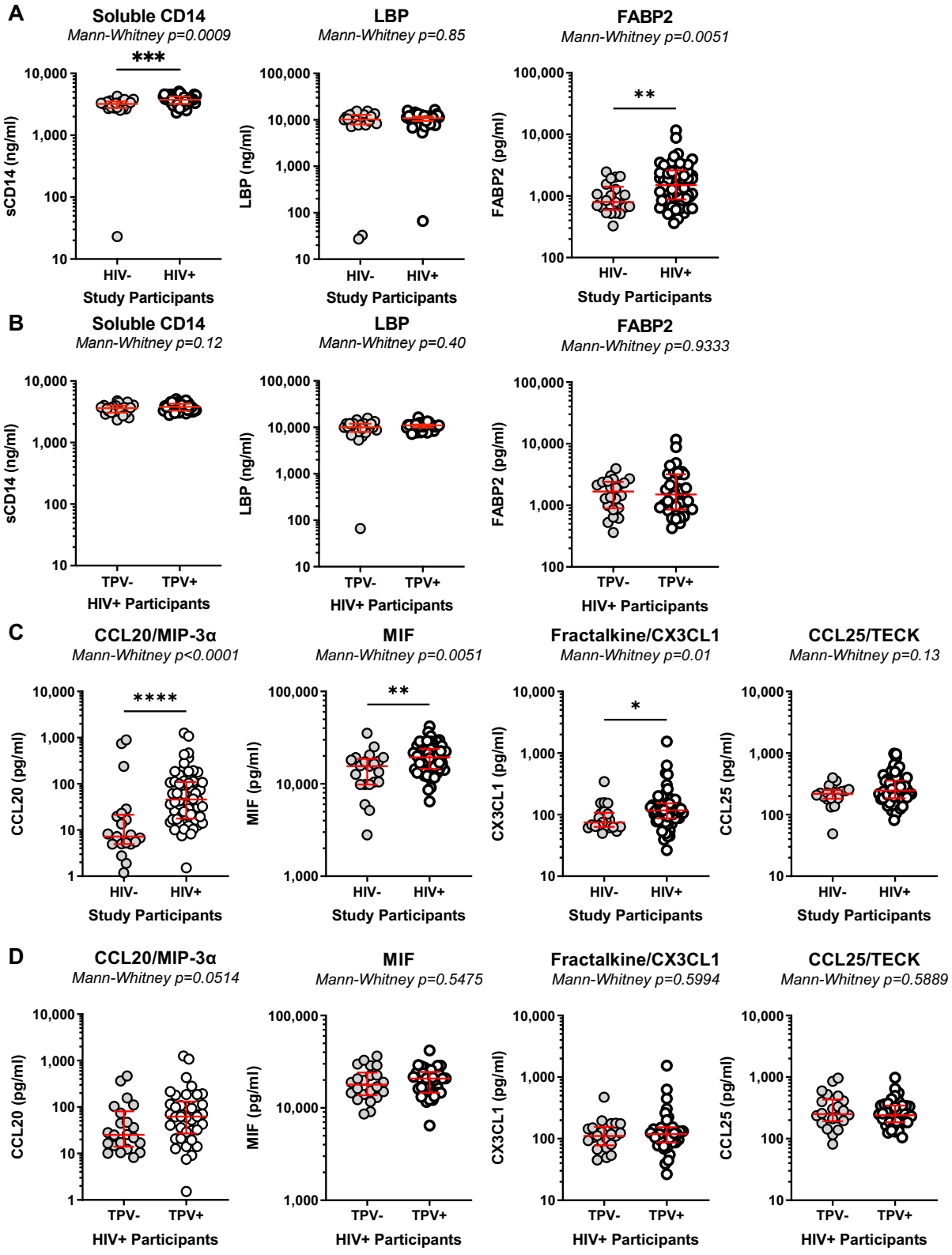
Target	Clone	Fluorochrome	Supplier
Th17/Treg cocktail			
CD3	UCHT1	Alexa Fluor 700	Biolegend
CD4	RPA-T4	PerCP/Cyanine5.5	Biolegend
CD45RA	HI100	APC-eFluor 780	eBioscience
CCR6	11A9	PE	BD Pharmingen
CCR7	3D12	BV786	BD Horizon
CD25	M-A251	BV421	BD Horizon
CD127	HIL-7R-M21	PE-Cy7	BD Pharmingen
CD26	M-A261	APC	BD Pharmingen™
CD161	DX12	BV650	BD Horizon
FOXP3	PCH101	Alexa Fluor 488	eBioscience
LIVE/DEAD™ Fixable Aqua Dead Cell Stain			Thermo Fisher Scientific
Monocyte cocktail			
CD3	UCHT1	Alexa Fluor 700	Biolegend
CD4	RPA-T4	PerCP/Cyanine5.5	Biolegend
CD1c	F10/21A3	BV786	BD OptiBuild
CD14	M5E2	APC	BD Pharmingen
CD16	3G8	PE-Cy7	BD Pharmingen
HLA-DR	L243	APC/Cyanine7	Biolegend
CCR6	11A9	PE	BD Pharmingen
M-DC8/Slan	DD-1	FITC	Miltenyi
CCR2	K036C2	PE/Dazzle 594	Biolegend
CX3CR1	2A9-1	BV421	BD Horizon
LIVE/DEAD™ Fixable Aqua Dead Cell Stain			Thermo Fisher Scientific
Monocyte and DC cocktail			
CD3	UCHT1	Alexa Fluor 700	Biolegend

CD4	RPA-T4	PerCP/Cyanine5.5	Biolegend
CD1c	F10/21A3	BV786	BD OptiBuild
CD14	M5E2	APC	BD Pharmingen
CD16	3G8	PE-Cy7	BD Pharmingen
HLA-DR	L243	APC/Cyanine7	Biolegend
CCR6	11A9	BV421	BD Horizon
CCR9	112509	PE	BD Pharmingen
CD123	6H6	PE/Dazzle 594	eBioscience
CD303 (BDCA2)	AC144	FITC	Miltenyi
LIVE/DEAD™ Fixable Aqua Dead Cell Stain			Thermo Fisher Scientific

SUPPLEMENTAL FIGURES



Supplemental Figure 1: Subclinical atherosclerosis in HIV-uninfected and ART-treated PLH participants. A slice computed tomography (CT) scanner was used to perform noncontrast cardiac CT and coronary computed tomography angiography to measure (A) total plaque volume (TPV) and (B) low attenuated plaque volume (LAPV) in a cohort of asymptomatic CVD risk-matched HIV-uninfected (HIV-; n=21) and ART-treated PLH (HIV+; n=61) participants. Differences among groups were determined by Mann-Whitney rank test. P-values and statistical significance are indicated in the Figures. (C) The correlation between TPV and LAPV in HIV+ (open symbols) and HIV- (grey symbols) participants was studied using the Spearman correlation model, with p and r values indicated on the graphs.


A
Soluble CD14
Mann-Whitney p=0.0009
***
sCD14 (ng/ml)
10,000
1,000
100
10
HIV-
HIV+
Study Participants
LBP
Mann-Whitney p=0.85
LBP (ng/ml)
100,000
10,000
1,000
100
10
HIV-
HIV+
Study Participants
FABP2
Mann-Whitney p=0.0051
**
FABP2 (pg/ml)
100,000
10,000
1,000
100
HIV-
HIV+
Study Participants
B
Soluble CD14
Mann-Whitney p=0.12
sCD14 (ng/ml)
10,000
1,000
100
10
TPV-
TPV+
HIV+ Participants
LBP
Mann-Whitney p=0.40
LBP (ng/ml)
100,000
10,000
1,000
100
10
TPV-
TPV+
HIV+ Participants
FABP2
Mann-Whitney p=0.9333
FABP2 (pg/ml)
100,000
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1,000
100
TPV-
TPV+
HIV+ Participants
C
CCL20/MIP-3α
Mann-Whitney p<0.0001
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CCL20 (pg/ml)
10,000
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HIV-
HIV+
Study Participants
MIF
Mann-Whitney p=0.0051
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MIF (pg/ml)
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HIV-
HIV+
Study Participants
Fractalkine/CX3CL1
Mann-Whitney p=0.01
*
CX3CL1 (pg/ml)
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1,000
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10
HIV-
HIV+
Study Participants
CCL25/TECK
Mann-Whitney p=0.13
CCL25 (pg/ml)
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HIV-
HIV+
Study Participants
D
CCL20/MIP-3α
Mann-Whitney p=0.0514
CCL20 (pg/ml)
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TPV-
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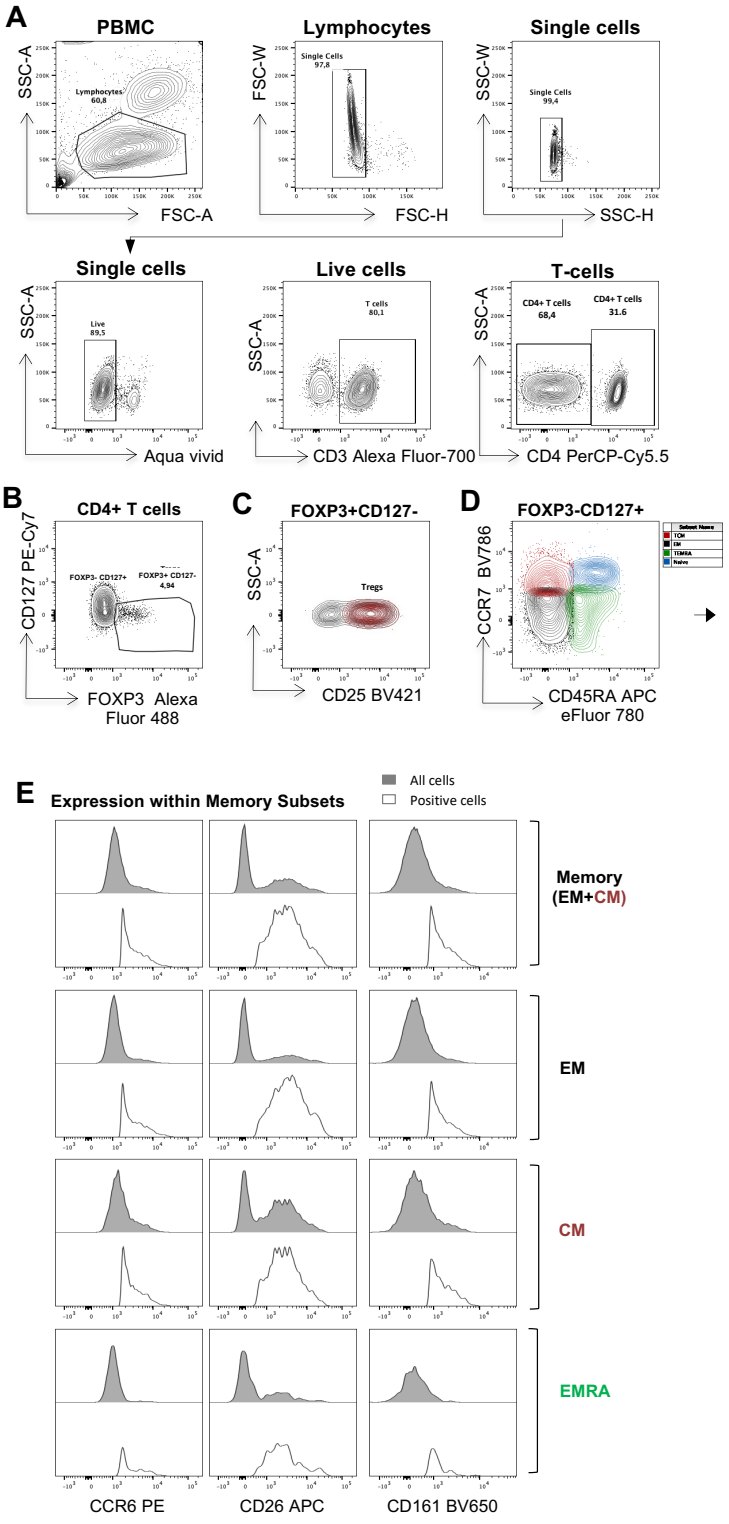
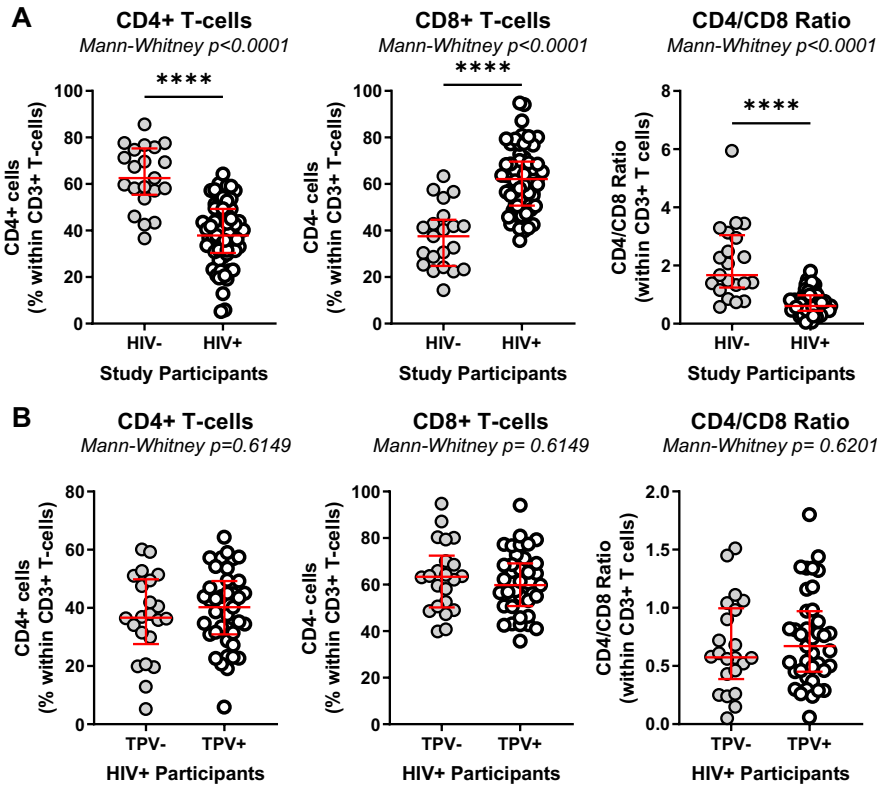
HIV+ Participants
MIF
Mann-Whitney p=0.5475
MIF (pg/ml)
100,000
10,000
1,000
TPV-
TPV+
HIV+ Participants
Fractalkine/CX3CL1
Mann-Whitney p=0.5994
CX3CL1 (pg/ml)
10,000
1,000
100
10
TPV-
TPV+
HIV+ Participants
CCL25/TECK
Mann-Whitney p=0.5889
CCL25 (pg/ml)
10,000
1,000
100
10
TPV-
TPV+
HIV+ Participants

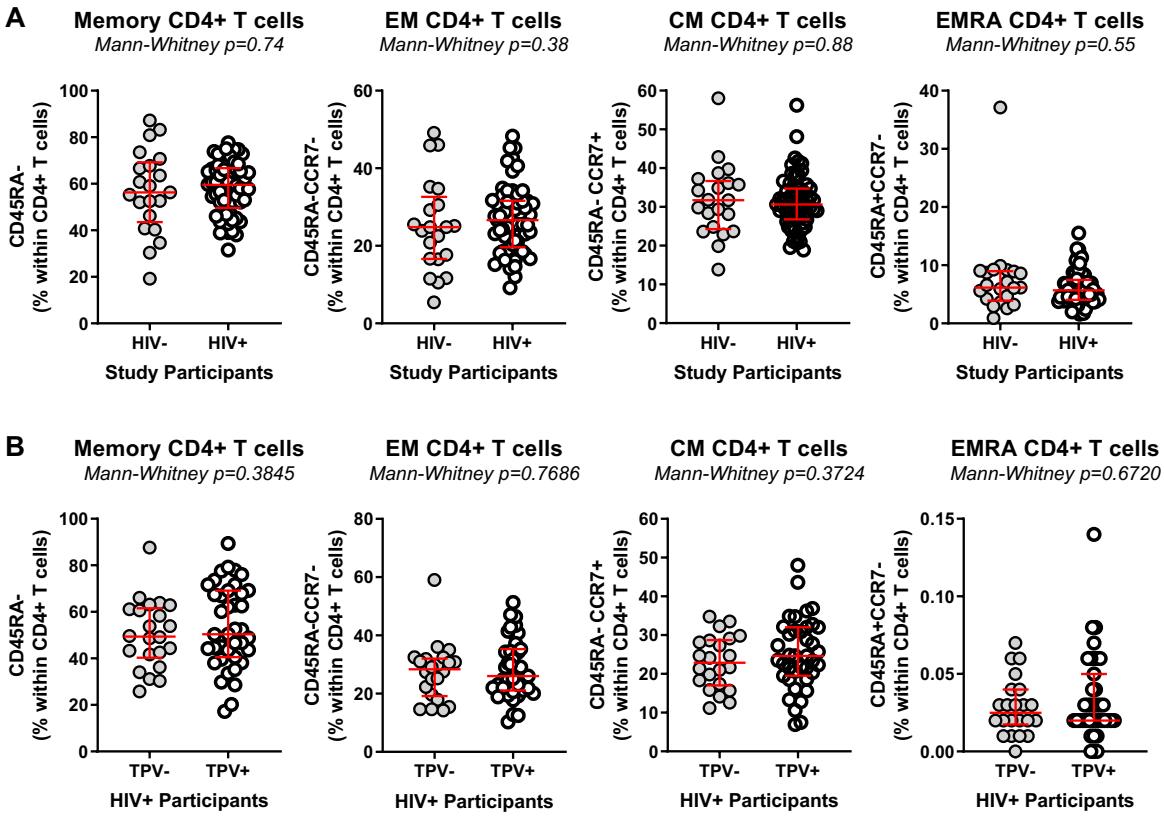
Supplemental Figure 2: Serological markers of microbial translocation, mucosal damage, and chemokines in HIV- and HIV+ individuals with or without coronary plaque. Markers of microbial translocation and gut damage (sCD14, LBP and FABP2; A-B) as well as markers of systemic inflammation (CCL20/MIP-3α, MIF, CX3CL1/FKN, CCL25/TECK; C-D) were quantified in the plasma of HIV+ (n=61) versus HIV- (n=21) participants (A and C) and also in the plasma of HIV+ participants with (TPV+; n=39) and without subclinical signs of atherosclerosis (TPV-; n=22) (B and D). Median and IQ range are indicated in red as horizontal lines. Differences among study groups were determined by Mann-Whitney rank test. P-values and statistical significance are indicated in the figures (*, $p < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).


A
PBMC
Lymphocytes
Single cells
SSC-A
FSC-A
FSC-W
FSC-H
SSC-W
SSC-H
Lymphocytes 60,8
Single Cells 97,8
Single Cells 99,4
Single cells
Live cells
T-cells
Live 89,5
T cells 80,1
CD4+ T cells 68,4
CD4+ T cells 31.6
Aqua vivid
CD3 Alexa Fluor-700
CD4 PerCP-Cy5.5
B
CD4+ T cells
CD127 PE-Cy7
FOXP3- CD127+
FOXP3+ CD127- 4,94
FOXP3 Alexa Fluor 488
C
FOXP3+CD127-
Tregs
CD25 BV421
D
FOXP3-CD127+
CCR7 BV786
CD45RA APC eFluor 780
E
Expression within Memory Subsets
All cells
Positive cells
Memory (EM+CM)
EM
CM
EMRA
CCR6 PE
CD26 APC
CD161 BV650

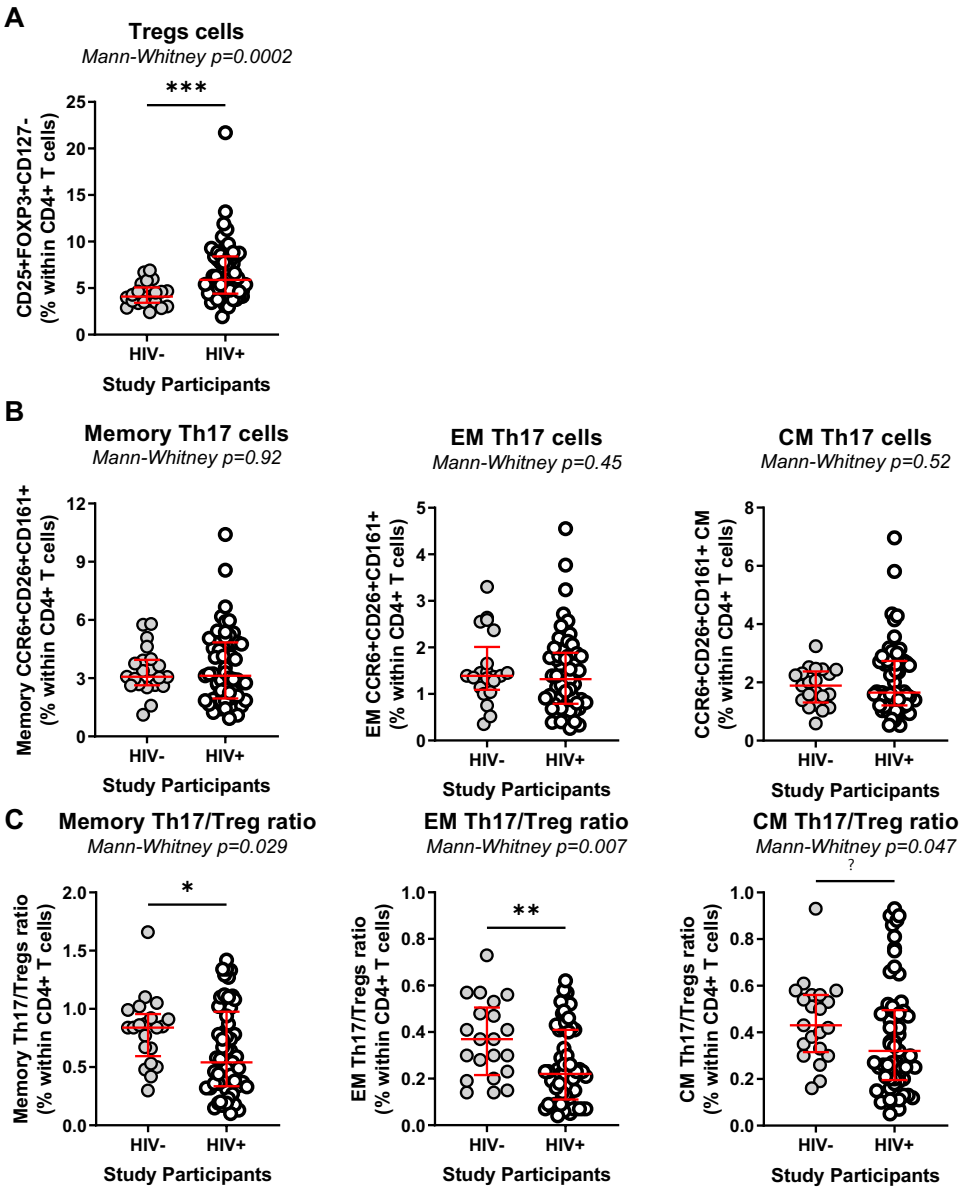
Supplemental Figure 3: Gating strategy for the identification of CD4+ T-cell subsets. PBMCs from HIV+ and HIV- participants were stained with fluorochrome-conjugated CD3, CD4, CD45RA, CCR7 CD25, CD127, CCR6, CD26, CD161, and FOXP3 Abs. Shown is the gating strategy to identify different T-cell subsets. Briefly, doublets and dead cells were excluded based on the FSC-W/FSC-H/SSC-W/SSC-H parameters and the viability dye Aqua Vivid, respectively, and CD4+ (CD3+CD4+) or CD8+ (CD3+CD4-) T-cells were identified based on the CD3/CD4 Abs staining (A). CD4+ T-cells were then divided into FOXP3+CD127- and FOXP3-CD127+ CD4+ T-cells (B), with Tregs defined as CD25+FOXP3+CD127- (C). Further, CD127+FOXP3- CD4+ T cells were subdivided in central memory (CM) CCR7+CD45RA-, effector memory (EM) CCR7-CD45RA-, terminally differentiated EM cells expressing CD45RA (EMRA) CCR7-CD45RA+, and naïve CCR7+CD45RA+ cells (D). Shown are histograms reflecting the expression of CCR6, CD26, and CD161 on EM, CM, and EMRA subsets. Th17 cells were identified by Boolean combination of gates between CCR6, CD26 and CD161 and defined as CCR6+CD26+CD161+ in each memory subset. Grey histograms represent the expression of CCR6, CD26 and CD161 within the whole population of each memory subset, whereas white histograms illustrate uniquely the positive population for each marker (E).



Supplemental Figure 4: CD4+ and CD8+ T-cell frequencies in HIV+ and HIV- individuals with/without subclinical atherosclerosis. T-cell subsets were identified as in Supplemental Figure 1. The frequencies of total CD4+ (left panels) and CD8+ T-cells (middle panels) and the CD4/CD8 ratios (right panels), were compared between HIV+ (n=61) and HIV- (n=21) participants (A) and among HIV+ participants with (TPV+; n=39) and without (TPV-; n=22) subclinical atherosclerosis (B). Median and IQ range are indicated in red as horizontal lines. Differences among study groups were determined by Mann-Whitney rank test. P-values and statistical significance are indicated in the figures (*, $p < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

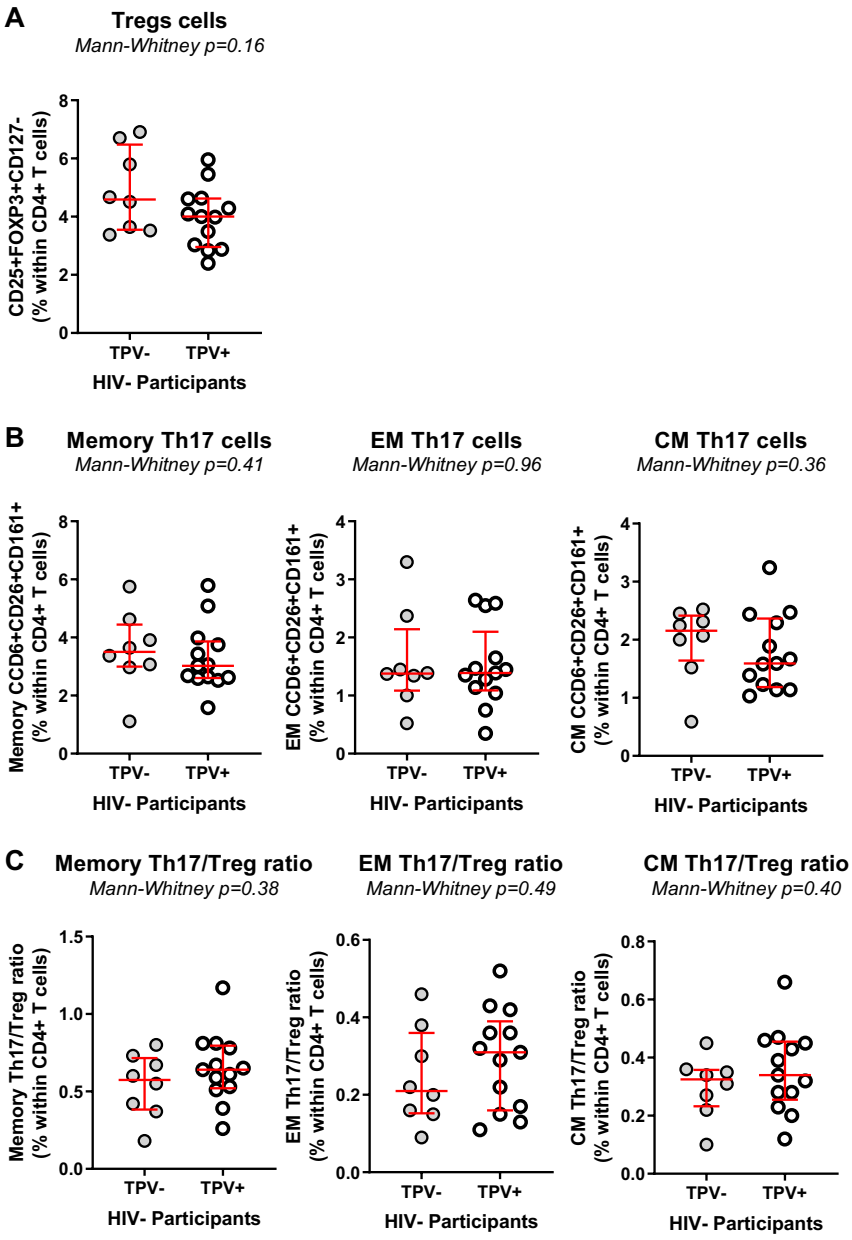


Supplemental Figure 5: Frequency of memory CD4+ T-cell subsets in HIV- and HIV+ participants with and without subclinical signs of atherosclerosis. T-cell subsets were identified as in Supplemental Figure 1. The frequency of CD4+ T cells with a total memory (CD45RA-), as well as EM (CD45RA-CCR7-), CM (CD45RA-CCR7+), EMRA (CD45RA+CCR7-) phenotypes were compared between HIV+ (n=61) and HIV- (n=21) participants (A) and among HIV+ participants with (TPV+; n=39) and without (TPV-; n=22) subclinical atherosclerosis (B). Median and IQ range are indicated in red as horizontal lines. Differences among study groups were determined by Mann-Whitney rank test. P-values and statistical significance are indicated in the figures (*, $p < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).



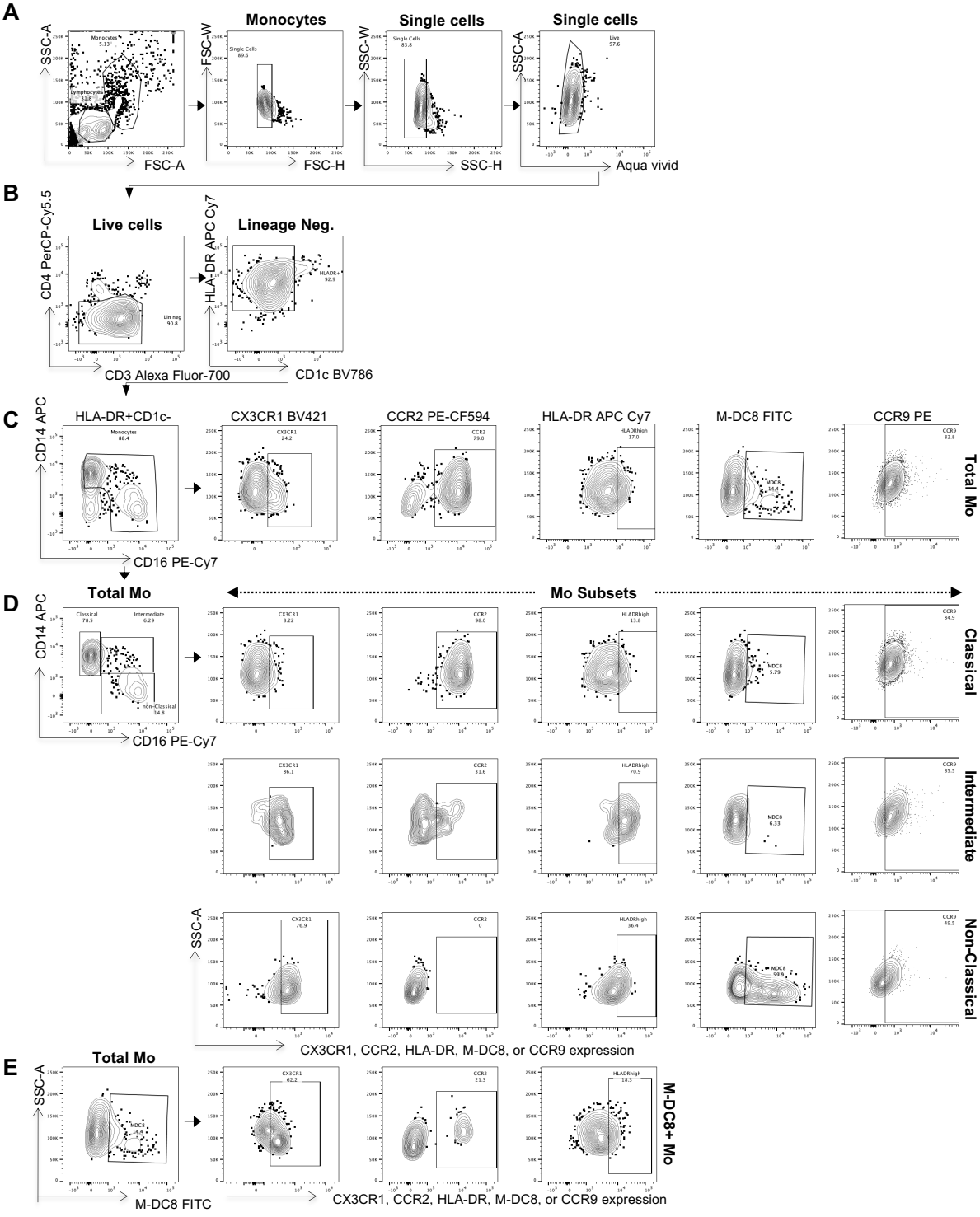
Supplemental Figure 6: Frequency of Tregs and Th17 subsets in HIV+ and HIV- individuals. The frequencies of Tregs (CD25+FOXP3+CD127-CD4+) (A), as well as Th17-polarized CD4+ T-cells (CCR6+CD26+CD161+) with total memory (CD45RA-), EM (CD45RA-CCR7-) and CM (CD45RA-CCR7+) phenotypes (B) were compared between HIV+ and HIV- participants. (C) The Th17/Treg ratios within the memory, EM and CM Th17 subsets were also compared among HIV+ and HIV- participants. Median values and IQ ranges are indicated in red as horizontal lines. Differences among study groups were determined by Mann-Whitney rank test. P-values and statistical

significance is indicated in the figures (*, $p < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Sample size HIV+ n=61 and HIV- n=21.



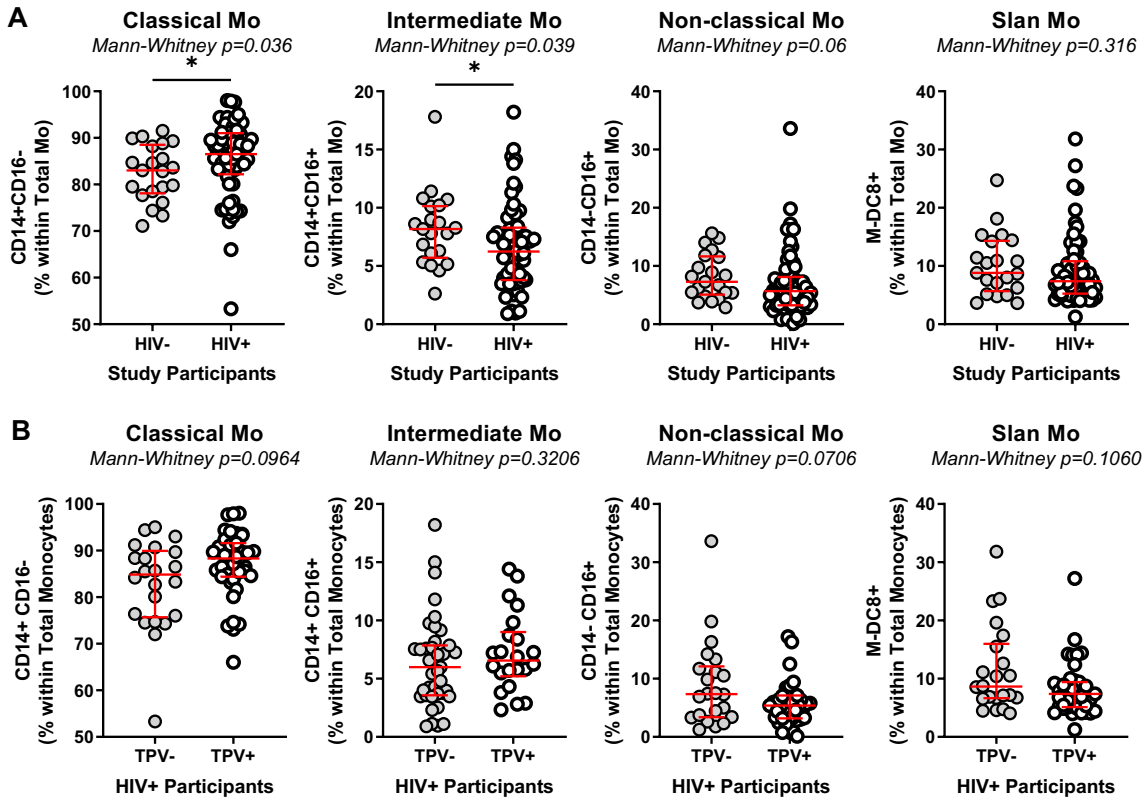
Supplemental Figure 7: Frequency of Tregs and Th17 subsets in HIV+ individuals with and without subclinical signs of atherosclerosis. The frequencies of Tregs (CD25+FOXP3+CD127-CD4+) (A), as well as Th17-polarized CD4+ T-cells (CCR6+CD26+CD161+) with total memory (CD45RA-), EM (CD45RA-CCR7-) and CM (CD45RA-CCR7+) phenotypes (B) were compared between HIV+ participants with (TPV+) an without (TPV-) subclinical signs of atherosclerosis. (C) The Th17/Treg ratios within the memory, EM and CM Th17 subsets were also compared among TPV+ and TPV- HIV+ participants. Median values and IQ ranges are indicated in red as horizontal

lines. Differences among study groups were determined by Mann-Whitney rank test. P-values and statistical significance is indicated in the figures (*, $p < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Sample size TPV+ n=39 and TPV- n=22.

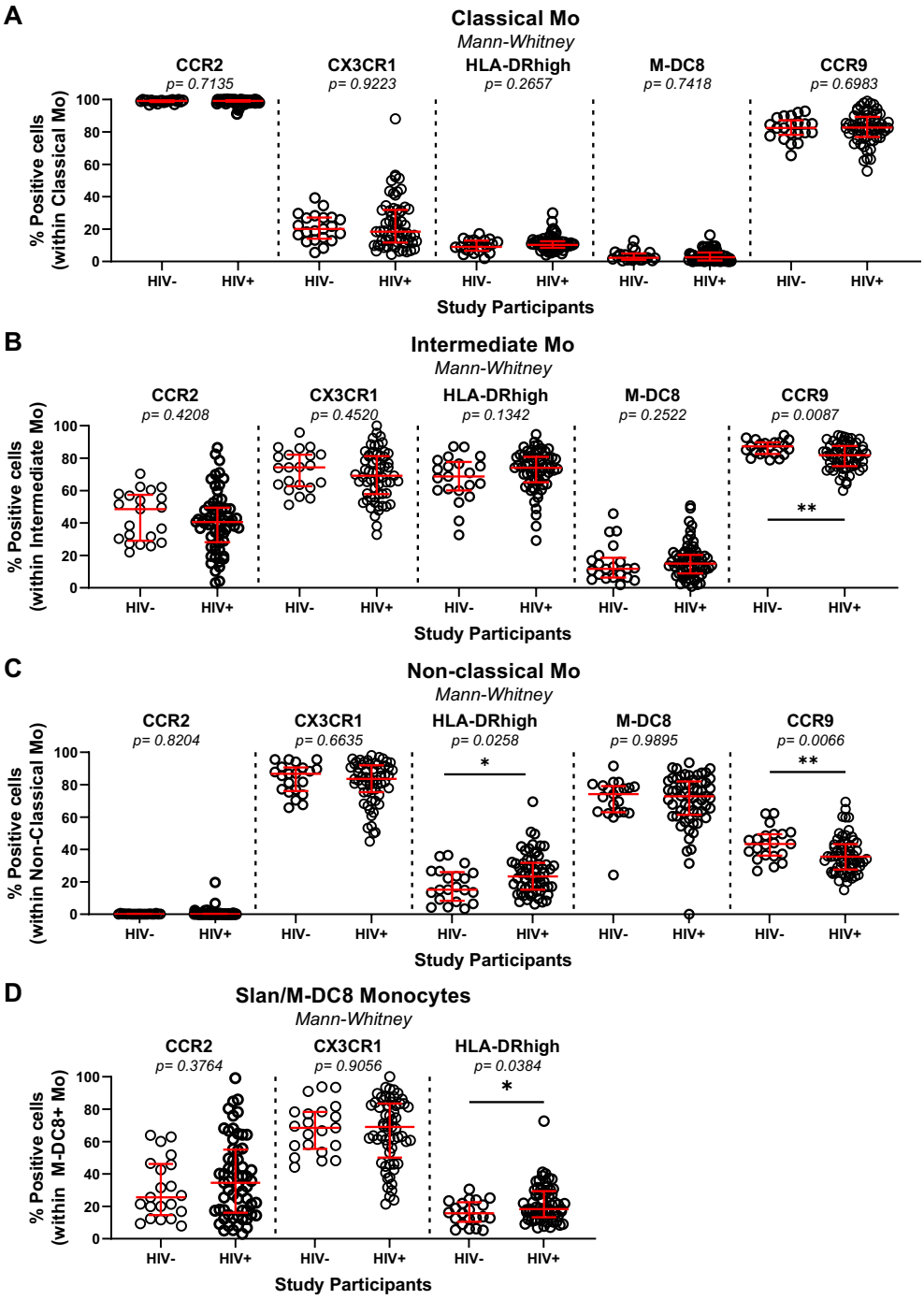


Supplemental Figure 8: Gating strategy for the identification of monocyte subsets. PBMCs from HIV+ and HIV- participants were stained with cocktails of fluorochrome-conjugated CD3, CD4, CD1c, CD14, CD16, HLA-DR, CCR6, Slan (M-DC8), CCR2 and CX3CR1 Abs (Monocyte cocktail) or

CD3, CD4, CD1C, CD14, CD16, HLA-DR, CCR6, CCR9, CD123 and CD303 Abs (Monocyte and DC cocktail; Supplemental Table 1). Shown is the gating strategy to identify monocyte (Mo) subsets. Briefly, doublets and dead cells were excluded as in Supplemental Figure 3 (A). Total monocytes within cells with a CD4-CD3-CD1c-HLD-DR+ phenotype (B) were further divided into classical (CD14+CD16-), intermediate (CD14+CD16+), non-classical (CD14lowCD16+) and Slan/M-DC8+ monocytes (C-E). The expression of CX3CR1, CCR2, CCR9, HLA-DR, and M-DC8 were evaluated on total, classical, intermediate, non-classical and M-DC8+ monocytes. Combination of gates using the Boolean tool for CCR2, CX3CR1, and HLA-DRhigh or CCR9 and HLA-DRhigh within monocyte subsets was performed using the single marker gates presented here.

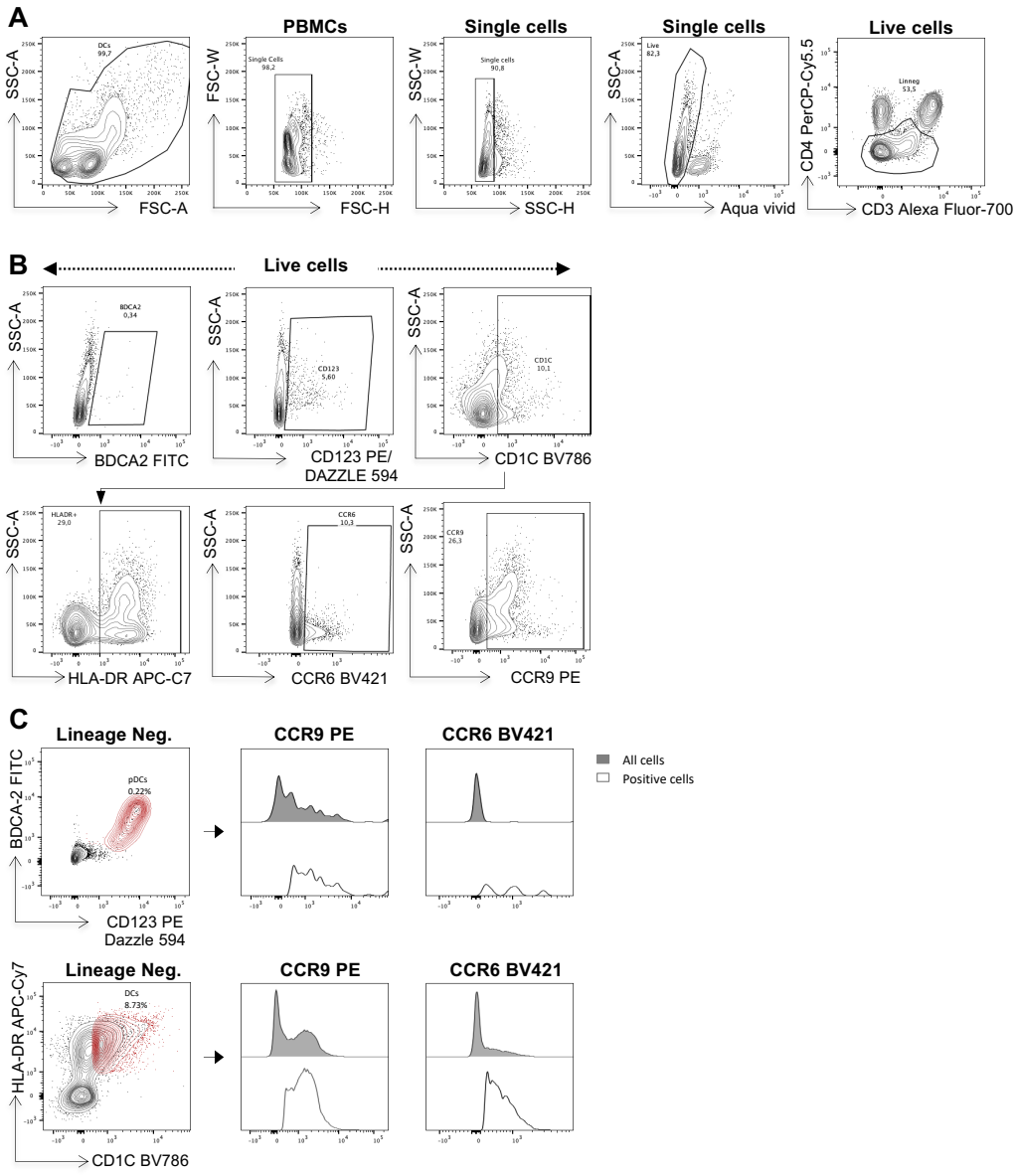


Supplemental Figure 9: Frequency of monocyte subsets in HIV+ and HIV- individuals. The frequencies of classical, intermediate, non-classical and Slan/M-DC8+ monocytes were compared between HIV+ (n=61) and HIV- (n=21) participants (A), as well as between TPV+ (n=39) and TPV- (n=22) HIV+ participants (B). Median and IQ range are indicated in red as horizontal lines. Differences among study groups were determined by Mann-Whitney rank test. P-values and statistical significance is indicated in the figures (*, $p < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).



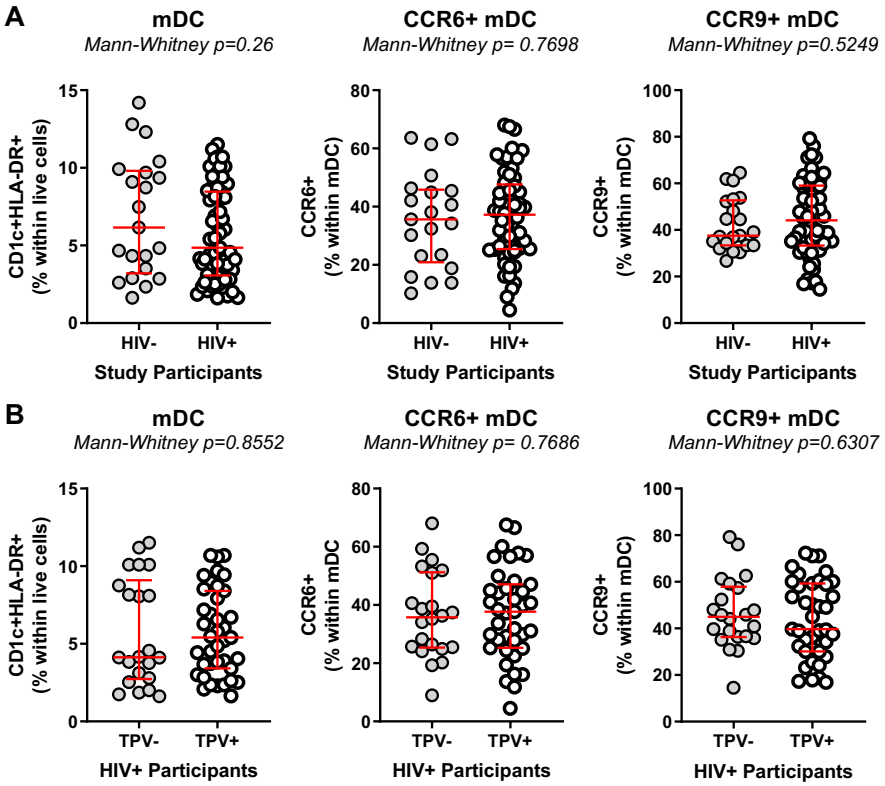
Supplemental Figure 10: Phenotypic analysis of monocyte subsets in HIV+ and HIV- individuals. The frequency of CCR2, CX3CR1, CCR9, HLA-DR, and M-DC8 on classical (A), intermediate (B), and non-classical monocytes (C), as well as the expression of CCR2, CXCR3, and HLA-DR on Slan/M-DC8 monocytes (D) were compared between HIV+ (n=61) and HIV- (n=21) participants. Median values and IQ ranges are indicated in red as horizontal lines. Differences among study

groups were determined by Mann-Whitney rank test. P-values and statistical significance is indicated in the figures (*, $p < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

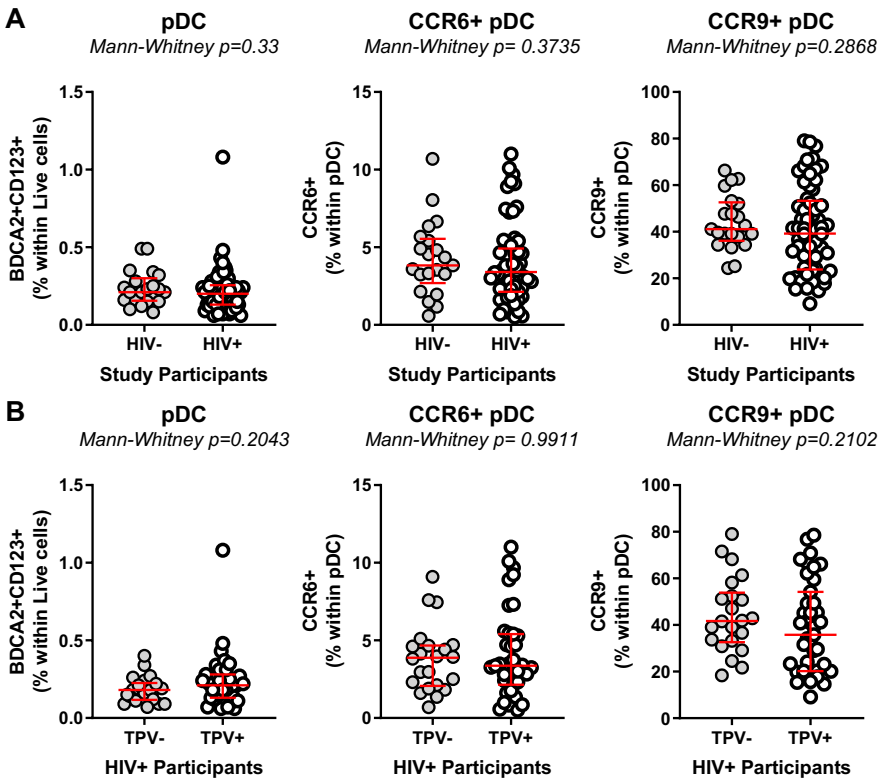


Supplemental Figure 11. Gating strategy for the identification of DC subsets. PBMCs from HIV+ and HIV- participants were stained with a cocktail of fluorochrome-conjugated CD3, CD4, CD1C, CD14, CD16, HLA-DR, CCR6, CCR9, CD123 and BDCA2 Abs (Supplemental Table 1). Show is the gating strategy to identify plasmacytoid (pDCs) and myeloid DC (mDC). (A) Doublets and dead cells were excluded, and Lineage negative (Linneg) cells were determined as CD3low/- and CD4low/-. (B) Shown are gates to determine the surface expression of BDCA2, CD123, CD1C, HLA-DR, and CCR6 within Live cells. (C) Boolean gating using gates set in B were used to identify pDCs (CD123+CD303+Linneg) (top panels) and mDCs (HLA-DR+CD1C+Linneg) (bottom panels). CCR6 and CCR9 expression was evaluated in both subsets. Grey histograms represent the expression

of CCR6 and CCR9 within the whole population of each memory subset, whereas white histograms illustrate uniquely the positive population for each marker.



Supplemental Figure 12. Dynamics of mDC in HIV- and HIV+ individuals with or without coronary plaque. The frequencies of total, CCR6+ and CCR9+ mDC (CD11c+HLA-DR+) were compared between HIV+ (n=61) and HIV- (n=21) participants (A), as well as between TPV+ (n=39) and TPV- (n=22) HIV+ participants (B). Median values and IQ ranges are indicated in red as horizontal lines. Differences among study groups were determined by Mann-Whitney rank test. P-values and statistical significance are indicated in the figures (*, $p < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).



Supplemental Figure 13. Dynamics of pDC in HIV- and HIV+ individuals with or without coronary plaque. The frequencies of total, CCR6+ and CCR9+ pDC (BDCA2+CD123+) were compared between HIV+ (n=61) and HIV- (n=21) participants (A), as well as between TPV+ (n=39) and TPV- (n=22) HIV+ participants (B). Median values and IQ ranges are indicated in red as horizontal lines. Differences among study groups were determined by Mann-Whitney rank test. P-values and statistical significance are indicated in the figures (*, $p < 0.05$; **, $P < 0.01$; ***, $P < 0.001$).

Chapter 6: Discussion and Perspectives

The general objective of my doctoral studies was to investigate the role of Th17 cells in the pathogenesis of HIV infection at the intestinal level and in the context of non-AIDS comorbidities such as CVD, with the final goal to identify novel Th17-specific therapeutic targets for HIV remission/cure.

1. Th17 cells at the interface between innate and adaptive immunity and intestinal level

The discovery of Th17 cells as a new lineage of helper CD4+ T-cells provided a new understanding of the division of labor within the immune system aimed to develop appropriate immune responses against specific pathogens at specific anatomic sites(277, 351).

Th17 cells play a critical role in maintaining the epithelial barrier integrity and immunity homeostasis at various levels including the intestinal mucosa(316, 547, 548). Their differentiation in vivo is dependent on the presence of specific microbiota components, such as the Segmented filamentous bacteria (SFB)(321, 555-557). Once differentiated, Th17 cells, via the interplay with mucosal epithelial cells, regulate the composition of the commensal microbiota and protect against pathogenic bacteria and fungi(558, 559). Th17 cells can also adopt a pro-inflammatory program and become pathogenic, as observed in Crohn's disease, axial spondyloarthritis, and psoriasis(560). Most recent studies demonstrated that encephalitogenic pathological Th17 cells originate from stem cell-like intestinal Th17 cells(299). Alternatively, the acquisition of Th17 pathologic functions is dependent on hypoxic metabolic niches in deep tissues(561). Targeting IL-17A, a signature cytokine produced by Th17 cells, has been proven to be an effective therapeutic strategy for psoriasis and axial spondyloarthropathies(562, 563), whereas targeting IL-23 improved Crohn's disease(561). The blockage of IL-17 during Crohn's disease was ineffective and increased disease severity(564). Similar findings were observed in an experimental model of colitis where IL-17 neutralization exacerbated the condition(565). These observations may be explained by the well-reported ability of IL-17 to induce the expression of tight junctions in the

intestine, which fortify the mucosal barrier, and by the production of antimicrobial peptides(566, 567). These studies emphasize the complexity of targeting Th17 effector cytokines in vivo while avoiding deleterious effects. The possibility of specifically targeting Th17 cells for HIV infection was unexplored at the moment when I started my doctoral studies.

As described in the introduction, one of the preferential sites of HIV replication during HIV infection is the gastrointestinal tract(197, 568). This results in the depletion of CD4+ T-cells and more precisely Th17 cells in the lamina propia which leads to intestinal dysfunction(200). The paucity of Th17 cells is caused by its increased permissiveness to HIV infection and by their disrupted trafficking into mucosal sites(337, 341). The direct damage of enterocytes has also been proposed as a possible mechanism driving intestinal mucosa alterations. For example, the envelope protein HIV-gp120 increases intracellular calcium, which is associated with a decreased ability of enterocytes to maintain ionic balance; also the accessory protein HIV Tat was reported to alter the glucose uptake at the level of enterocytes(568), thus impairing their functions.

In **the first article of this thesis**(553), we explored the role of IL-17A, the signature cytokine produced by Th17 cells, on the early steps of HIV transmission and replication, as well as on viral reservoir reactivation in CD4+ T-cells of ART-treated PLWH. These experiments were designed to mimic events that occur during primary HIV infection when epithelial cells are the first cells to encounter the virus (362), as well as during ART where the majority of CD4+ T-cells carrying integrated HIV-DNA are located in the GALT(345, 569). Our results demonstrated that IL-17A acts in synergy with TNF-α to boost IEC production of CCL20, a Th17-attractant chemokine, and to promote the transmission of HIV from inflamed IEC to CD4+ T-cells. IL-17A mediated this effect by counteracting IFN-I-mediated responses, thus supporting a pivotal role played by IL-17A-producing cells during primary HIV infection. In line with this, we also observed that IL-17A promoted viral outgrowth from reservoir cells of ART-treated PLWH. This effect was partially explained by the capacity of IL-17A to abrogate the TNF-mediated Type I interferon immune responses, including the expression of HIV restriction factors (BST2, IFIT1, and ISG15). Other mechanisms by which inflamed intestinal epithelial cells sustain the reactivation of HIV reservoirs

in gut-infiltrating and/or homing CD4+ T-cells remain to be discovered and may represent new targets for therapeutic interventions. Toward this goal, the large sets of DEG identified by RNA-Seq(553) is currently under investigation by Dr. Ancuta's team. The co-culture of CD4+ T-cells of ART-treated PLWH with IL-17A-activated intestinal epithelial cells represents the basis of a new viral outgrowth assay currently under development by her team. In addition to its capacity to interfere with type I IFN response, IL-17A-activated intestinal epithelial cells may produce soluble factors and/or express membrane-bound molecules that may be involved in this process. Of particular notice, IL-1α and IL-1β were preferentially upregulated in intestinal epithelial cells activated by TNF and IL-17A(553), pointing to the potential contribution of these cytokines in viral reactivation. In line with this possibility, IL-1 isoforms represent important growth factors for T cells(570). In addition, other recent publications demonstrated the involvement of ICAM1 and VCAM1 expressed by epithelial cells in promoting HIV replication in CD4+ T-cells(571). In our RNA-Seq data ITGA8 and ITGA1 were upregulated on IL-17A-activated intestinal epithelial cells, while ICAM-1 and VCAM-1 were mainly induced by TNF-α and decreased in the presence of IL-17A(571). Future studies *in situ* or *ex vivo* are needed to validate the role of IL-1 isoforms and integrins in the interplay between intestinal epithelial cells and CD4+ T-cells in ART-treated PLWH in view of their potential use as therapeutic targets in HIV remission/cure.

As of today, the dynamics and interactions between Th17 responses and Type I interferons have not been exhaustively explored in the context of HIV infection despite this component of the immune system's critical role in HIV pathogenesis. During acute SIV infection, type I interferon signaling is detected at late time points and coincides with the depletion of Th17 cells and their effector functions(379). Then, during the chronic phase, there is a persistent increase of Type I interferon and depletion of Th17 cells(572).

Therapeutic interventions have targeted IFN responses and Th17 associated cytokines to alter the course of HIV infection. The administration of IFN-α2a to rhesus macaques before SIV infection upregulated the expression of ISGs and prevented systemic disease. In contrast, a delay of the IFN response induced by the blockage of IFN receptor reduced the expression of antiviral

genes and increased the size of the viral reservoir(573). It is noteworthy that the continued administration of IFN-α2a induced IFN-I desensitization and rapid loss of CD4+ T-cells due to a lack of viral control(572). The blockade of IFN during chronic infection did not affect inflammation and viral reservoir. Unfortunately, Th17 responses were not evaluated in detail in the previous studies but it was suggested that Th17 responses and Type I interferon do not overlap during HIV infection.

Concomitantly, the administration of IL-21, another cytokine produced by Th17 cells and exhibiting antiviral features(574), to SIV-infected rhesus macaques improved the restoration of Th17 cells in the gut, reduced immune activation and microbial translocation, and reduced levels of plasma viremia and cell-associated SIV DNA. Additionally, the levels of microbial translocation were inversely correlated with levels of intestinal Th17 cells(575, 576). Type I interferon responses were not evaluated in the latter studies. Based on our observation on the fact that IL-17A can modulate the response to Type I interferon, it is pertinent to assess the modulation of Type I interferon signaling when re-establishing the Th17 compartment in the context of HIV infection.

In a broader perspective, previous reports have linked Th17 responses with tissue injury and/or viral burden in infections other than HIV. Among those viral infections are Influenza, SARS Cov-2 and HCV. In the first case, it was reported that Th17 mediators, IL-17 and IL-17-responsive cytokines were elevated in infected patients with the 2009 H1N1 strain(577). The administration of anti-IL-17 antibodies protected H1N1-infected mice against lung injury and showed lower levels of IL-17-responsive cytokines. Similar results were observed in IL-17-deficient mice infected with H1N1 influenza(577). Since the interferon production and signaling pathway are critical for the antiviral host response, these observations could be due to the capacity of IL-17 to attenuate the Influenza virus infection-induced expression of Type III interferons(578). In fact, it was reported that Type III interferons are upregulated on bronchial epithelial cells after exposure to the Influenza virus. The production of IFN-λ induced by TLR-3 ligand poly I:C and influenza A virus

in bronchial epithelial cells is suppressed by IL-17A, which increases the expression of suppressor of cytokine signaling(SOCS)1 and SOCS3. This results in the attenuation of IFN-λ(578).

Regarding SARS-Cov-2 infection, the migration of neutrophils to the lung is among the immune alterations driving lung damage(579). It was found that CD16+ neutrophils were enriched in patients who died of COVID-19(580). As described earlier, IL-17 induces the secretion of chemokines on lung epithelial cells that chemoatrack neutrophils(581). As an enhanced frequency of Th17 cells is also observed in patients with poor outcomes, these cells may contribute to this altered neutrophils distribution (580).

Finally, it was reported in HCV infection that IL-17A has the ability to dampen the response to Type I interferons. More precisely, the pretreatment with IL-17A of HepG2 cells expressing Hepatitis B virus followed by IFN-α decreased the levels of ISGs(ISG15, ISG20, and Mx1) and increased the levels of HBsAg, HBeAg, and HBV DNA. As observed in influenza, IL-17A mediated its mechanism by inducing the expression of SOCS1, SOCS3, and USP18, which are negative regulators of the critical transcriptional ISGF3 complex. The ISGF3 transcriptional complex activated the transcription of ISGs. The block of the IL-17A pathway by TRAF6 inhibitor abrogated the effect of IL-17A in response to interferon(582). These results suggest that blocking IL-17A may boost the IFN response against viral infections.

2. From autoimmunity to HIV-1 infection: New Th17-specific therapeutic interventions

The pathogenic role of Th17 cells during autoimmunity prompted the development of Th17-targeted interventions(583). Such interventions include the blockade of IL-17 with secukinumab and ixekuzynab and the blockade of IL-17R with brodalumab(584). These therapies are currently FDA-approved for treating psoriasis, psoriatic arthritis, and ankylosing spondylitis. Since the pathogenic role of Th17 cells is not only driven by IL-17 but also by other pro-inflammatory cytokines such as IL-17F, IL6, and GM-CSF, targeting the Th17 lineage may be more effective than

targeting a single cytokine(583). RORC2 together with T-bet, IL-6/STAT2, TGF-β, IL-1β A, and IL-23 drives the generation of pathogenic Th17 cells and has been considered a potential target via small molecules(583, 585, 586). It is of note that the regulation of RORC2 is dependent of its natural ligands, which are molecules of the cholesterol pathway, such as oxysterols. In patients, with progressive multiple sclerosis, oxysterol levels increased from baseline to 5-year follow-up(587, 588), pointing to RORC2 as a drugable target. In that aspect, in mice with EAE, the RORC2 inhibitor SR1001 diminished the severity of EAE and repressed the expression of IL-17 in spinal cord and splenocytes(589, 590).

In inflammatory bowel disease, there is an abundance of Th17 cells in the lamina propia of patients and mice. Targeting only IL-17 therapeutically has been shown ineffective in these patients and resulted, in some cases, in adverse events including a degradation of their condition. These adverse events could be explained due to the importance of IL-17 in maintaining mucosal homeostasis(589-591). Another explanation is that targeting only IL-17 does not prevent Th17 cells from secreting other cytokines involved in the development of inflammatory bowel disease. Therefore, the use of RORC2 inhibitors should be considered as an option(590). In line with this, the use of the oral RORC2 inverse agonist TAK-828F in a murine model of ulcerative colitis decreased disease severity and improved the expression of tigh junction proteins, which play a crucial role in barrier function of intestinal mucosa(592).

Additionally, current clinical trials are testing the effectiveness of RORC2 inhibitors for treating psoriasis in humans(593). Considering the relevance of RORC2 inhibitors in multiple sclerosis, inflammatory bowel disease, and psoriasis and taking in consideration out discovery that Th17 cells are highly permissive to HIV replication and harbor viral reservoirs under ART, it could also be of interest to test these inhibitors in that context.

In **the second article of this thesis**(594), we originally demonstrated that RORC2, the master transcription regulator of Th17 cells, is a critical positive regulator of HIV infection. We observed

that pharmacological inhibition of this transcription factor using specific compounds directed against its LBD reduced HIV replication in *in vitro* infected CD4+ T-cells and HIV outgrowth in CD4+ T-cells from ART-treated PLWH. Furthermore, we found an enrichment of HIV-1 DNA in CCR6+RORC2+ CD4+ T-cells compared to CCR6-RORC2- T-cells of ART-treated PLWH. Mechanistic studies, including ChIP experiments, indicated that RORC2 directly promotes HIV-1 gene expression by a mechanism likely involving binding to the NRRE in the HIV-1 LTR. These results indicated that this transcription factor might be a cell-specific target for HIV-1 therapy. These investigations are currently continuing in the laboratory of Dr. Ancuta as part of collaboration with GSK USA and collaborators within the pan-Canadian collaboratorium CanCURE 2.0. One major objective is to provide pre-clinical evidence for the antiviral effects of RORC2 inhibitors in a humanized mice model of HIV-infection.

As of today, the concept of pathogenic and non-pathogenic Th17 cells has not been fully explored in the context of HIV infection. Pioneering work performed by our team showed that memory CD4+ T-cells with CCR4+CCR6+ (Th17, producing IL-17A) and CXCR3+CCR6+ (Th1Th17, producing IL-17A and IFN-γ) phenotypes are highly permissive to HIV-1 infection(337). In addition to RORC2, transcriptional profiling identified pathways and molecular targets of pathogenic and non-pathogenic Th17 cells to regulate HIV replication(339, 595). Among those targets, the ligand-dependent nuclear receptor PPARγ, preferentially expressed in Th1Th17 cells(595), was identified as a negative regulator of HIV replication(595, 596). The downregulation of PPARγ via small interfering RNA led to increased HIV replication, whereas the pharmacological activation of PPARγ with rosiglitazone inhibited HIV replication(595). In line with these observations, the inhibition of PPARγ with the antagonist T0070907 increased HIV transcription and intra-cellular HIV-p24 but unexpectedly limited HIV outgrowth in cells from PLWH receiving ART. PPARγ antagonism with T00709076 upregulated the expression of HIV-restriction factors and downregulated HIV permissiveness factors such as CCR5 or furin, which could explain this observation(596). These results point to the complexity of targeting PPARγ as a host factor to counteract HIV infection.

In addition to PPARγ, the transcription factor AhR has been identified as a signature molecule of non-pathogenic Th17 cells by studies performed in mice and humans in the context of autoimmunity(322, 585). Its modulation can inhibit the expression of IL-17, IL-22 and other pro-inflammatory cytokines(322, 585, 597). AhR also regulates the expression of IL-10(585), which contributes to viral reservoir establishment and persistence in SIV-infected macaques(214, 585). The modulation of AhR has been a topic of active research to treat autoimmune diseases such as psoriasis. The most recently approved treatment for psoriasis is the AhR ligand agonist Tapiranof(598). In imiquimod-induced psoriasiform inflammation in mice, tapiranof decreased the tissue expression of IL-17A, IL-23, and IL-1β(598).

During my doctoral studies I participated to multiple collaborative studies in the laboratory, including one aimed at evaluating AhR's role in HIV infection. CRISPR/Cas9-mediated AHR knockout facilitated HIV replication in *in vitro* infected primary memory CD4+ T-cells from HIV-uninfected volunteers(599). We further tested the effect of AHR agonist FICZ and AhR antagonist CH223191. FICZ increased IL-22 and IL-10 but did not alter the production of IL-17A or IFN-γ, whereas CH223191 decreased the production of IL-17A, IL-22, and IL-10 but not IFN-γ. In line with what was observed after CRISPR/Cas9-mediated AhR knockout, we observed that the pharmacological inhibition of AHR favored HIV replication *in vitro* and promoted HIV outgrowth in CD4+ T-cells of ART-treated PLWH. Accordingly, it was also observed that AhR agonist blocks HIV-1 replication in macrophages by indirectly increasing the catalytic activity of SAMHD1 known to reduce dNTP levels and HIV DNA synthesis during reverse transcription(600). These results indicate that AhR promotes an antiviral program in both macrophages and CD4+ T-cells.

Despite the recent progress in this field, future studies are needed to establish a link between AhR activation and residual transcription *versus* latency in HIV reservoirs of ART-treated PLWH. Ligands for AhR include xenobiotics from the environment, cigarette smoke, and drugs, as well as metabolites from microbiota and cellular tryptophane metabolism(601-603). Also, the evaluation of AhR activation in various deep tissues such as intestine, lungs, liver and lymph nodes will be of great relevance for understanding how AhR activation shapes the persistence of

latent HIV reservoirs and whether AhR antagonists can be considered as adjuvant therapy to boost HIV outgrowth in "*shock and kill"l* HIV cure strategies.

3. Th17 cells: from mucosal gate-keepers and sites of HIV replication to culprits of cardiovascular disease in the gut/heart axis

It has been suggested that alterations in the gut microenvironment could play a role in the development of atherosclerosis. For example, the microbiota composition imbalance is characterized by the decreased presence of *Roseburia Intestinalis* and *Faecalibactem prausnitzii.* These bacteria species are significant producers of the short-chain fatty acid butyrate, an essential energy source for colonocytes. Lower levels of butyrate can result in alterations of the mucosal integrity and microbial translocation(604). The local inflammation occasioned by microbial translocation fuels systemic inflammation, which echoes in the atherosclerotic plaque within the artery wall(605).

As mentioned previously, PLWH have an increased cardiovascular risk compared to the general population. The systemic inflammation and chronic immune activation observed in PLWH have been associated with establishing atherosclerosis(606) and one of the drivers of this systemic inflammation is microbial translocation from the intestine as a result of the depletion of Th17 cells(607, 608). In the **third article of this thesis**, we evaluated the immunological signature associated with CVD in a cohort of ART-treated PLWH enrolled in the Canadian HIV/Aging Cohort (CHACS). We observed that PLWH with subclinical atherosclerosis in the coronary arteries showed increased plasma fibrinogen levels, decreased Th17 frequencies and Th17/Treg ratio, and high frequencies of CCR9lowHLA-DRhigh non-classical monocytes compared to PLWH without coronary plaque. This study suggests that alterations in the mucosal barrier integrity driven by the depletion of Th17 cells may contribute to the establishment of atherosclerosis in ART-treated PLWH. Overall, the results presented in this thesis strengthened the relevance of Th17 cells in HIV pathogenesis and co-morbidities.

The persistent immune activation driven by the mucosal damage observed in PLWH receiving ART is accompanied by a pro-coagulative state(609). D-dimer is a product of the coagulation pathway, which is increased in PLWH and has been associated with increased cardiovascular risk(610). Fibrinogen, a precursor of D-dimer, is a strong independent predictor of mortality in HIV-infected adults(611). The coagulation cascade activation led to the activation of thrombin and cleavage of fibrinogen in fibrin that, upon polymerization, form a fibrin clot. D- dimer is the degradation product of fibrin. We observed that PLWH with coronary plaque had increased levels of fibrinogen in comparison with PLWH without plaque. Our results suggest that a subgroup of PLWH have an increased potential to form clots despite ART and is accompanied by an activation of the coagulative cascade reflected by increased levels of D-dimer(610). The treatment of PLWH with edoxaban, a factor X inhibitor and blocker of the coagulative pathway, showed a significant decline in D-dimer but no changes in inflammation or immune activation(612). These results suggest that targeting the coagulation pathway may not ameliorate the state of immune activation.

Fibrinogen is primarily produced by hepatocytes in the liver in response to IL-6 during the acute response to inflammation(613). IL-6 predicts morbidity and mortality in PLWH and has been considered a therapeutic target to reduce cardiovascular inflammation(614). Targeting IL-6 may improve systemic inflammation and reduce the pro-coagulative state of PLWH since it is the primary driver of fibrinogen production. In patients with rheumatoid arthritis, tocilizumab significantly reduced fibrinogen levels compared to those not treated with tocilizumab(615). In PLWH, the administration of tocilizumab reduced the indices of inflammation but increased the levels of total plasma lipids(616). It was not reported if changes in D-dimer or fibrinogen were observed. This alteration in the lipid profiles and the changes in the pro-coagulative state of PLWH requires further study.

In addition to IL-6, markers of activity of the IL-1 system were associated with cardiovascular events in PLWH(617). IL-1β is an essential upstream inflammatory cytokine in establishing

inflammatory disorders, including atherosclerosis(617, 618) and it is also a positive regulator of IL-6 production. In HIV-negative individuals with established cardiovascular disease, the inhibition of IL-1β with canakinumab(antibody that targets IL-1β) reduced the risk of cardiovascular events(619). In a small group of 10 PLWH, the administration of canakinumab reduced systemic markers of inflammation, such as IL-6, C-reactive protein, and sCD163. There were no changes in T-cell activation or monocyte subsets but a reduction of monocytes producing IL-6 and IL-1β. Canakinumab also reduced arterial inflammation, measured by fluorodeoxyglucose-positron-emission tomography/computed tomography(620). It would also be necessary to address how this immunological intervention may impact intestinal immunity since, according to our findings, the depletion of Th17 may contribute to atherosclerosis.

As mentioned earlier, there is robust evidence that early ART implementation reduces morbidity in PLWH(621). The Strategic Timing of Antiretroviral Therapy (START) and Temprano trials proved that immediate ART initiation reduced the risk of AIDS and non-AIDS events compared to delayed ART initiation(621-623). In line with this, early ART initiation effectively attenuated systemic inflammation(327), normalized levels of sIL-6R and D-dimer, and partially preserved intestinal Th17 cells(327, 624). The levels of systemic inflammation were also lower when ART was initiated during acute HIV infection rather than in the chronic phase. Despite this improvement, C-reactive protein and sCD14 levels decreased but were still higher than in HIV-negative individuals(624). The re-establishment of Th17 cells and amelioration of systemic inflammation points to the direct contribution that Th17 could have in establishing non-AIDS co-morbidities such as atherosclerosis. Therefore, the supplementation of ART with Th17-specific drugs (*e.g.,* RORC2 inhibitors) may preserve this pool of cells from HIV-mediated depletion and facilitate their positive role in the maintenance of gut-barrier integrity thus reducing the long-term CVD risk in ART-treated PLWH.

Chapter 7: Conclusion

The original work performed throughout the three manuscripts presented in the context of this thesis allowed us to have a deeper understanding of the immunopathology of HIV on the Th17 compartment. Indeed, our results highlight the importance that Th17 cells play in HIV infection at different stages of the disease, and this allows us to propose therapeutic strategies to counteract the pathogenic effect of HIV through these cells. In that aspect, it is of essence to keep in mind the complexity of the HIV infection and to nuance the possible benefits that a therapeutic intervention can have depending on the stage of the disease and the immune state at that precise time point (Figure 7).

In the acute phase, the fact that Th17 cells are highly permissive to the virus and attenuate the response to type I interferon suggests that using RORC2 inhibitors would decrease the replication of the virus in these cells (Figure 7A). In fact, as Th17 cells are the first to be infected, it is suggested that these cells could be an important vehicle of viral amplification despite their fundamental role as protectors of the mucosa. Inhibition of RORC2 could thus be beneficial by lowering Th17 depletion, reducing damage to the intestinal mucosa, and limiting the dissemination of HIV. Additionally, by blocking the production of IL-17, the response to interferon would be improved, limiting the spread of the infection. That being said, the administration of RORC2 inhibitors should be done with caution in people living with HIV due to the precedent that blocking IL-17 in patients with inflammatory bowel disease resulted in adverse effects. However, this may differ based on observations in mice where the use of RORC2 inhibitors improved the integrity of the intestinal barrier by mechanisms that have yet to be determined.

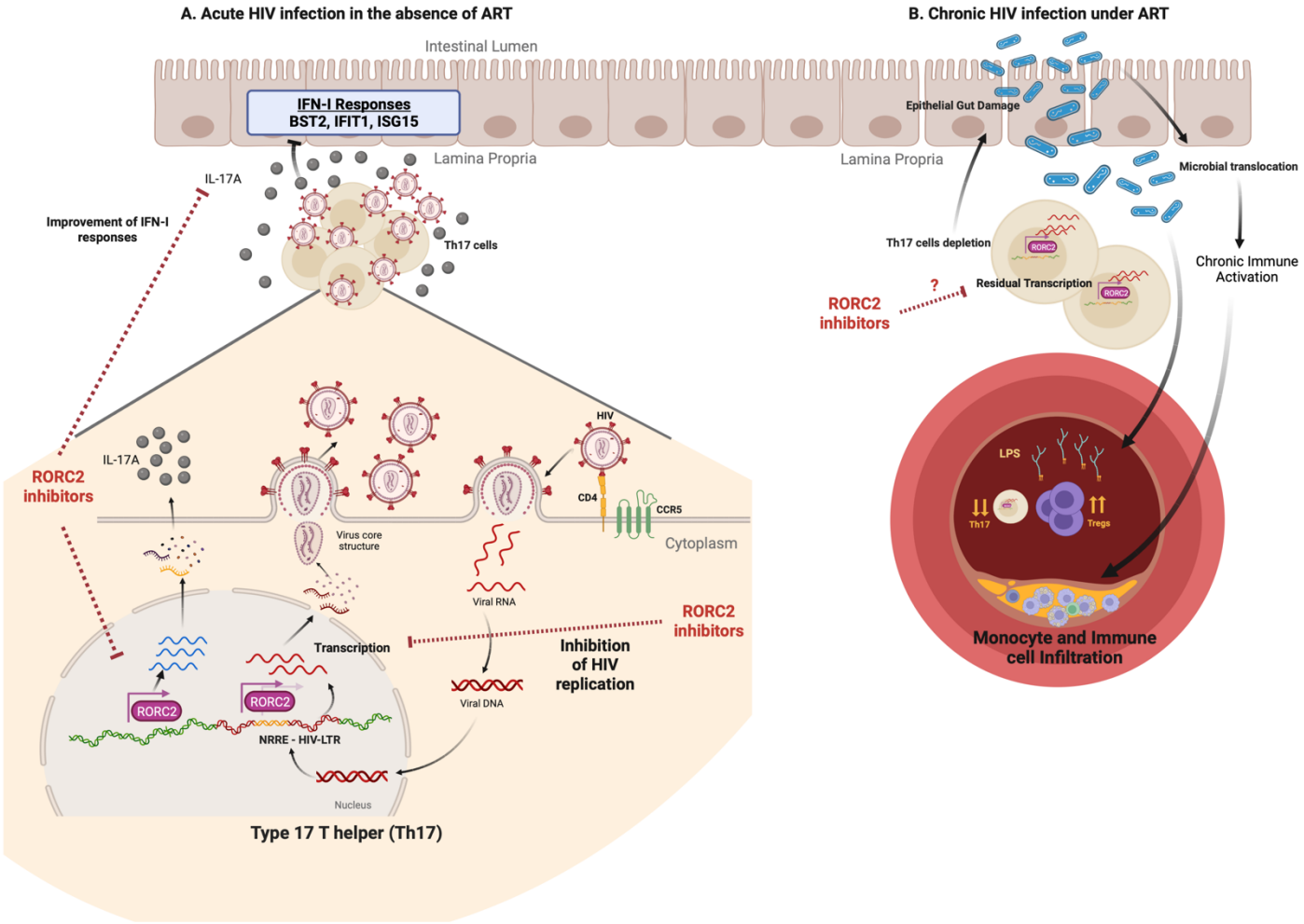
At a later stage of HIV infection, when people are under ART, we observed that the level of depletion of Th17 cells is associated with the presence of cardiovascular disease. The initiation of early antiretroviral therapy with RORC2 inhibitors would prevent the depletion of Th17 cells and, therefore, the damage to the intestinal mucosa, maintaining mucosal integrity and preventing the chronic inflammation that promotes the development of cardiovascular disease. In the context where the administration of RORC2 inhibitors would be done subsequently to ART

initiation, it could contribute to blocking residual HIV replication and transcription, thus reducing once again the chronic inflammation due to viral products (Figure 7B). Therefore, there would be important benefits in blocking Th17 functions both at the acute and chronic phases of HIV infection.

Despite those potential advantages of inhibiting Th17 functions, the important role that these cells play in maintaining the integrity of the mucosal barrier cannot be forgotten. Considering the fact that the alteration of the gut barrier also has a fundamental impact on inflammation and that the Th17 compartment is not completely restored under ART, another alternative would be to rather promote the reconstitution of the Th17 pool to re-establish intestinal homeostasis. Since active virus replication is blocked in patients receiving ART, the chances of re-infecting Th17 cells would be minimal.

In other words, the modulation of the Th17 function during HIV infection is a delicate balance of risks and benefits. Considering their permissiveness to the infection, but also their vital function to gut homeostasis, therapies targeting Th17 cells in HIV infection should carefully consider the stage of the disease in which they are given, and the work presented in this thesis can be of great utility in this objective.

Figure 7: Modulation of Th17 responses through RORC2 inhibition as a potential therapeutic target in acute and chronic HIV infection.



Chapter 8: Perspectives in the SARS-CoV-2 Era

Large numbers of discoveries performed in the HIV field were extrapolated to other pathological conditions. Some examples include AMD3100, a CXCR4 inhibitor designed for HIV entry inhibition(625), is currently used to mobilize stem cells from the bone marrow into the blood in the context of transplantation(626). Also, Maraviroc, a CCR5 inhibitor designed to block HIV entry, is currently used for improving the clinical outcomes upon brain stroke. Similarly, we consider some of the discoveries included in this thesis may be extrapolated to pathological conditions other than HIV, especially those associated with deleterious Th17 functions. One example is SARS-CoV-2 infection for which we presented our opinion in the review article below.

Targeting the Interleukin-17 Pathway to Prevent Acute Respiratory Distress Syndrome Associated with SARS-CoV-2 Infection

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Targeting the Interleukin-17 Pathway to Prevent Acute Respiratory Distress Syndrome Associated with SARS-CoV-2 Infection

The novel coronavirus causing severe acute respiratory syndrome (SARS-Cov-2) is responsible for coronavirus disease 2019 (COVID-19) pandemic. This is spurring a global response and accelerating trials of a panoply of antivirals, antibiotics, cell therapies, anticoagulants, convalescent plasma infusion, and immune modulation using steroids and anti-cytokine therapies. In the absence of SARS-Cov-2-specific interventions to treat the infection, there are urgent needs to identify adjunctive treatments that prevent or counteract the "cytokine storm" underlying the severe acute respiratory distress syndrome (ARDS) manifestations (1, 2). Trials underway using immune modulation focus on targeting interleukin (IL)-6, IL-6 receptor (IL-6R), tumor necrosis factor-alpha (TNF-□), IL-1R, granulocyte-macrophage colony-stimulating factor (GM-CSF), and janus kinase (JAK) inhibition among others. Here, we provide the rationale for considering clinical trials testing of IL-17 blockade as a therapeutic strategy for overt pulmonary inflammation caused by SARS-CoV-2 infection.

IL-17 plays a key role in the "cytokine storm" observed in ARDS of any cause and is associated with alveolar inflammation and a poor prognosis (3-5). In mouse models, both the direct IL-17 blockade and the upstream blockade of histone acetyltransferase p300 (p300) and transcription factor retinoic acid receptor-related orphan receptor gamma t (RORγt), which up-regulate IL-17 production, resulted in an attenuation of the lung injury (6, 7). Consistently, peripheral blood mononuclear cells (PBMCs) from ARDS patients have an increased expression of p300 and RORγt, especially among non-survivors (7).

In severe compared to non-severe COVID-19, different studies found increased levels of IL-17-regulated cytokines, including IL-6, MCP-1, IL-8, G-CSF, MIP-1α, and TNF-□; however, IL-17 was only increased in severe cases compared to non-infected controls (2, 8, 9). Another study observed that IL-17 distinguished between mild and severe cases and correlated positively with

an increased lung injury severity score (10). A pathological assessment found a high frequency of peripheral Th17 in a patient with severe COVID -19 who did not survive (11). Furthermore, IL-17 plays a role in facilitating early neutrophil recruitment into the lungs, a deleterious phenomenon associated with poor prognosis in severe cases of COVID-19 (12).

Activation of the IL-17 pathway is also a marker of severity in various other known viral infections. Infections due to the MERS-CoV outbreak in 2012 were associated with a proinflammatory Th1 and Th17 cytokine profile and IL-17 responses (13-15). In the 2009 Influenza A (H1N1) pandemic, the IL-17 response played a detrimental role in lung injury and was higher in patients with ARDS who did not survive (16, 17). In childhood Respiratory Syncytial Virus (RSV) infections, high IL-17 expression was associated with a poor interferon (IFN) production, abrogated type I IFN (IFN-I) responses, and RSV infection severity (5, 18, 19). IFN-I is implicated in reducing viral spread, and high levels of IFN-I seem particularly relevant in the early infection phase for disease control. Indeed, in mice, early IFN-I administration was protective against MERS-CoV lung disease (20, 21). Noteworthy, our observations revealed that IL-17A decreased IFN-I responses in intestinal epithelial cells, thus favoring Human Immunodeficiency Virus type 1 (HIV-1) cell-to-cell spread (22). Similarly, in Simian Immunodeficiency Virus (SIV) infections, mucosal IFN-I responses only developed at late time points post-infection and coincided with a vanished IL-17 response (23). These results point to the detrimental role of IL-17 in mounting a rapid IFN-I-mediated antiviral response. However, the beneficial impact of IFN-I on human lung diseases remains under investigation. A recent trial in ARDS patients reported an absence of benefit (24), although this was not in the context of a viral infection.

Finally, while IL-6 has garnered much interest as a potential target to improve COVID-19 outcomes (25, 26), it is important to emphasize that it has interdependent relationships with IL-17. IL-6, along with proinflammatory IL-23 and other molecules, are upstream inducers of T-helper 17 cell (Th17) differentiation and subsequent IL-17 production. IL-17 then has diverse downstream proinflammatory effects increasing neutrophil activity, TNF-□ secretion, and inducing IL-6 production (5). This all leads to a positive inflammatory feedback loop (27). In fact,

the rationale for the current interest with JAK blockade in COVID-19 derives from its role in mediating cytokine production, with JAK 2 mediating Th17 responses (28). Moreover, IL-17 and IL-6 synergistically promote viral persistence (29). In mice, IL-17 blockade improved H1N1-induced acute lung injury and decreased the levels of cytokines IL-1β, G-CSF, MCP-1, MIP-1α, MIP-1β, and TNF-□ (16). Additionally, in viral myocarditis, IL-17 blockade abolished viral replication and decreased levels of IL-6 (30). Noteworthy, in COVID-19, myocarditis was observed in the context of ARDS (31). Thus, IL-17 blockade may be beneficial in controlling the "cytokine storm" while boosting antiviral IFN-I responses during SARS-CoV-2 infection (Figure 1). Consideration of IL-17 blockade is strengthened by the relative absence of adverse inflammatory lung manifestations when these therapies are used for autoimmune conditions such as psoriasis (32, 33). Since the initial submission of this Commentary, other groups supported the idea of blocking IL-17 for controlling overt inflammation in COVID-19 patients (34, 35). In addition to its pro-inflammatory role, our most recent studies originally support a pro-viral role of IL-17 by interfering with IFN-I production/responses (22). Therefore, we consider that early IL-17A blockade will also boost the control of viral replication by the host.

In conclusion, as we rapidly explore existing immunotherapies to be repurposed as adjunctive treatments for SARS-CoV-2-associated ARDS, IL-17 blockade may represent an interesting avenue that deserves testing, especially in people with pre-existent pathologies associated with exacerbated IL-17 responses. Monoclonal antibodies against IL-17A (Secukinumab, Ixekizumab) and IL-17r (Brodalumab) may represent possible therapeutic options.

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FIGURES

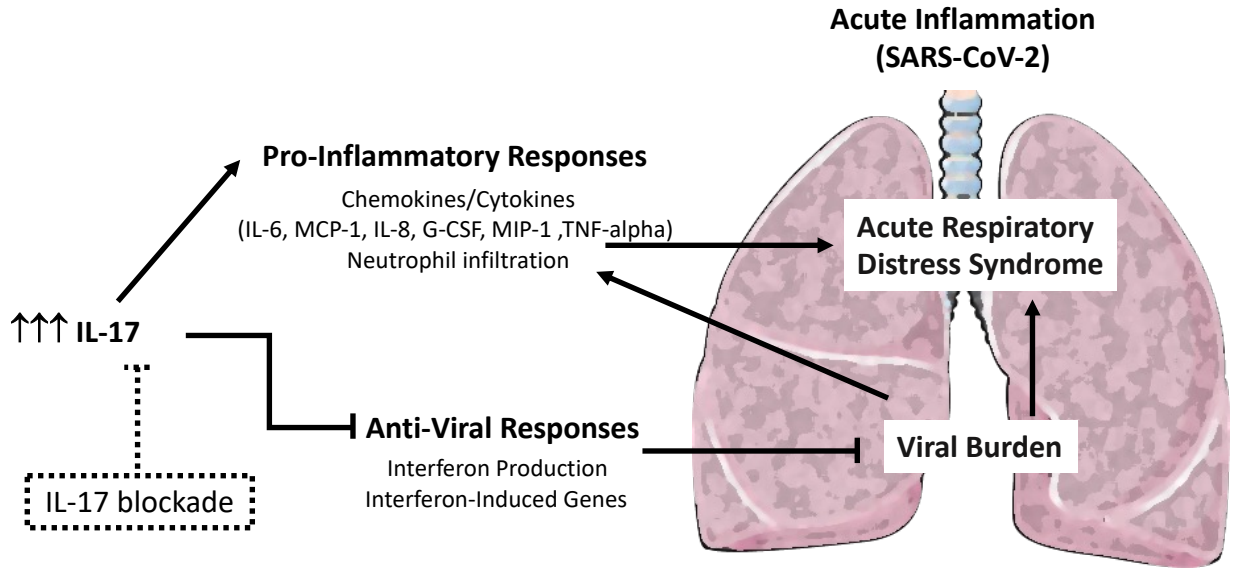

Figure 1. Blocking interleukin (IL)-17 as a strategy to boost antiviral responses and diminish exacerbated lung inflammation leading to acute respiratory distress syndrome in severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections.

Annexes

Annex 1 : Publications

First Author Publications

Wiche Salinas TR#, Zhang Y#, Sarnello D, Zhyvoloup A, Marchand LR, Fert A, et al. Th17 cell master transcription factor RORC2 regulates HIV-1 gene expression and viral outgrowth. Proc Natl Acad Sci U S A. 2021;118(48).#equal contribution

Wiche Salinas TR, Gosselin A, Raymond Marchand L, Moreira Gabriel E, Tastet O, Goulet JP, et al. IL-17A reprograms intestinal epithelial cells to facilitate HIV-1 replication and outgrowth in CD4+ T cells. iScience. 2021;24(11):103225.

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Co-author Publications

Chatterjee D#, Zhang Y#, Wiche Salinas TR, Ngassaki-Yoka C-D, Chen H, Smail Y, et al. Identification of Aryl Hydrocarbon Receptor as a Barrier to HIV-1 Infection and Outgrowth in CD4+ T-Cells. bioRxiv. 2022:2022.10.17.512596.#equal contribution

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Annex 2: Scientific presentations

Oral Presentations

(2022) An immunological signature for subclinical atherosclerosis in people living with HIV-1 receiving antiretroviral therapy. The 24th International AIDS Conference. Montreal, Canada, July 29-August 2, 2022.

(2021) Th17 cell master transcription factor RORC2 regulates HIV-1 gene expression and viral outgrowth. Keystone Symposia HIV Pathogenesis and Cure. Colorado, USA, June 1-4,2021.

Poster Presentations

(2021) Th17 Cell Master Transcription Factor RORC2 Regulates HIV-1 Gene Expression and Viral Outgrowth. 30th Annual Canadian Conference on HIV/AIDS Research (CAHR), Virtual, Canada, May 5-7, 2021.

(2021) Th17 Cell Master Transcription Factor RORC2 Regulates HIV-1 Gene Expression and Viral Outgrowth. Conference on Retrovirus and Opportunistic Infections (CROI) 2021, Virtual, USA, March 6-10, 2021.

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