Université de Montréal

Mécanismes moléculaires de permissivité à l'infection par le VIH dans les lymphocytes T CD4$^+$

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Thèse présentée
en vue de l'obtention du grade de Docteur
en Microbiologie et Immunologie

Juin, 2019

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RÉSUMÉ

Le virus de l'immunodéficience humaine de type 1 (VIH-1) cause une infection chronique caractérisée par la perte progressive des cellules T CD4+ et une susceptibilité accrue aux infections opportunistes. Les cellules T CD4+ polarisées Th17 jouent un rôle important dans l'immunité des muqueuses et le maintien de l'intégrité de la barrière intestinale. Cette population cellulaire est aussi la cible principale du VIH et est rapidement épuisée au niveau intestinal après l'infection. La thérapie antirétrovirale (TAR) ne permet pas de restaurer la fréquence et les fonctions des cellules Th17 intestinales, ce qui accroit le risque de comorbidités non-SIDA chez les sujets infectés sous TAR. Il est donc important d'identifier des stratégies thérapeutiques supplémentaires pour restaurer l'immunité de la muqueuse intestinale chez les personnes vivant avec le VIH recevant la TAR. Des études précédentes au laboratoire ont montré que le VIH persiste préférentiellement dans les cellules T CCR6+ à profil de polarisation Th17 du colon chez les sujets avirémiques sous TAR. De plus, l'induction du tropisme intestinal via l'acide rétinoïque (AR) augmente la permissivité à l'infection par le VIH des cellules T CCR6+.

Par le biais de mon projet doctoral, nous avons exploré les mécanismes par lesquels les cellules T CCR6+ à tropisme intestinal supportaient la réplication virale pour ainsi pouvoir identifier des nouvelles cibles thérapeutiques antirétrovirales et immuno-régulatrices. La première partie de ces travaux de thèse ont permis de montrer que l'induction du tropisme intestinal via l'AR active la voie Akt/PI3K/mTORC1 dans les cellules T CCR6+. Nous avons démontré que les inhibiteurs du complexe mTORC1 (rapamycin) ou mTORC1/2 (INK128) abrogent les effets de l'AR sur la réplication/réactivation du VIH dans les cellules T CCR6+. De plus, de hauts niveaux d'expression de CCR5 et de mTOR phosphorylé ont été observés dans les cellules T CCR6+ qui ont infiltré le colon des individus sous TAR, ce qui fournit une explication à la permissivité et à la persistance préférentielle du VIH dans ces cellules.

Sur la base de ces travaux démontrant que le VIH cible sélectivement les cellules CCR6+Th17 à tropisme intestinal via des mécanismes mTOR-dépendants, nous avons testé l'administration clinique de la metformine, un agent inhibiteur de mTOR chez des sujets vivant avec le VIH sous TAR dont les résultats constituent la deuxième partie de cette thèse. Dans le cadre de ces travaux, nous avons exploré les effets de la metformine sur la taille des réservoirs du VIH dans le colon et sur la diminution de l'inflammation persistance chez les participants. Malgré une réduction subtile des marqueurs plasmatiques de l'inflammation et des altérations de la barrière intestinale, la metformine a diminué de manière significative plusieurs paramètres dans le colon incluant la fréquence/activation des lymphocytes T CD4+ et l'expression du mTOR phosphorylé dans les cellules T CCR6+. Ces modulations sont associées, chez 68% des participants, à une réduction de la transcription résiduelle du VIH dans le colon.

Finalement, dans une stratégie alternative, nous avons exploré la possibilité de restaurer les fonctions effectrices des cellules Th17 dans le but de rétablir l'immunité mucosale chez les personnes traitées séropositives pour le VIH. Des études précédentes au laboratoire ont identifié PPARγ, exprimé dans cellules Th17, comme un régulateur négatif de la transcription du VIH et de RORC, le facteur de transcription spécifique aux cellules Th17. Dans la troisième partie de cette thèse, nous avons exploré les effets de l'inhibition pharmacologique de l'activité de PPARγ dans une stratégie de type « shock and kill » pour éradiquer le VIH de l'organisme tout en promouvant les fonctions effectrices des cellules Th17. Nos résultats montrent que l'antagoniste de PPARγ T0070907 entraîne une augmentation de la production d'IL-17A et de la transcription du VIH. Contre toute attente, T0070907 inhibe également la

libération des virions et la réplication du VIH *in vitro*. Les études du profil transcriptionnel au niveau du génome entier ont révélé de multiples voies de signalisation et transcrits modulés par l'antagoniste de PPARγ, incluant : i) une diminution des facteurs impliqués dans la permissivité au VIH (e.g., CCR5, furine); ii) une augmentation de ceux relatifs à la restriction par le VIH (e.g., TRIM22, BST2, miR29) et iii) une augmentation des fonctions effectrices Th17 (e.g., RORγt, STAT3, BCL6, IL-17A/F, IL-21). D'une importance majeure, T0070907 augmente considérablement la production d'IL-21, une cytokine essentielle à la survie des cellules Th17, présentant des propriétés d'une part immuno-régulatrices et d'autre part antivirales.

En conclusion les travaux de cette thèse désignent les cellules T CCR6+ comme étant des cibles thérapeutiques de choix dans les stratégies de guérison/rémission du VIH, notamment au niveau de l'intestin. Ces études proposent l'utilisation des drogues avec des effets immunomodulateurs, notamment des inhibiteurs de mTOR ou des antagonistes de PPARγ, pour des stratégies de rémission/éradication du VIH chez les personnes sous TAR.

Mots-clés : VIH-1, Th17, CCR6, intestin, acide rétinoïque, metformine, PPARγ

ABSTRACT

Human immunodeficiency virus type 1 (HIV-1) causes a chronic infection characterized by a progressive loss of CD4+ T-cells and an increased susceptibility to opportunistic infections in people living with HIV (PLWH). Th17-polarized CD4+ T-cells play an important role in mucosal immunity and in the maintenance of the intestinal barrier integrity. Mucosal Th17 cells are also the first and main targets of HIV infection and are rapidly depleted after infection, especially in the intestinal mucosa. Antiretroviral therapy (ART) does not restore the frequency and function of intestinal Th17 cells. The paucity of Th17 cells increases the risk of non-AIDS comorbidities in ART-treated PLWH. Thus, it is important to identify additional therapeutic strategies to restore mucosal immunity in these individuals. Previous laboratory studies have shown that HIV persists preferentially in colon- infiltrating CCR6+Th17-polarized T-cells from aviremic subjects on ART. In addition, induction of intestinal tropism via retinoic acid (RA) preferentially increases the permissiveness to HIV infection of CCR6+ T-cells.

At the beginning of my Ph.D. training, molecular mechanisms underlying HIV permissiveness in gut-homing CCR6+ T-cells remained to be elucidated in view of the identification of new antiviral and immune-regulatory therapeutic strategies.The first part of this thesis demonstrated that the induction of intestinal tropism via AR activates the Akt/PI3K/mTORC1 pathway in CCR6+ T-cells. We have shown that mTORC1 (rapamycin) or mTORC1/2 (INK128) inhibitors abrogate the effects of AR on HIV replication/reactivation in CCR6+ T-cells. In addition, high levels of CCR5 and phosphorylated mTOR were observed in colon infiltrating CCR6+ T-cells from ART-treated PLWH, providing an explanation for the permissiveness and preferential persistence of HIV in these cells.

This work demonstrating that HIV selectively targets gut-homing CCR6+ T-cells via mTOR-dependent mechanisms, prompted us to investigate the clinical benefits of metformin, an indirect mTOR inhibitory well-tolerated drug, in ART-treated PLWH. Thus, in the second part of this thesis, we explored the effects of metformin on the size of HIV reservoirs in the colon and on the persistent inflammation. Despite a subtle reduction in plasma markers of inflammation and alterations of the intestinal barrier, metformin significantly decreased several parameters in colon infiltrating T-cells, including a decrease of the frequency/activation of CD4+ T-cells and a decrease of the phosphorylated mTOR expression. These modulations were associated, in 68% of the participants, to a reduction of the residual HIV transcription in the colon.

Finally, in an alternative strategy, we explored the possibility of restoring the effector functions of Th17 cells in order to restore mucosal immunity in ART-treated PLWH. Previous studies in the laboratory identified the expression of PPARγ in Th17 cells as a negative regulation of HIV and RORC transcription. In the third part of this thesis, we explored the effects of the pharmacological inhibition of PPARγ activity as a « shock and kill » strategy for the HIV eradication and amplification of Th17 effector functions. Our results demonstrated that the PPARγ antagonist T0070907 leads to an increase in IL-17A production and in HIV transcription. Unexpectedly, T0070907 inhibited virion release and HIV replication *in vitro*. Whole genome transcriptional profile studies revealed multiple signaling genes/pathways modulated by the PPARγ antagonist, including: ***i)*** a decrease expression of factors involved in HIV permissiveness (e.g., CCR5, furin); ***ii)*** an increase expression of HIV restriction/resistance (e.g. TRIM22, BST2, miR29) and ***iii)*** an increase of genes related to Th17 effector functions (e.g. RORγt, STAT3, BCL6, IL-17A / F, IL-21). Of major importance, PPARγ antagonism significantly increases the production of IL-21, a cytokine with properties acting as immunoregulatory and antiviral shares.

In conclusion the work of this thesis designates the CCR6+ T cells as being key therapeutic targets, especially in the intestine and proposes to further explore the clinical benefits of using immune-modulatory drugs such as mTOR inhibitors or PPARγ antagonists for HIV cure/remission strategies in ART-treated PLWH.

Keywords : HIV-1, Th17, CCR6, retinoic acid, gut-homing, metformin, PPARγ

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LISTE DES SIGLES

A

ABCA1; ATP- binding cassette sub-family B member 1
ADN ; acide désoxyribonucléique
ALIX; ALG2-interacting protein X
APOBEC3G; apolipoprotein B MRNA Editing Enzyme Catalytic Subunit 3G
AR; acide rétinoique
ARN ; acide ribonucléique
ARVs; antirétroviraux
ATRA; all-trans retinoic acid
AZT; azidothymidine (zidovudine)

B

bNAbs; broadly neutralizing antibody
BST-2; bone marrow stromal antigen 2 (tetherin)

C

CA; capside (p24)
CCR ; C-C chimiokine
CD4 ; lymphocytes T portant le cluster de différenciation (CD)4
CDK9 ; Cyclin-dependent kinase 9
cGAS ; cyclic GMP-AMP synthase
CMH ; complexe majeur d'histocompatibilité
CMV ; cytomegalovirus
CPA ; cellule présentatrice d'antigènes
CPI ; complexe de pré-intégration
CRISPRCas9; Clustered Regularly Interspaced Short Palindromic Repeats Cas associated protein- 9
CXCR ; CXC chemokine receptors
CypA ; cyclophiline A

D

DC ; cellule dendritique
dNTP; désoxynucléosides triphosphates

E

EAE ; Experimental Autoimmune Encephalomyelitis
EI ; inhibiteur du site de l'entrée (*entry inhibitors*)
Env ; protéine d'enveloppe
ESCRT-I, ESCRT-III; endosomal sorting complex required for transport I et III

G

GALT; tissus lymphoïdes associés à l'intestin (*gut-associated lymphoid tissues*)
GLUT1 ; glucose transporter 1
gp ; glycoprotéine

H

HAART ; thérapie antirétrovirale hautement active (*highly active anti-retroviral therapy*)
HDAC; histones désacétylase
HIV-1; Human immunodeficiency virus type 1
HPV ; human papilloma virus
HSV; herpes simplex virus
HTLV-1 ; virus T-lymphotropique humain 1

I

ICOS; inducible T-cell costimulator
IFIT16 ; interferon-γ inducible protein 16
IFITM; interferon-induced transmembrane proteins
IKK-b; inhibitor of nuclear factor kappa-B kinase subunit beta
IL; interleukine
IN; intégrase
INSTIs ; inhibiteurs de l'intégrase (*integrase inhibitors*)
Iono; ionomycine
iPPP; phosphates inorganiques
IRF3; interferon regulatory factor 3
ISGs ; IFN-stimulated genes

L

LAG3; lymphocyte-activation gene 3
LAV; lymphadenopathy associated virus
LEDGF/p75; lens epithelium-derived growth factor
LFA-I; lymphocyte function-associated antigen 1
LRA; latency reversing agent
LTR; long terminal repeat

M

MΦ ; macrophages
MA; matrice [p17]
MadCAM ; mucosal vascular addressin cell adhesion molecule 1
MDR1 ; multidrug resistance protein 1
MS; multi-épissé (*multiply spliced*)
mTOR ; mechanistic target of rapamycin

N

NC ; nucléocapside [p7]
NES; signal d'export nucléaire (*nuclear export signal*)
NF-κB ; nuclear factor-kappa B
NFAT ; nuclear factor of activated T-cells
NNRTIs ; inhibiteurs non nucléosidiques de la transcriptase inverse (*non nucleoside reverse transcriptase inhibitors*)
NRTIs ; inhibiteurs nucléosidiques et nucléotidiques de la transcriptase inverse (*nucleoside reverse transcriptase inhibitors)*

Nup153; nucleoporine 153

P

PAMPs ; motifs moléculaires associés aux pathogènes (*pathogènes-associated molecular pattern*)
PD-1 ; programmed cell death 1
PIC ; complexe de pré-intégration (*pre-integration complexe*)
Pis ; Inhibiteurs de la protéase (*protease inhibitors*)
PKC ; protéine kinase C
PLWH; people living with HIV
PMA; phorbol 12-myristate 13-acetate
PPARγ; Peroxisome Proliferator-activated Receptor-gamma
PPRE ; peroxisome proliferator responsive element
PR ; protéase virale
PrEP; prophylaxie préexposition
PRR; pattern recognition receptor

R

RA; retinoic acid
RANBP2; RAs-related nuclear protein binding protein 2
RAR ; récepteur de l'acide rétinoïque
région TAR ; trans-activation response RNA element
RIG-I; retinoic-acid induced genes I
RING; really interesting new gene
RORγt; RAR-related orphan receptor gamma
RRE ; Rev responsive element
RT; transcriptase inverse (*reverse transcriptase*)
RTC ; complexe de rétro-transcription (*reverse transcription complexe)*
RXR ; récepteur X des rétinoïdes
RZG; rosiglitazone
SAMHD1; SAM domain and HD domain-containing protein 1
SIDA ; syndrome de l'immunodéficience acquise
STING; stimulator of interferon genes

T

TAK-1; TGF-β activated kinase 1
TAR ; thérapie antirétrovirale
TBK1; TANK-binding kinase 1
T_{CM} ; T mémoire centrale
TCR; T cell receptor
T_{EM} ; T mémoire effectrice
T_{FH}; T helper folliculaires
TGF-β; tumor growth factor-β
Th ; cellules T helper
TIGIT; T cell immunoreceptor with Ig and ITIM domains

TILDA; Tat/rev Induced Limiting Dilution Assay
T_N ; T naïve
TNF-β ; facteur de nécrose tumorale (*tumor necrosis factor*)
TNPO3; nuclear import factors transportin 3
TRAF2 ; TNF receptor associated factor 2
TRAF6 ; TNF receptor associated factor 6
T_{REG} ; cellule T régulatrice
TREX1; three prime repair exonuclease 1
TRIM5; tripartite motif-containing protein 5
T_{SCM} ; T mémoire stem cell
T_{TM} ; T mémoire transitionnelle

V

VIH-1 ; virus de l'immunodéficience humaine de type 1
VIHΔVif ; VIH déficient pour Vif
VIS ; virus de l'immunodéficience simien
VIS_{cpz} ; VIS infectant les chimpanzés
VIS_{SM} ; VIS infectant les sooty mangabey

LISTE DES ABRÉVIATIONS

Etc.; Et cætera

e.g.; exempli gratia

vs; versus

REMERCIEMENTS

Je tiens à remercier ma directrice de thèse Petronela Ancuta pour m'avoir permis de réaliser ces travaux. Merci pour votre soutient continuel pendant ces cinq années et votre enthousiasme contagieux pour la recherche. Nos discussions, qu'elles aient été professionnelles ou personnelles, m'ont fait beaucoup grandir. Merci pour tous ces partages et votre générosité.

Je tiens à remercier les membres du jury: Dr Jean-Sébastien Delisle, Dr Tarik Möröy, Dr Pierre Delobel et Dre Nathalie Arbour pour leur temps passé à lire ma thèse.

À tous les membres passés ou présents du laboratoire, vous avez tous contribué de près ou de loin à la réalisation de ces travaux. Je vous en remercie. Yuwei et Annie, vous avez été mes piliers pendant mes 3 premières années de thèse et encore aujourd'hui je ne fais pas une expérience sans penser à vos précieux conseils. Tomas, merci pour les discussions tardives que nous avons pu avoir ensemble. Je te remercie également pour m'avoir patiemment écouté et avoir réalisé les diagnostics de mes maladies imaginaires. Debashree, je ne pensais pas te remercier un jour pour cela, mais les 30 heures de marathon au P3 resteront une expérience mémorable. Merci d'avoir rendu cela possible.
Augustine, merci d'avoir été un binôme si serein et efficace... Un merci particulier à Laurence et Amélie. Je n'aurai jamais réussi à finir ce manuscrit sans vous. Merci pour les heures de relecture, le soutien moral et bien évident tous ces moments de rire et d'échanges… et ce n'est pas terminé…

Je tiens également à remercier certains membres du laboratoire voisin. Nicolas, de par les discussions scientifiques que nous avons pu avoir lors de nos pauses santé, tu as largement contribué à mes études (je ne te remercie par contre pas de m'avoir spoilé volontairement la moitié de mes séries…). Merci à Remi, Marta, Julia, Pierre et Amélie pour m'avoir continuellement écouté, guidé et aidé à générer mes résultats. Amélie, je te remercie encore pour ton expertise, ton aide et ta patience si précieuse dans l'étude LILAC.

Dr Routy je vous remercie de m'avoir fait confiance pour l'étude LILAC et également pour toutes vos contributions à nos études. Josée, ta générosité inspirante et ton aide ont contribué à tous les travaux de notre laboratoire. Je t'en remercie.

Car on ne fait pas une thèse sans avoir un soutien moral hors pair, je tiens à remercier mes amis et ma famille :
Mes amis du vindredi, merci pour votre grande générosité, vos précieux conseils et tous ces moments de grand délire… parfois non sans conséquence.
Mes amis du 210chill, merci pour votre amitié et votre soutien permanent durant les moments difficiles.

Je voudrais finir par remercier mes parents pour leur support moral et pour avoir supporté mon caractère des 4 derniers mois… bon, des 30 dernières années également. Je vous dédie ce manuscrit... que vous ne lirez jamais mais dont je vous sais fiers.

CHAPITRE I : INTRODUCTION

1.1. CONNAISSANCES ACTUELLES SUR LE VIH-1

1.1.1. DÉCOUVERTE DU VIH ET GÉNÉRALITÉS

Le virus de l'immunodéficience humaine de type 1 (VIH-1) appartient à la famille des *retroviridae* du genre lentivirus. L'infection par le VIH-1, si elle n'est pas traitée, cause le syndrome de l'immunodéficience acquise (SIDA) qui se caractérise par une diminution progressive du taux de lymphocytes T portant le cluster de différenciation (CD) 4 (<200 cellules/µl de sang) résultant en une plus grande susceptibilité face aux infections opportunistes [1] [2].

Les premiers cas de SIDA ont été observés aux États-Unis au début des années 1980 et furent rapportés dans le New York Times dès l'été 1981. C'est au sein de la communauté homosexuelle que les premiers signes de cette nouvelle maladie infectieuse apparaissent. Les symptômes les plus courants sont des pathologies pulmonaires (pneumocystose pulmonaire), digestives, des infections buccales graves et surtout une détérioration rapide et importante du système immunitaire [2].

Le virus fut isolé pour la première fois à l'Institut Pasteur de Paris à partir de cultures de cellules isolées de biopsies de ganglions lymphatiques d'une personne atteinte de lymphadénopathie généralisée. Barré-Sinoussi *et al*. détectent la présence d'ARN et d'une activité de transcriptase inverse dans le surnageant de culture de ces cellules. La visualisation, par microscopie électronique, de particules naissant à la surface des cellules en culture confirme la présence et la réplication d'un rétrovirus chez ce sujet [3]. La piste d'une infection par le virus T-lymphotropique humain 1 (HTLV-1), premier rétrovirus infectant l'humain découvert en 1977 et isolé en 1980 [4], a rapidement été écartée. Le nouveau virus est donc nommé LAV pour Lymphadenopathy Associated Virus. Ce virus ayant un tropisme sélectif pour les lymphocytes T $CD4^+$ fut séquencé en 1985 [5] [6]. En mai 1986, le comité international de taxonomie des virus annonce que l'agent infectieux causant le SIDA sera officiellement appelé VIH pour Virus de l'Immunodéficience Humaine [7].

Les virus de l'immunodéficience présentent une grande diversité génétique. Ils sont classés en 2 types : VIH-1 et VIH-2, chacun subdivisés en groupes et les groupes pouvant être subdivisés en sous-types. Le VIH-1, à l'origine de la pandémie, est le plus répandu à travers le monde. Il est génétiquement proche du virus de l'immunodéficience simien (VIS) infectant les chimpanzés (VIS_{cpz}) en Afrique de l'ouest. Le VIH-1 et le VIS_{cpz} sont responsables du SIDA chez l'humain et le chimpanzé respectivement. Le VIH-1 est très infectieux et pathogène et il possède une grande diversité génétique. Il en existe quatre groupes (M, N, O et P). Chacun d'eux est issu d'une transmission indépendante du VIS à l'humain [8]. Neuf sous-types (A, B, C, D, F, G, H, J et K) sont décrits au sein du groupe M (major), le plus fréquent en Europe et Amérique du Nord [9]. Les groupes N (new) et O (outlier) sont retrouvés chez les individus infectés en Afrique centrale [10]. Quant au groupe P (« *pending the identification of further human cases* »), il a été identifié plus récemment chez les femmes infectées au Cameroun [11]. Le VIH-2 est principalement retrouvé en Afrique de l'ouest et est génétiquement proche du VIS infectant le sooty mangabey (VIS_{SM}) [12]. La progression de la maladie est beaucoup plus lente suite à une infection par le VIH-2 comparativement au VIH-1 [13].

1.1.2. ÉPIDÉMIOLOGIE (GLOBAL HEALTH OBSERVATORY [GHO] DATA)

Depuis le début de l'épidémie, plus de 70 millions de personnes ont été infectées par le VIH causant le décès d'environ 35 millions d'entre elles. En 2017, 36.9 millions [31.1 millions–43.9 millions] d'individus vivaient avec le VIH et seulement 21.7 millions [19.1 millions–22.6 millions] avaient accès à la thérapie antirétrovirale (TAR). Il est estimé qu'uniquement 75% des personnes réellement infectées connaissent leur statut sérologique. Au cours de 2017, 1.8 millions [1.4 millions–2.4 millions] nouvelles infections ont été recensées et 940 000 individus [670 000–1.3 millions] sont décédés de maladies associées au SIDA. La tuberculose reste la cause de mortalité la plus élevée chez les sujets vivant avec le VIH. En 2016, environ 10% des personnes ayant développé la tuberculose étaient infectées par le VIH [14].

1.1.3. STRUCTURE DU VIH

Le VIH-1 est un rétrovirus enveloppé d'environ 100 nm de diamètre. L'enveloppe du virus est composée d'une double couche lipidique dérivée de la membrane cellulaire de la cellule hôte. Les glycoprotéines (gp) gp41 (transmembranaire) et gp120 (de surface) forment des trimères dans l'enveloppe virale. À l'intérieur de l'enveloppe, une matrice formée des protéines p17 entoure une capside constituée de protéines p24. Le génome du virus, contenu dans la capside, est constitué d'un simple brin d'ARN en double exemplaire. Les protéines virales telles que les enzymes transcriptase inverse et intégrase sont aussi retrouvées dans la capside [15] (Figure 1).

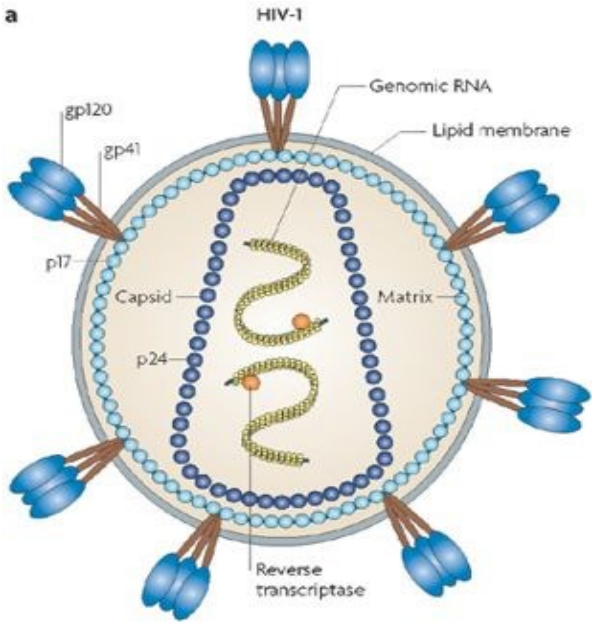

Figure 1. Structure du VIH-1.
L'enveloppe du virus est constituée des glycoprotéines gp120 et gp41 et renferme une matrice (protéines p17) et une capside (protéines p24) contenant deux copies d'ARN virale simple brin et les enzymes virales (intégrase et transcriptase inverse) [16]. Reproduite avec la permission de Nature - Licence number : 4558351507717.

1.1.3.1. Génome du VIH

Le génome du virus de 9 kilobases encode neuf gènes et est flanqué de chaque côté de séquences terminales longues (LTR; long terminal repeat). Ces séquences sont essentielles

pour l'intégration de l'ADN (*acide désoxyribonucléique*) viral dans l'ADN de la cellule hôte et contiennent des sites de liaison pour la protéine régulatrice Tat, impliquées dans la transcription du génome viral. La transcription de l'ADN proviral en ARN (*acide ribonucléique*) viral non-épissé génère les polyprotéines Gag et Gag-Pol. L'épissage alternatif de l'ARN viral génère quant à lui la protéine Env ainsi que les protéines accessoires [17] (Figure 2).

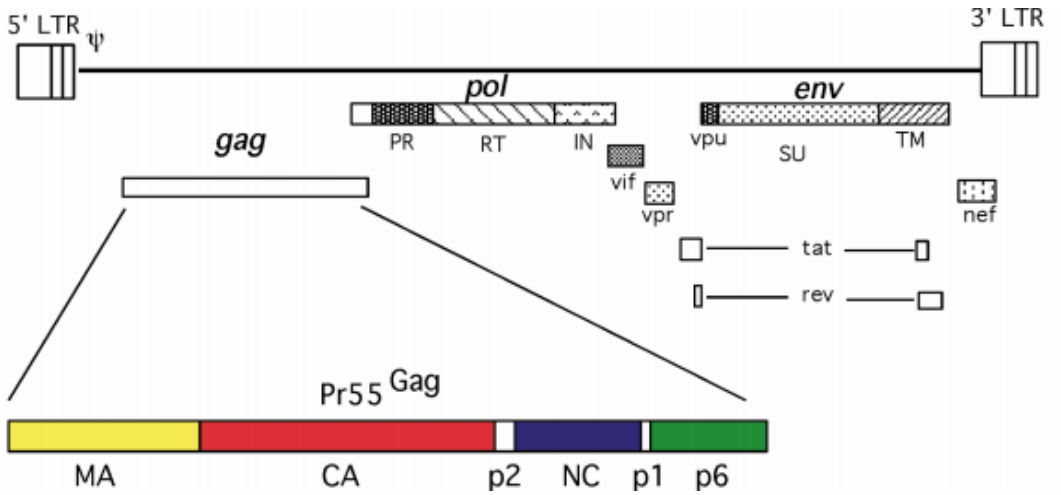

Figure 2. **Génome du VIH-1.**
Illustration des protéines structurelles, régulatrices et accessoires codés par le génome du VIH-1 [18]. Reproduite avec la permission de Virology - Numéro de licence : 4519040708652.

1.1.3.2. Protéines structurelles

Le gène *gag* code pour une polyprotéine précurseur, appelée p55, qui est traduite à partir de l'ARN viral non-épissé [19]. La polyprotéine p55 s'associe à la membrane de la cellule et recrute les deux copies d'ARN viral ainsi que les protéines virales et cellulaires impliquées dans le bourgeonnement des virions à la surface de la cellule infectée [19]. Lorsque les particules virales bourgeonnent à la membrane cellulaire, le processus de bourgeonnement, la polyprotéine p55 est clivée par la protéase virale (PR) en 6 protéines : MA (matrice [p17]), CA (capside [p24]), p6, NC (nucléocapside [p7]) et deux peptides « spacer » p1 et p2 [20]. Les protéines de la matrice forment une couche matricielle sous l'enveloppe du virus et stabilisent la particule [19]. Les protéines de la capside se regroupent entre elles pour former une structure conique qui entoure le génome viral [19]. La protéine p6 est impliquée dans

l'interaction entre p55 et la protéine virale accessoire Vpr afin d'assurer l'internalisation de Vpr dans le virion bourgeonnant [21]. Elle est aussi importante pour assurer la libération du virion à partir de la cellule infectée [19]. Quant à la protéine p7 de la NC, elle se trouve associée à l'ARN viral à l'intérieur de la particule tout comme l'intégrasse et la transcriptase inverse [19]. Elle est essentielle à l'internalisation spécifique de l'ARN viral dans le virion bourgeonnant et assure un assemblage et une maturation adéquate du virion en formation [22]. Entre les séquences de la CA et de la NC ainsi qu'entre celles de la NC et de la p6 se trouvent deux régions, p1 et p2 respectivement, codant pour des peptides spacer de quelques acides-aminés. Ces peptides jouent un rôle dans l'assemblage et l'infectiosité des virions bourgeonnant à la surface de la cellule infectée [23] [24].

Le précurseur Gag-Pol code pour les enzymes virales : protéase, intégrase (IN) et transcriptase inverse (RT; *reverse transcriptase*) [25]. L'intégrase permet l'insertion de l'ADN viral dans le génome de la cellule infectée grâce à ses trois activités : exonucléase, endonucléase et ligase [26]. En ce qui concerne la protéase aspartique, elle est nécessaire au clivage des polyproteines précurseurs Gag et Gag-Pol durant la maturation du virion [27]. La transcriptase inverse, elle, a une activité d'ADN polymérase ARN-dépendante et assure la transcription inverse de l'ARN viral en ADN double brin. Cette protéine possède une activité ribonucléase qui permet la dégradation de la matrice d'ARN durant le processus de transcription inverse. Elle ne possède cependant pas d'activité de relecture (« *proof reading* »), ce qui induit des mutations dans les nouvelles copies d'ADN viral synthétisées. C'est la raison pour laquelle le VIH a une grande variabilité génétique [28].

La protéine Env (gp160) est synthétisée à partir de l'ARN viral épissé. Après sa synthèse dans le réticulum endoplasmique et sa glycosylation dans le Golgi, Env migre au niveau de la membrane plasmique. Elle est alors clivée en gp41 et gp120 grâce à des protéines cellulaires comme la furin [29]. Les protéines gp41 et gp120 sont localisées à la surface de la cellule infectée et se retrouvent à la surface du virion produit par la cellule. La gp120 interagit avec les récepteurs CD4 et CCR5/CXCR4 de la cellule cible afin d'entrer dans la cellule cible. La gp41

est impliquée dans la fusion de la membrane virale et la membrane plasmique de la cellule cible [30] (*voir section 1.1.5 Cycle de réplication*).

1.1.3.3. Protéines régulatrices

Les protéines accessoires Tat et Rev sont générées à partir de l'ARN viral multi-épissé.
Tat est un transactivateur transcriptionnel essentiel à la réplication du VIH [31]. Par ailleurs, Tat est aussi impliqué dans la régulation de l'expression de plusieurs facteurs cellulaires incluant TNF-β (*tumor necrosis factor-β)*, TGF-β (*tumor growth factor-β)*, IL-6, Bcl-2, MIP-1α [32].

De son côté, Rev est une protéine ayant une séquence spécifique de liaison à l'ARN ce qui lui permet de s'attacher sur le site RRE (*Rev responsive element*) de l'ARN viral [33] [34]. Rev contient un signal d'export nucléaire permettant ainsi le transfert du complexe Rev/ARN viral du noyau vers le cytoplasme indispensable à l'export nucléaire des transcrits non-épissés ou semi-épissés [35].

1.1.3.4. Protéines accessoires

Bien que les protéines accessoires (Vif, Vpu, Nef et Vpr) soient nécessaires à la réplication virale dans certains types cellulaires, leurs rôles exacts sont longtemps restés incompris. Vif est impliqué dans l'assemblage et la maturation du virus [36], alors que Vpu, qui est exclusif au VIH-1, joue un rôle dans la dégradation de CD4 et dans la libération des particules virales [37]. Vpr est, de son côté, impliquée dans le transport nucléaire du complexe de pré-intégration (CPI) et facilite la transcription inverse [38]. Quant à Nef, cette protéine présente diverses activités incluant la down-régulation de CD4 et du complexe majeur d'histocompatibilité (CMH) de classe I et II à la surface de la cellule infectée, l'activation des cellules T et l'infectiosité du VIH [39] [40] [41]. C'est en étudiant le rôle de ces protéines accessoires dans la réplication virale que la plupart des facteurs impliqués dans les mécanismes de défense antiviraux intrinsèques ont été découverts (*voir section 1.1.7.2 Facteur de restriction*).

1.1.4. CIBLES CELLULAIRES DU VIH

Il est indispensable pour que le VIH entre dans la cellule que celle-ci exprime à sa surface le récepteur CD4 et les corécepteurs CCR5 ou CXCR4 [42] [43].Toutes les cellules exprimant ces récepteurs sont ainsi des cibles potentielles de l'infection. De ce fait, malgré un tropisme cellulaire majeur du VIH pour les lymphocytes T $CD4^+$, ce virus est capable d'infecter d'autres cellules immunitaires incluant les cellules dendritiques (DCs), les macrophages (MΦ) et les monocytes. Les MΦ et les DCs font partie du système immunitaire inné et sont les premières lignes de défense face aux infections [44]. Ce sont des cellules présentatrices d'antigènes qui permettent d'enclencher la réponse ciblée du système adaptatif grâce à la reconnaissance de signatures spécifiques [44]. Alors que la contribution directe des cellules myéloïdes dérivées des monocytes aux réservoirs du VIH sous TAR est exceptionnellement faible [45], la contribution des cellules myéloïdes d'origine embryonnaires, résidants dans tous les tissus incluant le cerveau (microglies), les poumons (MΦ alvéolaires) et le foie (cellules de Kupffer), reste un sujet en plein essor d'investigation. [46] [47] [48] [49] [50].

Les lymphocytes T $CD4^+$ par leur capacité à recruter les neutrophiles au niveau du site d'infection ou d'inflammation, par la sécrétion de cytokines et de chimiokines, leur capacité à renforcer l'immunité médié par les cellules B et à activer les MΦ pour une action microbicide, jouent un rôle important dans l'orchestration des réponses immunitaires adaptatives contre divers pathogènes [44].

L'identification des récepteurs de chimiokines CCR5 (*récepteur à C-C chimiokine de type 5; CD195*) et CXCR4 (*récepteur à C-X-C chimiokine de type 4; CD184*) comme les corécepteurs majeurs du VIH nécessaires à l'entrée des virus dans la cellule a permis une classification des souches virales [42] [43]. Ainsi, le VIH peut être divisé en trois classes selon son tropisme pour un certain type de corécepteur : souches R5 pour les virus qui utilisent le CCR5, souches X4 pour ceux utilisant le CXCR4 et souches à double tropisme R5/X4 pour les virus pouvant utiliser l'une ou l'autre des molécules [51]. L'interaction avec l'un ou l'autre ou les deux corécepteurs dépend de la séquence de la protéine d'enveloppe du virus. Indépendamment de ce tropisme,

le VIH infecte préférentiellement les lymphocytes T $CD4^+$ activés qui permettent la production massive de particules virales et ainsi la propagation efficace de l'infection à travers les différents sites anatomiques et cellulaires. Bien que la plupart de ces cellules infectées meurent en quelques jours, quelques-unes persistent pendant plusieurs années [52] [53].

1.1.5. CYCLE DE RÉPLICATION

Le cycle de réplication du VIH peut être divisé en deux phases : la phase précoce et la phase tardive.

1.1.5.1. Phase précoce

La phase précoce englobe les événements du cycle de réplication compris entre l'attachement du virus à la membrane cellulaire et l'intégration de l'ADN viral dans le génome de la cellule hôte [54].

L'entrée de virus dans la cellule cible se fait grâce à l'interaction spécifique entre la gp120 de l'enveloppe virale et la molécule CD4 exprimée à la surface de la cellule cible. Cette interaction entraîne un changement conformationnel de la gp120, permettant alors son interaction avec l'un des récepteurs aux chimiokines, CCR5 ou CXCR4. Un second changement de conformation de la gp120 conduit ensuite à la libération de la gp41 qui pénètre dans la membrane cellulaire pour induire la fusion des membranes virale et cellulaire [30] [51]. La capside virale, contenant les 2 copies d'ARN viral génomique, les enzymes RT et IN ainsi que les protéines accessoires, est alors libérée dans le cytoplasme de la cellule [55]. L'ARN viral est alors rétro-ranscrit en ADN viral double brin formant ainsi le complexe de rétro-transcription . La migration le long du réseau de microtubule de la cellule permet au génome viral d'atteindre le noyau. Cette relocalisation vers le noyau cellulaire est dépendante de la protéine CA, mais l'intégrité structurelle de la capside, à cette étape, est controversée. En effet, plusieurs scénarios de décapsidation sont encore proposés dans la littérature ; la capside pourrait se désintégrer i) immédiatement après la fusion de virus à la surface de la cellule cible, ii) graduellement durant sa migration vers le noyau ; ou iii) plus tardivement au niveau des pores nucléaire permettant ainsi une protection du génome viral vis-à-vis des senseurs d'ADN présents dans le cytoplasme

[56]. L'utilisation des techniques d'imagerie de cellules vivantes a toutefois apporté une idée plus précise de la cinétique et de la localisation de la décapsidation [57 , 58]. Des travaux récents montrent une décapsidation graduelle et dépendante de l'initiation de la transcription inverse [58]. Les auteurs indiquent que les protéines CA ainsi que leurs interactions avec les protéines de l'hôte (*voir section 1.1.8 Facteur de permissivité*) sont importantes pour assurer la migration et la pénétration du VIH au noyau cellulaire et que la capside joue un rôle protecteur durant ces étapes [58]. Dès lors que la rétro-transcription est terminée, le complexe de pré-intégration passe au travers des pores nucléaires afin d'être importé dans le noyau [56]. Alors, le génome viral s'intègre au sein de la chromatine de la cellule cible. Les étapes et les détails précis de ce processus sont cependant très peu connus. Plusieurs études ont identifié la protéine CA également impliquée dans le processus d'intégration de l'ADN viral dans le génome de la cellule hôte. Ceci suggère que quelques protéines CA persistent et protège le PIC (pre-integration complex) jusqu'au noyau [59]. Il faut aussi souligner que le VIH s'intègre préférentiellement au niveau des gènes transcriptionnellement actifs, incluant des gènes reliés à l'activation des cellules T et le métabolisme cellulaire [60] [61]. Certaines protéines de l'hôte sont impliquées dans l'intégration du HIV dans le génome de la cellule. Par exemple, LEDGF/p75 (*Lens Epithelium-derived growth factor*) peut lier simultanément l'intégrase du VIH et la chromatine afin de guider l'intégration du virus vers le site transcriptionnellement actif [62]. LEDGF interagit également avec les composants de la machinerie d'épissage ce qui permet au VIH d'être intégré au niveau de régions hautement épissées [63].

À la suite de cette intégration, le provirus code pour les protéines virales nécessaires à la génération de virions, ce qui correspond à la phase tardive du cycle de réplication du VIH-1.

1.1.5.2. Phase tardive

Pendant la phase tardive du cycle de réplication, l'ADN proviral est transcrit en ARN messager [54]. La transcription du VIH est dépendante de la protéine virale Tat et de plusieurs facteurs cellulaires. Tat est localisé dans le noyau de la cellule infectée et se lie au complexe cellulaire p-TEFβ qui comprend une sous-unité régulatrice Cyclin T et une sous-unité catalytique CDK9. Tat/p-TEFβ se lie à la région TAR (*trans-activation response RNA element*), structure en tige et

boucle située en 5' des transcrits viraux naissants [64]. CDK9 phosphoryle la région C-terminal de l'ARN polymérase II et active ainsi le processus de transcription/élongation. Cyclin T facilite la reconnaissance du complexe Tat/Cyclin T au niveau de la région TAR [65].

L'ARN messager est ensuite exporté du noyau vers le cytoplasme de la cellule cible où les protéines virales sont synthétisées. Grâce au processus d'épissage, plus de 100 différents transcrits sont générés servant ainsi de source pour les ARN messagers de toutes les protéines virales [66]. Dans un premier temps, ce sont les transcrits multi-épissés (MS; multiply spliced) qui sont produits et exportés dans le cytoplasme [66]. Ils codent pour les protéines régulatrices (Tat, Rev et Nef). Rev assure l'export nucléaire de transcrits non épissés codant pour les protéines de structure et accessoire du VIH [35]. La traduction du transcrit non épissé donne naissance à deux polyprotéines précurseurs : Gag et Gag-Pol. Ces deux poly-protéines migrent vers la membrane cytoplasmique et sont clivées dans le bourgeon naissant, pour générer les protéines MA, CA, NC, p6, RT, IN et PR (*voir section 1.1.3. Structure du VIH)*. La traduction des transcrits ARN ayant subi un seul épissage donneront naissance aux glycoprotéines de l'enveloppe (gp120, gp41) (*voir section : 1.1.3. Structure du VIH*). Une fois les étapes de transcription/épissage/traduction complétées, Gag recrute l'ARN viral au niveau de la membrane plasmique de la cellule hôte et s'insère au niveau de rafts lipidiques [67]. Les protéines gp120 et gp41 sont insérées dans la membrane plasmique de la cellule hôte et se retrouvent à la surface de la particule virale alors prête à bourgeonner [68]. Le complexe *endosomal sorting complex required for transport I et III* (ESCRT-I, ESCRT-III) et *ALG2-interacting protein X* (ALIX) est recrutés au niveau de la particule virale en formation afin de promouvoir le bourgeonnement et assurer la scission de la membrane qui est essentielle à la libération du virion. La maturation des virions se poursuit après sa libération avec le clivage par la protéase virale des précurseurs Gag et Gag-Pol en protéines fonctionnelles [68].

1.1.6. PATHOGÉNÈSE : EFFETS DU VIH-1 SUR LE SYSTÈME IMMUNITAIRE.

L'infection par le VIH-1 induit un dysfonctionnement du système immunitaire. Ceci résulte directement de la déplétion des lymphocytes T $CD4^+$ qui est observée dès les premières semaines suivant l'infection [69]. En l'absence de traitement, l'infection évolue en trois

phases : la phase aigüe de primo-infection, la phase chronique asymptomatique, et la phase SIDA [70, 71] (Figure 3)

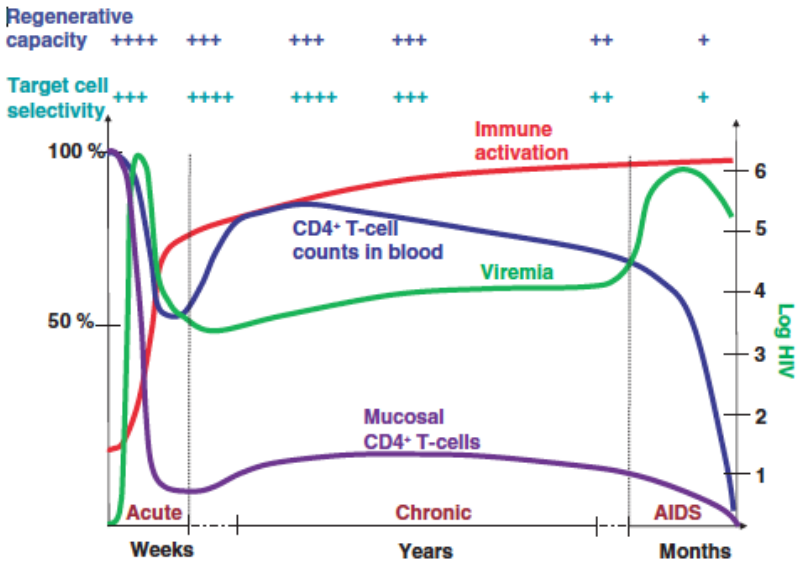

Figure 3. **Phases Clinique de l'infection au VIH-1.**
La progression de la maladie suite à une infection par le VIH-1 peut être découpée en 3 phases : aigue, chronique et SIDA. La figure illustre le taux de lymphocytes dans le sang périphérique et les muqueuses, le niveau d'activation immunitaire ainsi que la virémie pendant la progression de la maladie. [71]. Reproduite avec la permission de Nature Medicine - Numéro de licence : 4558361460280.

La phase aiguë de primo-infection dure quelques semaines durant lesquelles le virus se réplique de façon active et soutenue. Les lymphocytes T $CD4^+$, cibles principales du VIH, sont majoritairement localisés au niveau de la muqueuse intestinale qui est un site majeur de réplication virale [72]. Dès les premières semaines d'infection, l'augmentation de la charge virale est associée à une forte diminution de nombre de lymphocytes T $CD4^+$ dans le sang périphérique et au niveau des tissus lymphoïdes associés à l'intestin (GALT; *gut-associated lymphoid tissues*). À la fin de cette première phase d'infection, la réplication virale devient contrôlée par le développement de réponses immunitaires spécifiques impliquant les lymphocytes T $CD8^+$ et les lymphocytes B. Ceci mène à une réduction de la charge virale et à une légère augmentation du nombre de lymphocytes T $CD4^+$ dans le sang périphérique. Toutefois, la déplétion des lymphocytes T $CD4^+$ au niveau du GALT persiste et est associée à

une perturbation significative de l'homéostasie de la muqueuse intestinale incluant i) une altération de la barrière épithéliale, ii) une translocation microbienne et iii) une activation immunitaire chronique [73] [74].

La phrase aigüe fait ensuite place à la phase d'infection chronique qui est asymptomatique et qui peut durer plusieurs années. Elle est caractérisée par une réplication virale à faible niveau mais persistante, ainsi que par une activation importante des lymphocytes T $CD4^+$ et $CD8^+$ [70, 71]. Durant cette phase, il existe un certain contrôle de la réplication virale par le système immunitaire, et la déplétion des lymphocytes T $CD4^+$ est lente mais persistante. Outre l'infection en elle-même de ces cellules, le dysfonctionnement thymique réduisant la production de nouvelles cellules naïves, la maturation accélérée des cellules naïves et l'épuisement des acteurs immunitaires contribuent également à cette perte lymphocytaire [75, 76] [77]. De pair avec l'élimination graduelle des populations T $CD4^+$, l'expansion des lymphocytes T $CD8^+$ conduit à une diminution du ratio CD4/CD8 qui est un prédicteur clinique de la progression de la maladie [78].

Au moment où le taux de cellules T $CD4^+$ dans le sang périphérique descend sous le seuil de 200 cellules/µL (500 à 1500 cellules/µL chez les sujets non-infectés), généralement après plusieurs années d'infection, la phase SIDA est déclarée [79] [2]. Celle-ci est associée à une augmentation plus rapide de la charge virale et à une déplétion massive des lymphocytes T $CD4^+$. La perte d'intégrité du système immunitaire permet aux microorganismes et aux virus normalement peu virulents, d'infecter l'hôte plus efficacement et de devenir des pathogènes dangereux. Ces infections opportunistes comprennent des infections fongique et bactériennes (e.g., *Candida albicans, Staphylococcus aureus, Escherichia coli, Mycobacterium Tuberculosis et Pseudomonas aeruginosa*), virales (e.g., Cytomegalovirus (CMV), human papilloma virus (HPV), Herpes simplex virus (HSV) et parasitaires [70] [80] [81].

1.1.7. MÉCANISMES ANTIVIRAUX

1.1.7.1. Détection du VIH

Le système immunitaire inné constitue la première ligne de défense face aux pathogènes. Il répond à l'intrusion d'un micro-organisme pathogène de façon non-spécifique et rapide via l'activation de la voie des interférons et des réponses inflammatoires [82]. Afin de détecter la présence d'un pathogène, la cellule est équipée de récepteurs

de reconnaissance de motifs moléculaires (PRR; *pattern recognition receptor*) qui détectent des motifs moléculaires associés aux pathogènes (PAMPs; *pathogènes-associated molecular pattern*) [83]. La reconnaissance d'un PAMP par un PRR stimule l'expression des interférons de type I, induisant l'expression de centaines de différents gènes, les ISGs (*IFN-stimulated genes*) [82, 83]. Dans le cadre d'une infection virale, la plupart de ces ISGs ont pour fonction de freiner la réplication du virus en bloquant diverses étapes du cycle viral [84].

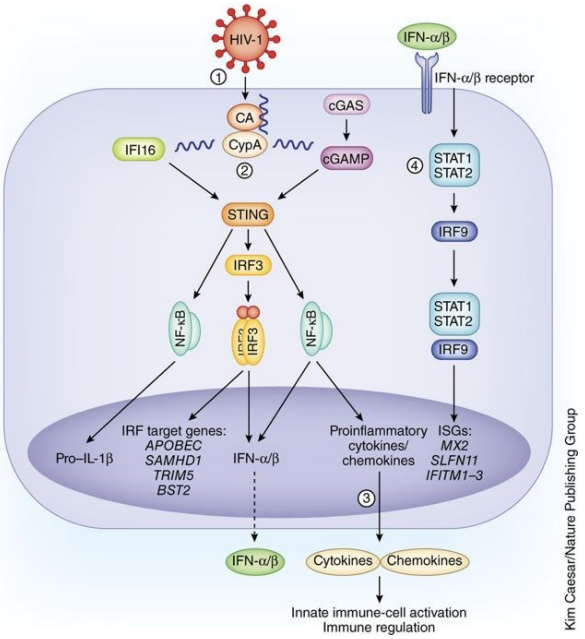

Figure 4. **<u>Mise en place de la réponse immunitaire innée face à l'infection au VIH.</u>**
Le génome du VIH est reconnu par le système immunitaire par cGAS et IFI16 ce qui permet d'enclencher une réponse antivirale via la production d'interféron de type I et des facteurs de restriction [85]. Reproduite avec la permission de Nature Immunology - Numéro de licence : 4521420886326

Le VIH est décelé par le système immunitaire inné via la reconnaissance de l'ADN viral, issu de la transcription inverse, par les PRRs : cGAS (*cyclic GMP-AMP synthase*) dans les macrophages et les DC [86] [87] et IFIT16 (*interferon-γ (IFNγ)-inducible protein 16*) dans les macrophages et les cellules T $CD4^+$ [88] (Figure 4). La détection de l'ADN viral par cGAS ou IFIT16 engage l'adapteur STING (*stimulator of interferon genes*) au niveau du réticulum endoplasmique qui se dimérise et s'associe avec TBK1 (*TANK-binding kinase 1*). L'activation du complexe TBK1-IKK-b permet la phosphorylation d'IRF3 (*interferon regulator factor 3*) et la translocation nucléaire de NF-κB (*nuclear factor-kappa B*), induisant ainsi l'expression des IFN-α et IFN-β [89]. La détection du VIH par le système immunitaire inné induit une réponse pro-inflammatoire via la sécrétion d'IL-1β et une activation immunitaire. Ceci conduit à la mort de la cellule infectée par apoptose et celle des cellules voisines par pyroptose [90]. Il est à noter que ce processus de mort cellulaire est en grand partie responsable de la déplétion des lymphocytes T $CD4^+$ après l'infection par le VIH [85]. Par ailleurs, plusieurs travaux suggèrent que RIG-I (*retinoic-acid induced genes I*) reconnait l'ARN du VIH, ce qui induit la production d'IFN et des réponses antivirales [91] [92, 93]. L'induction de RIG-I par des dérivés des acides rétinoique (AR) tels que l'acitretin, augmente la reconnaissance de l'ARN du VIH et déclenche l'apoptose des cellules infectées [94].

Bien que le système immunitaire inné ait recours à plusieurs mécanismes de reconnaissance contre les pathogènes dont le VIH, ce dernier fait usage de nombreuses stratégies afin de contourner ces mécanismes de détection, incluant la protection du génome viral par la capside, comme discuté précédemment, et en limitant la quantité des retro-transcrit viraux via SAMHD1 (*SAM domain and HD domain-containing protein 1*) et TREX1 (*Three prime repair exonuclease 1*) afin d'éviter leur détection par le système immunitaire [95].

1.1.7.2. Facteurs de restriction

Outre la reconnaissance du VIH, les mécanismes innés de défense de l'hôte permettent de bloquer certaines étapes du cycle de réplication de ce virus. Ces médiateurs de la réponse antivirale sont appelés facteurs de restriction et leur expression est le plus souvent induite en réponse aux IFN de type I [96, 97]. L'obstruction d'une étape du cycle viral par les facteurs de restrictions a des conséquences sur la mort cellulaire et la présentation des antigènes viraux au système immunitaire [98]. L'activité des facteurs de restriction est, la plupart du temps, contrecarrée par le virus lui-même [96, 97]. En effet, afin de se répliquer, le virus doit s'adapter à la complexité de la cellule cible et empêcher les facteurs de restriction intracellulaires de jouer leur rôle antiviral est l'une des principales fonctions des protéines virales accessoires. Plusieurs facteurs de restriction ont été mis en évidence pour le VIH/VIS: APOBEC3G, SAMHD1, SERCIN2/5, Mx2, ITFIM, TRIM5α, et BST-2 [96, 97] (Figure 5)

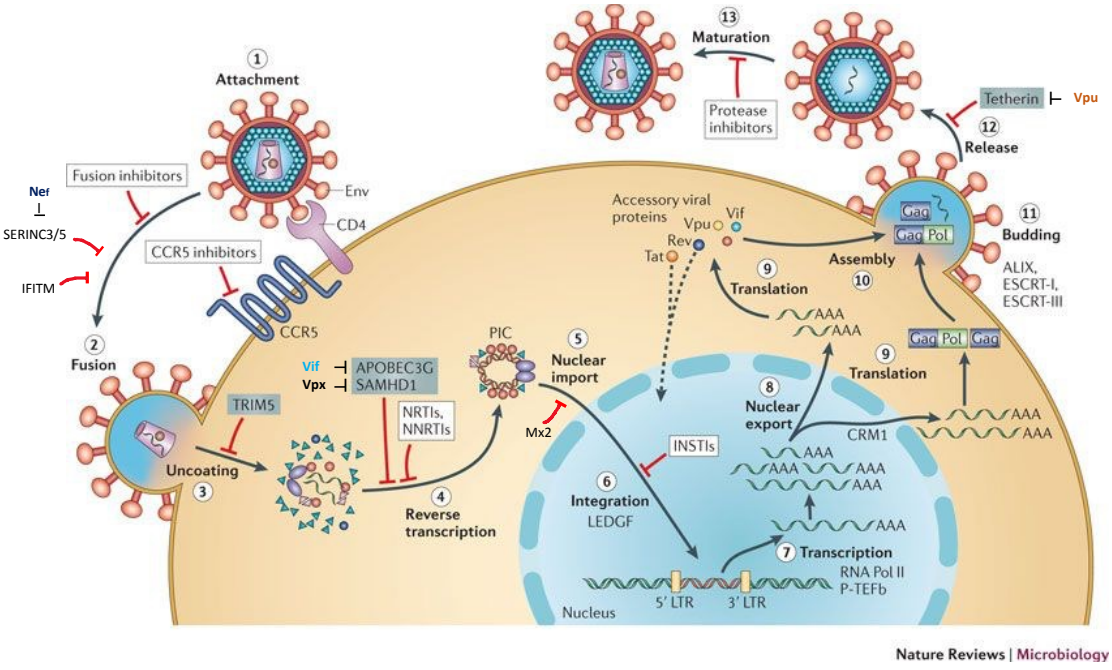

Figure 5. Régulation du cycle de réplication du VIH via les facteurs de restrictions de la cellule hôte et les antirétroviraux (ARVs).

La figure illustre les multiples étapes du cycle de réplication du VIH. Les étapes interrompues par une restriction d'hôte ainsi que les facteurs ciblés par les stratégies thérapeutiques

antirétrovirales sont indiqués. Adapté de [90] avec la permission de Nature Microbiology Review - Numéro de 4574330721502

APOBEC3G

C'est en étudiant le rôle de la protéine virale Vif dans l'infectiosité des virions produits par une cellule infectée que APOBEC3G (*apolipoprotein B MRNA Editing Enzyme Catalytic Subunit 3G*) a été identifié comme facteur de restriction du VIH-1 [36] [99]. Gabuzda *et al*. ont identifié plusieurs lignées cellulaires susceptibles à l'infection par le VIH wild-type (WT), mais résistantes à l'infection par un VIH déficient pour Vif (VIHΔVif), suggérant l'existence d'un mécanisme de défense de la cellule hôte contrecarré par Vif [36] [100]. Plusieurs années de recherche sur le rôle de Vif ont été nécessaires afin d'identifier APOBEC3G comme étant responsable de la résistance à l'infection par le VIHΔVif [99]. APOBEC3G est une cytidine désaminase qui cible l'étape de transcription inverse du VIH dans les cellules T $CD4^+$ et les MΦ [101]. Elle est d'abord incorporée dans les virions produits par une cellule infectée. Puis, au cycle d'infection suivant, APOBEC3G, lorsqu'il n'est pas dégradé par Vif, désamine les cytosines en uracil (C → U) du brin négatif de l'ADN viral introduisant ainsi des hypermutations [102]. Ces rétro-transcrits aberrants peuvent être reconnus par la cellule hôte et être immédiatement dégradés. Ils peuvent aussi s'intégrer dans le génome de la cellule hôte, mais les mutations compromettent largement l'intégrité de la séquence du provirus et donc la production de virions infectieux [102].

APOBEC3G agit principalement sur le virus en introduisant des hypermutations létales au niveau de son génome, mais peut aussi bloquer la réplication virale indépendamment de son activité désaminase [103] [104]. Ces mécanismes incluent l'altération ***i)*** du processus de transcription inverse ***ii)*** de l'élongation de l'ADNc viral ou encore ***iii)*** de l'intégration de l'ADN viral dans le génome de la cellule hôte [105-107].

Vif interagit simultanément avec APOBEC3G et un complexe d'ubiquitine ligase E3, entraînant son ubiquitination et sa dégradation, permettant ainsi une réplication efficace du VIH [108] [109].

SAMHD1

La principale distinction génétique entre le VIH-1 et le VIH-2 est l'expression respective des protéines accessoires Vpu et Vpx. C'est justement cette différence qui a mené à l'identification de SAMHD1 comme facteur de restriction, et ce, en comparant la permissivité et la résistance de différents types cellulaires à l'infection au VIH-1 et au VIH-2 [110, 111]. En fait, Vpx est requis pour permettre au VIH-2 d'infecter des cellules myéloïdes et dendritiques [110]. Considérant la capacité de Vpx à former un complexe avec la E3 ubiquitin ligase pour induire la dégradation de plusieurs composants cellulaires, des études protéomiques ont permis d'identifier SAMHD1 parmi les facteurs intrinsèques dégradés en présence de Vpx [110]. L'inhibition de l'expression de SAMHD1 dans les cellules dendritiques est, tout comme l'insertion de Vpx, associée à une augmentation de la permissivité à l'infection au VIH-1, confirmant le rôle antiviral de SAMHD1. De façon similaire, l'introduction de la séquence Vpx dans le génome du VIH-1 est associé à une augmentation de l'infection des cellules T quiescentes par le virus [112, 113].

SAMHD1 restreint le VIH-1 par le biais de son activité phosphohydrolase qui convertit les désoxynucléosides triphosphates (dNTP) en phosphates inorganiques (iPPP) et en 2'-désoxynucléoside, ce qui permet de réguler le pool de dNTPs intracellulaire [114]. Lors de la retro-transcription, la RT utilise les dNTP intracellulaire pour générer l'ADN viral double brin. En présence de SAMHD1, la concentration de dNTP est insuffisante pour supporter la retro-transcription du VIH. De plus, SAMHD1 possède une activité de RNAse impliquée dans la dégradation de l'ARN viral [115].

Vpx retient SAMHD1 dans le noyau de la cellule infectée et inhibe ainsi son activité ([116].

BST-2 / Tetherin

L'importance de Vpu dans la maturation des virions est connue depuis longtemps avec une accumulation des virions, incapables de se détacher, à la surface de la cellule infectée observées lors d'infection *in vitro* avec un virus VIH-1Δ*Vpu* [117]. Ce blocage dans la libération des virions est induit par les IFN de type I et n'est observé que dans certains types cellulaires

[118]. Un screening des protéines membranaires induites par les IFN de type I et spécifiquement exprimées dans les cellules résistantes au VIH-1 a permis d'identifier BST-2 (*bone marrow stromal antigen 2*) [119]. La transfection de BST-2 dans les cellules permissives bloque la libération des virions VIH-1Δ*Vpu,* mais pas celle des virions VIH-1 WT confirmant que ce facteur de restriction est contré par Vpu [119] [120].

BTS-2, ou tetherin, est une protéine transmembranaire possédant, en N-terminal, une portion transmembranaire ancrée à la membrane plasmique de la cellule et, en C-terminal, un lien glycosyl-phosphatidylinositol pouvant s'insérer dans la membrane virale ce qui empêche ainsi le virion d'être libéré.

Tetherin est aussi un senseur de l'infection virale agit comme un récepteur de reconnaissance de motifs moléculaires activant le système immunitaire inné [121]. En effet, après infection avec le VIH-1, l'interaction de tetherin avec la membrane virale en processus de bourgeonnement induit la phosphorylation de tetherin et le recrutement des adaptateurs TRAF2, TRAF6 et TAK-1 qui à leur tour vont activer la voie NF-κB et induire l'expression des IFN de type I [121].

Vpu séquestre tetherin dans la région périnucléaire provoquant son endocytose et sa dégradation par le protéasome [122-124].

TRIM5α

Des études génétiques de criblage ont permis d'identifier TRIM5α (*tripartite motif-containing protein 5 α*) comme étant responsable de la résistance à l'infection au VIH-1 chez les Singes de l'Ancien Monde [125]. En effet, la protéine TRIM5α du Singe de l'Ancien Monde bloque efficacement les étapes précoces de l'infection alors que la protéine TRIM5α humaine ou provenant des Singes du Nouveau Monde a une efficacité moindre voire nulle [126].

Une étude récente a identifié TRIM5α comme étant impliqué dans le mécanisme de protection naturelle au VIH des élites contrôleurs (sujet VIH+ qui contrôlent la réplication du VIH sans ARVs (*voir section1.3.2.3 Stratégie de guérison fonctionnelle*). [127].

TRIM5α interagit avec la capside virale dans le cytoplasme de la cellule nouvellement infectée, ce qui conduit à la dégradation protéasomale des protéines virales (CA, RT) et à une

décapsidation prématurée [125]. TRIM5α inhibe aussi l'intégration de l'ADN viral via un mécanisme indépendant du protéasome [128].

Par ailleurs, ce facteur de restriction agit comme un récepteur de reconnaissance de motifs moléculaires activant le système immunitaire inné. En effet, son interaction avec la capside virale active la voie NF-κB, ce qui induit un état antiviral général de la cellule infectée [129]. De plus, il a été montré que l'activité antivirale de TRIM5α est augmentée lorsqu'il interagit avec le facteur cellulaire Cyclophiline A (*CypA*) via un mécanisme non élucidé [130]. TRIM5 appartient à la famille des protéines à motif tripartite caractérisée par la présence d'un domaine RING (*really interesting new gene*) et d'un domaine B-box et coiled-coil (RBCC) [96, 97].

Plusieurs membres de la famille TRIM participent à la résistance à l'infection de différents lentivirus et sont induits par les IFN de type I: TRIM1, TRIM19, TRIM22, TRIM32, and TRIM34 [131].

SERINC3/5

Comme discuté plus tôt, Nef est responsable de la diminution de l'expression de CD4 et CMH-I à la surface membranaire permettant aux cellules infectées d'échapper à la surveillance du système immunitaire [132].

De plus, par un processus longtemps resté incompris, Nef augmente l'infectivité des virions produits par une cellule infectée. Ce mécanisme fut révélé grâce à la mise en évidence d'un nouveau facteur de restriction ciblé par Nef.

Des études transcriptomiques dans plusieurs types cellulaires infectés par un VIH-WT *versus* VIHΔNef ont montré que les protéines membranaires SERINC5 et SERINC3 (serine incorporator 3 et 5) sont uniquement incorporées dans les virions VIHΔNef. De plus, l'infectiosité en absence de Nef corrèle avec le niveau d'expression de SERINC5/3 [133, 134]. Ces travaux ont permis d'identifier ces deux protéines comme des facteurs de restriction du VIH dont leur activité est contrecarrée par Nef.

Les protéines de la famille des SERINC sont des protéines transmembranaires qui régulent les composants lipidiques de la membrane plasmique. SERINC3 et 5 sont retrouvées dans les virions nouvellement produits par la cellule infectée et empêchent la fusion du virion avec la cellule cible [133, 134].

Nef induit la séquestration de SERCINC3/5 dans les endosomes empêchant ainsi son incorporation dans les virions.

Mx2

En étudiant le transcriptome de lignées de monocytes différant dans leur capacité à engager un mécanisme anti-VIH en réponse à un traitement aux IFN de type I, Mx2 a été identifié comme facteur de restriction [135]. L'expression de Mx2 est corrèle positivement avec le degré d'inhibition de l'infection au VIH-1[135].

Mx2 est une GTPase, localisée au niveau des pores nucléaires qui inhibe la décapsidation et empêche ainsi l'importation du PIC dans le noyau [136].

IFITM2 et 3

Le génome humain code pour au moins cinq protéines IFITMs (*interferon-induced transmembranaire proteins*). Trois d'entre elles présentent une activité antivirale : IFITM1, IFIT2 et IFITM3 [137] [138-140] [141]. Leur expression est induite par les IFN de type I et II [142]. En 2009, Brass *et al*. démontrent que les protéines IFITMs inhibent l'infection de plusieurs pathogènes viraux comme le virus de la dengue et le virus de l'influenza [137]. Une étude de criblage à grande échelle, visant à déterminer l'effet antiviral de 380 facteurs induits par les IFN de type I, a mis en évidence que IFITM2 et IFITM3 régulent négativement l'infection au VIH-1 [143].

Ces deux protéines, localisées au niveau de la membrane plasmique et des membranes des lysosomes, exosomes et autophagosomes [144], bloquent l'entrée du VIH-1 dans les cellules [145]. Elles modifient les propriétés physiques des membranes dans lesquelles elles sont incorporées et empêchent la fusion entre la membrane du virus et celle de la cellule cible [146,

147]. Les protéines IFITMs peuvent aussi être incorporées dans la membrane des virions naissants et empêcher leur fusion avec une nouvelle cellule [148].

1.1.8. FACTEURS DE PERMISSIVITÉ

Le VIH-1 code pour seulement 15 protéines et doit donc exploiter des facteurs de la cellule hôte afin d'assurer efficacement sa réplication. Plusieurs études de criblage à grande échelle ont permis d'identifier de nombreuses protéines de l'hôte dont leur expression est essentielle au cycle de réplication viral [149]. Ces protéines sont appelées des facteurs de permissivité au VIH.

L'entrée du VIH dans une cellule nécessite l'expression des glycoprotéines CD4 et des corécepteurs CCR5 ou CXCR4 mais aussi d'autres récepteurs qui sont utilisés par le virus afin de se propager de façon optimale. Par exemple, le récepteur à chimiokine CXCR6 (Bonzo/STRL33) a été rapporté comme étant un corécepteur mineur du VIH [150] [151, 152]. CXCR6 se lie à son ligand soluble ou membranaire CXCL16, ce qui favorise la formation de synapses immunologiques entre les cellules T $CD4^+$ et les DC [153]. Cela permet une dissémination du virus plus efficace [154]. Récemment, il a été démontré que CXCR6 était un corécepteur utilisé par les virus « transmitted founder » lors des étapes précoces de l'infection [155]. De façon importante on note une co-expression de CXCR6 avec CCR5 et aussi CCR6 [151], un marqueur de cellules Th17 hautement permissives à l'infection par le VIH [156].
L'intégrine α4β7 se lie à la protéine gp120 et active ainsi l'intégrine LFA-1 (*lymphocyte function-associated antigen*), impliquée dans la formation des synapses virologiques, afin de faciliter la transmission virale de cellule à cellule [157]. Le ligand de l'intégrine α4β7, MadCAM (*mucosal vascular addressin cell adhesion molecule 1*), étant exprimé à la surface des cellules endothéliales du GALT, l'incorporation de α4β7 dans les virions permet au VIH d'infiltrer efficacement les plaques de Peyer de l'intestin [158]. De plus, le signal induit par MadCAM via l'intégrine α4β7 augmente la réplication virale dans les cellules T $CD4^+$ [159].

L'importance des rafts lipidiques dans le cycle de réplication viral n'est pas non plus à négliger. En effet, la localisation des corécepteurs CCR5 et CXCR4 au niveau de rafts membranaires favorise l'entrée du virus dans la cellule cible [160]. Les composants lipidiques de la membrane cellulaire jouent un rôle clef dans l'organisation et la formation de ces rafts [161]. Ils jouent aussi un rôle crucial dans l'assemblage et la libération des virions [162, 163].

Un autre facteur important pour l'entrée du virus est la protéine Rab6. Elle régule le transport des protéines de l'appareil de Golgi vers le réticulum endoplasmique et elle se trouve nécessaire pour assurer la fusion de la particule virale à la membrane de la cellule cible [164].

Outre les facteurs de permissivité requis pour l'entrée ou la transmission des particules virales aux cellules, il existe aussi des acteurs cellulaires importants pour le transport intracellulaire du matériel viral. En effet, le transport du PIC dans le noyau exige la présence de plusieurs facteurs cellulaires incluant: TNPO3 (*nuclear import factors transportin 3*), RANBP2 (*RAs-related nuclear protein binding protein 2*) et Nup153 (*nucleoporine 153*) [165].

Comme discuté plus tôt, la transcription du génome virale nécessite aussi certains facteurs intrinsèques de l'hôte. Entre autres, l'interaction entre la protéine virale Tat et le complexe pTEF-B (CDK9 and Cyclin T) est essentielle à cette étape du cycle de réplication [141]. De plus, l'expression des gènes viraux est induite par les facteurs de transcription NF-κB et NFAT (*nuclear factor of activated T-cells*) [166].

Il est aussi intéressant de noter que le VIH cible préférentiellement les cellules avec une activité métabolique soutenue suggérant que le virus utilise certains acteurs du métabolisme cellulaire à son avantage [167]. Parmi ces acteurs, GLUT1 (*Glucose transporter 1*) et mTOR (*mechanistic target of rapamycin*) ont été identifiés comme jouant un rôle substantiel dans l'infection des cellules T $CD4^+$ et des macrophages [168] [169] [170]. Dans ce sens, je fais référence au chapitre 3 de ma thèse qui présente nos résultats montrant le rôle de mTOR dans la réplication

et la réactivation des réservoirs du VIH dans les cellules T CCR6^{+} à profil de polarisation Th17 [171] [172]

Le VIH utilise également des facteurs cellulaires afin de se protéger et d'éviter sa détection par le système immunitaire inné. CypA joue un rôle important dans la stabilisation de la capside virale permettant ainsi la protection du génome viral des senseurs d'ADN cytoplasmiques [173]. L'exonucléase cytosolique Trex1 digère l'excédent de matériel d'ADN cytoplasmique permettant ainsi d'éviter aux senseurs d'ADN de détecter le génome viral [174].

Le système de reconnaissance virale inné et la présence de ces facteurs de restriction ne sont pas suffisants à empêcher le virus d'infecter les cellules du système immunitaire et de se répliquer. La mise en évidence d'autres facteurs de permissivité au VIH et de leur rôle dans le cycle viral est essentielle à la mise en place de nouvelles stratégies thérapeutiques pour bloquer l'infection par le VIH.

1.1.9. RÉSERVOIRS CELLULAIRES ET ANATOMIQUES DU VIH SOUS THÉRAPIE ANTIRÉTROVIRALE.

1.1.9.1. Établissement et persistance des réservoirs du VIH

Lorsqu'une cellule T naïve rencontre un antigène pour lequel elle est spécifique, elle prolifère et se différencie en cellule effectrice mémoire capable d'éliminer le pathogène duquel est issu l'antigène. Le virus infecte préférentiellement ces lymphocytes T CD4^{+} activés qui vont produire une grande quantité de virus et mourir en quelques jours [52]. Quelques-unes de ces cellules mémoires actives vont toutefois retourner vers un état de repos et persister comme cellules mémoires capables de répondre au même antigène dans le futur. C'est alors que la latence du VIH s'établit, phénomène qui arrive très tôt après l'infection [175] [176-178]. Ces cellules représentent donc un site idéal pour que le virus puisse persister dans un état transcriptionnel silencieux mais capable de se réactiver et de propager l'infection après interruption de la TAR.

Il a été estimé qu'une durée moyenne de 70 ans de TAR serait nécessaire pour éliminer toutes les cellules infectées d'une personne VIH+ [179]. L'infection préférentielle des cellules à long durée de vie et la prolifération homéostatique permettent non seulement au réservoir de se réapprovisionner, mais aussi de persister pendant de nombreuses années [180].

Il est aussi possible que la prolifération des cellules mémoires en réponse à un antigène spécifique, permette au réservoir de se réapprovisionner.

De plus, il est possible que la concentration des antirétroviraux soit suboptimale dans certains tissus, entrainant une réplication du virus à de très faibles niveaux, mais suffisante pour réalimenter constamment le réservoir [181]. On parle alors de réservoir actif.

1.1.9.2. Ontogénie des lymphocytes T CD4+

Les cellules T $CD4^+$ endossent un rôle majeur et essentiel dans l'orchestration d'une grande variété de réponses immunitaires. En effet, de par les cytokines qu'elles sécrètent, elles ont la capacité de recruter des neutrophiles au niveau du site d'infection ou d'inflammation, de renforcer l'immunité médié par les lymphocytes B ainsi que d'activer l'effet microbicide des macrophages. L'ontogénie des lymphocytes T $CD4^+$ débute dans le thymus où la génération de leur TCR (*T cell receptor*) prend place de même que l'expression de la glycoprotéine CD4 à leur surface. C'est lorsque les cellules T $CD4^+$ naïves reconnaissent un peptide antigénique présenté par les molécules du CMH de classe II des cellules présentatrices d'antigènes (CPA), qu'elles se différencient en diverses sous-populations effectrices (Figure 6)

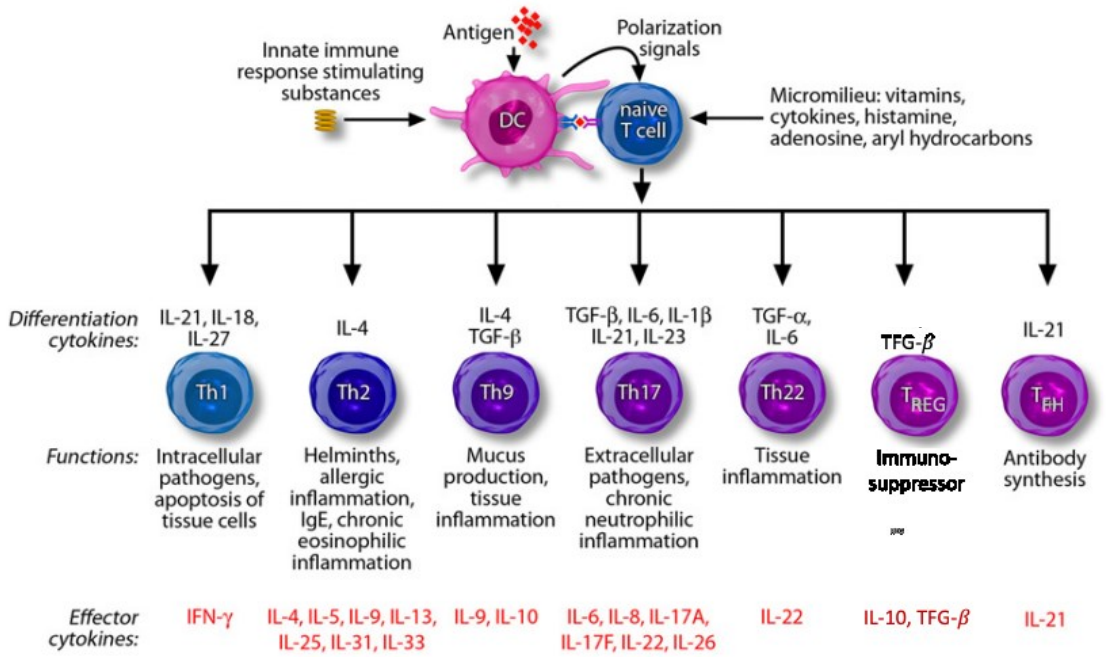

Figure 6. **Profil de polarisation des cellules T.**
Les cellules T CD4$^+$ naïves se différencient en lymphocytes T auxiliaires helper: Th1, Th2, Th9, Th17 et Th22, les T_{FH} et les T_{reg} en fonction des cytokines produites par la cellule présentatrice d'antigène. Chaque population de cellules T est caractérisée par des fonctions et un profil de cytokines sécrétées spécifiques. Reproduite avec la permission de Journal of Allergy and Clinical Immunology - Numéro de licence : 4574330511837

Lors de leur différenciation, ces cellules effectrices acquièrent l'expression de récepteurs impliqués dans le trafic cellulaire, leur permettant ainsi de migrer vers des sites d'inflammation où elles vont, de par les cytokines qu'elles sécrètent, induire la clairance de l'antigène. Lorsque l'infection est neutralisée, la grande majorité des cellules effectrices meure. Seule une population résiduelle à long durée de vie persiste et retourne dans un état de quiescence; ce sont les cellules mémoires capables de proliférer rapidement en cas de nouvelles rencontres avec l'antigène [182]. Ces cellules mémoires sont hétérogènes et peuvent être classées sur la base des récepteurs de surface qu'elles expriment. Ceux-ci reflètent leur état de différenciation qui est associé d'une part à leur capacité de migration entre le sang périphérique et les organes lymphoïdes et non-lymphoïdes et d'autre part à leurs aptitudes de prolifération de renouvellement et de fonctions effectrices.

Parmi les cellules mémoires se distinguent deux grandes classes : les cellules mémoires centrales (CM) et les cellules mémoires effectrices (EM) [183] [184]. On retrouve aussi les cellules mémoires transitoires (TM) qui constituent un sous-ensemble de lymphocytes mémoires dans un état de différenciation se situant entre les cellules T_{CM} et T_{EM} en termes de phénotype. Finalement, on trouve les lymphocytes en différenciation terminale (TD) (Figure 6) [185] [184] [186].

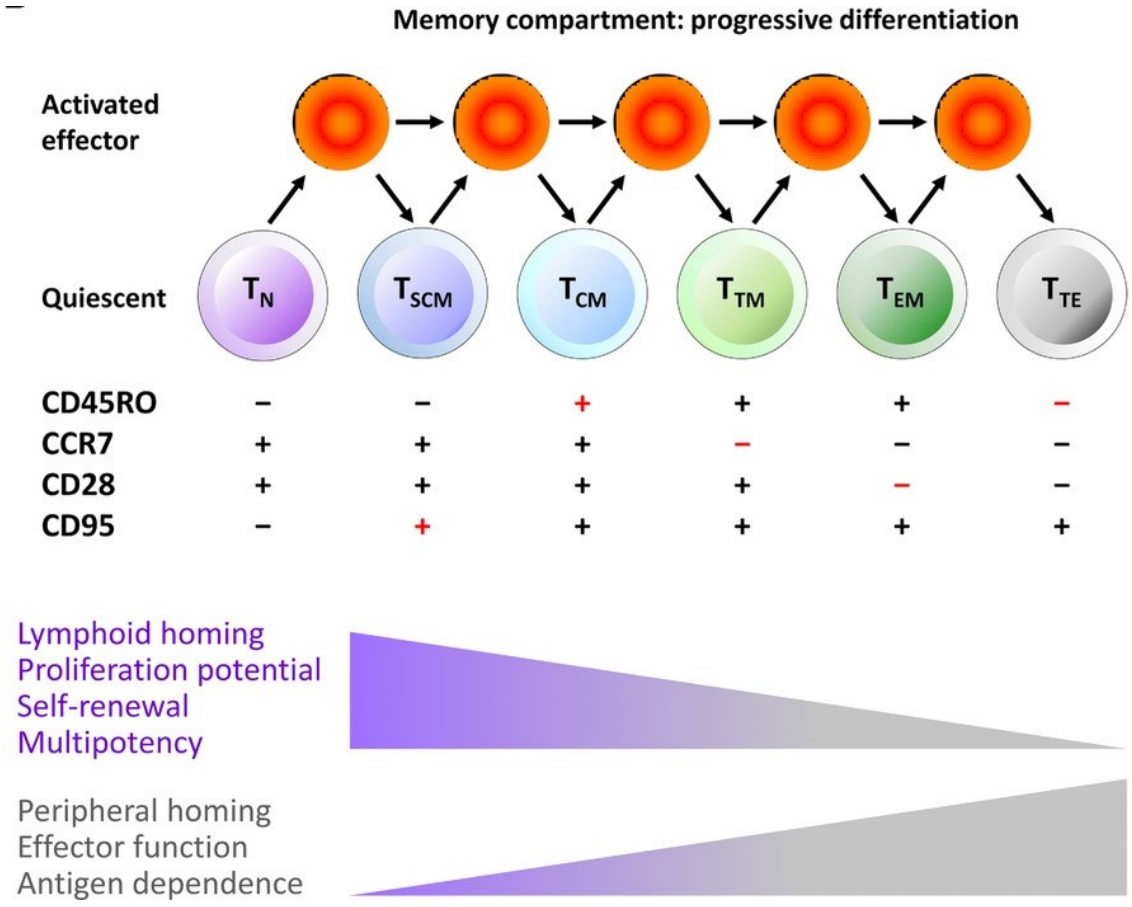

Figure 7. **Différents statuts de différenciation des cellules T.**
Il est illustré le model de différenciation progressive d'une cellule T du state naïve au stage terminal de différenciation. Reproduite avec la permission de European Journal of Immunology - Numéro de licence : 4574321416009

Cette classification des lymphocytes T mémoires a récemment été révisée pour inclure un nouveau sous-ensemble appelé lymphocytes T mémoire des cellules souches (T_{SCM}) [186]. La

population T_{SCM} ressemble phénotypiquement aux cellules T naïves, mais s'en distingue par le biais de sa co-expression de marqueurs mémoires, tels que CD95 et CXCR3 [186]. Les T_{SCM} possèdent des capacités d'auto-renouvellement, de prolifération et de longévité importantes, par rapport aux cellules T_{CM}, T_{TM} et T_{EM} [187]. Les T_{SCM}, qui sont à longue durée de vie, peuvent se différencier en cellules T_{CM} et/ou T_{EM} et, par conséquent, maintenir une immunité à long terme avec une reconstitution de tous les sous-ensembles de la mémoire des cellules T $CD4^+$ [187].

Les différentes sous-populations de cellules T $CD4^+$ naïves et mémoires peuvent être identifiées sur la base de leur expression différentielle de CD45R0, CCR7, CD28 et CD95 (Figure 7) En se différenciant (par activation) du stade T_{SCM} à T_{CM}, T_{TM}, T_{EM} pour aboutir à T_{TD}, les cellules T mémoires perdent ou acquièrent progressivement des fonctions spécifiques de prolifération, d'auto-renouvellement et de capacités migratoires (Figure 7).

1.1.9.3. Les sous-populations de lymphocytes T CD4+ mémoires

Il est bien établi aujourd'hui que les cellules T $CD4^+$ ayant expérimenté la rencontre de leur peptide auquel elles sont spécifiques peuvent se diviser en de multiples sous-ensembles de lymphocytes T auxiliaires (Th pour T helper): Th1, Th2, Th9, Th17 Th22, folliculaires (T_{FH}) et régulateurs (T_{reg}) (Figure 6) [188, 189] [190-193] [194] [195] [196]. Leur statut de différenciation dépend des cytokines et des molécules de co-stimulation produites par les CPA [197] et la polarisation en résultant est associée avec une expression spécifique de récepteurs aux chimiokines contrôlant leur migration et les types de réponse inflammatoire qu'elles déploieront [198].

En 1987, Thimothy Mosmann et al. décrivent, chez la souris, l'existence de deux groupes de cellules Th se distinguant par leur expression respective de certains facteurs de croissance [199]. Alors que les cellules Th1 produisent l'Il-2, et l'IFN-γ, les cellules Th2 génèrent de l'IL-4. Ces deux sous-populations cellulaires diffèrent également de par leur fonction immunitaire. D'un côté, les cellules Th1 dirigent la réponse immunitaire à médiation cellulaire et sont, par

conséquent, efficaces pour contrôler les agents pathogènes à multiplication intracellulaire. De l'autre, les cellules Th2 sont importantes pour contrôler les pathogènes extracellulaires via la production d'IL-4 qui est responsable de la commutation isotopique et de la production d'IgG, d'IgE et d'IgA par les cellules B [200]. Pendant près de 20 ans, la dichotomie Th1/Th2 a été considérée comme la pierre angulaire de la réponse immunitaire. Néanmoins, le paradigme Th1/Th2 a soulevé des questions et des contradictions provenant notamment d'études sur les maladies auto-immunes qui ont mis en lumière l'existence d'autres sous-populations au sein des cellules T $CD4^+$. En 2003, Cua et al. identifient pour la première fois l'IL-23 comme étant responsable de l'inflammation auto-immune chez le modèle de souris de l'EAE (*Experimental Autoimmune Encephalomyelitis*) [201]. L'IL-23 est une cytokine qui combine la sous-unité p40 et la sous-unité p19, tandis que l'IL-12 combine les sous-unités p40 et p35 [202]. En démontrant que les souris déficientes en IL-23 (p19 -/-) ou en IL-23 et IL-12 (p40 -/-) étaient résistantes à l'EAE, alors que celles privées uniquement d'IL-12 (p35 -/-) étaient susceptibles à l'EAE, les travaux de Cua et al., montre que l'IL-23 agit indépendamment de l'IL-12 pour contrer l'auto-immunité [201]. En parallèle, une étude menée par Aggarwal et al. a identifié l'IL-23, mais pas l'IL-12, comme étant responsable de l'induction de la production de la cytokine pro-inflammatoire IL-17 par les lymphocytes T mémoires [190]. Ceci conforte l'idée selon laquelle l'IL-23 agit sur une autre population de cellules T $CD4^+$ présentant des caractéristiques distinctes des profils Th1 et Th2. D'autres études ont permis de caractériser ces cellules T $CD4^+$ différenciées en présence d'IL-23, TGF-β et IL-6, et les ont associées à la production de cytokines inflammatoires (IL-17A, IL-17F, IL-6, IL-21, IL-22, IL-26, TNF) [203] [189, 204, 205]. Elles furent nommées les cellules Th17 et il a été démontré que leur différenciation est dépendante de l'expression de RORγt [206]. Via leur production d'IL-17A/F et d'IL-22, elles sont impliquées dans la sécrétion de molécules antimicrobiennes par les cellules épithéliales et dans le recrutement des neutrophiles, jouant ainsi un rôle de défense contre les bactéries pathogéniques [207] [208].

Parmi les autres sous-populations cellulaires mises en évidence se trouvent les cellules T_{reg} qui sont impliquées dans la tolérance aux antigènes du soi et dans la régulation de

l'activation/prolifération des lymphocytes T effecteurs grâce à leurs actions immunosuppressives [209]. La différenciation des cellules T_{reg} nécessite, comme pour les Th17, la présence de TGF-β. C'est la présence d'IL-6 qui favorise la différenciation en Th17 *versus* T_{reg} [210].

Quant aux lymphocytes T_{FH}, ils ont été découverts dans les années 2000 [195] [192, 194]. Les cellules T_{FH} sont co-localisées avec les cellules B dans les organes lymphoïdes secondaires et délivrent leur des signaux (IL-21, IL-4) qui induisent leur différenciation et la survie des lymphocytes B produisant des anticorps de haute affinité [192]. Le facteur de transcription Bcl-6 régule positivement la différenciation des cellules T naïves en cellules T_{FH} [211]. Les cellules T_{FH} expriment CXCR5, PD-1 (*programmed cell death 1*), ICOS (*inducible T-cell costimulator*) et sécrètent de l'IL-21 qui est importante pour la survie des lymphocytes Th17 et T_{FH} [212] [213] [214]. Les cellules T_{FH} des centres germinatifs sont hétérogènes et présentent une signature cytokinique qui correspond aux cytokines sécrétées par les différentes cellules Th effectrices (Th1, Th2 et Th17) pour une réponse donnée [215]. En dehors des centres germinatifs, il existe une population circulante de cellules T $CD4^+$ centrale mémoire exprimant CXCR5 qui, en co-culture avec des lymphocytes B, conduit à une forte sécrétion d'anticorps. Les cellules T $CD4^+CXCR5^+$ circulantes sont les équivalents mémoires des T_{FH} des centres germinatifs [216]. Tout comme ces dernières, elles sont hétérogènes et peuvent être distinguées en p (*periphérie*)$T_{FH}1$, p $T_{FH}2$ et p$T_{FH}17$ selon leur capacité à sécréter les mêmes cytokines que les cellules $CD4^+$ Th1, Th2 et Th17 respectivement [216].

En plus des cellules Th17, T_{reg} and T_{FH}, deux autres sous-ensembles lymphocytaires effectrices ont été découverts dans la dernière décennie: les cellules Th9 et Th22. Les cellules Th9 se différencient en présence de TGF-β et d'IL-4, et produisent de l'IL-9 et IL-10. Ces cellules sont impliquées les maladies auto-immunes ainsi que l'asthme et ont un rôle anti-tumoral [217] [218] [219]. De leur côté, les cellules Th22 sont caractérisées par la production d'IL-22 et contribuent à l'hyperplasie épidermique chez les sujets atteints d'une maladie inflammatoire de la peau [193] [220].

De façon importante, l'hétérogénéité au niveau du sang périphérique des sous-ensembles de lymphocytes T mémoires au profil de polarisation Th1/Th2/Th17/T_{FH}/T_{reg} ne révèle qu'une petite fraction de la complexité et la singularité de la distribution corporelle des lymphocytes T mémoires [221]. Ainsi, les cellules T_{EM} à profil de polarisation Th17 constituent le sous-ensemble prédominant de cellules T mémoires présentes dans l'intestin [222], alors que les ganglions lymphatiques par exemple renferment majoritairement des cellules T_{FH}. Cette distribution hétérogène des lymphocytes T $CD4^+$ au niveau des tissus soulève des questions importantes sur la recirculation en périphérie versus la résidence dans les tissus de certaines populations de cellules T.

1.1.9.4. Persistance des réservoirs cellulaires du VIH sous ART

Malgré une charge virale indétectable chez les sujets sous TAR, l'interruption du traitement est associée à un rebond virale rapide équivalent aux niveaux détectés avant l'initiation du traitement [223]. La TAR ne permet donc pas d'éradiquer le virus et les personnes infectées doivent être traitées à vie afin de maintenir le virus silencieux. Le VIH persiste pendant la thérapie antirétrovirale dans les réservoirs cellulaires, préférentiellement dans les cellules T $CD4^+$ quiescentes à long durée de vie, mais possiblement dans d'autres types cellulaires [175].

La proportion de cellules T $CD4^+$ infectées est très faible chez les patients sous TAR. On parle de 1 cellule infectée avec un virus compétant pour la réplication par 10^6 cellules T $CD4^+$ mémoires [224]. Malgré une charge virale indétectable, l'interruption du traitement est associée à un rebond viral rapide équivalent aux niveaux détectés avant l'initiation du traitement [223]. La TAR ne permet donc pas d'éradiquer le virus et les personnes infectées doivent être traitées à vie afin de maintenir le virus silencieux. Le VIH persiste, pendant la thérapie antirétrovirale, dans des réservoirs cellulaires, préférentiellement dans les cellules T $CD4^+$ quiescentes à long durée de vie, mais possiblement dans d'autres types cellulaires [175]. De nombreuses études visent à identifier ces cellules infectées en latence afin de mettre en évidence des thérapies ciblées.

Il est connu aujourd'hui que la sensibilité des lymphocytes T $CD4^+$ à l'infection à VIH varie selon leur stade de différenciation et de leur statut d'activation. Ainsi, les populations T_{CM} et T_{TM} à long durée de vie sont les principaux constituants du réservoir viral chez les sujets infectés sous TAR [53]. Les T_{SCM}, ont une durée de vie supérieure à celle des T_{CM} et T_{TM}, et affichent une fréquence très élevée d'infection [225 , 226]. Les séquences du génome viral dans les lymphocytes T_{SCM} en début d'infection et 4 à 8 ans plus tard sont parfaitement semblables, ce qui suggère que ces cellules contribuent au réservoir viral à très long terme [225].

Outre leur stade de différenciation et leur statut d'activation, la fonction effectrice des multiples sous-populations de cellules T $CD4^+$ dicte également leur permissivité à l'infection par le VIH et donc leur contribution à la persistance du VIH sous TAR. En effet, les cellules T_{FH} ($PD\text{-}1^+CXCR5^+$ productrices d'IL-21) sont hautement permissives au VIH et représentent le compartiment majeur de réplication viral chez les sujets virémiques [227] en plus de contribuer activement aux réservoirs viraux sous TAR [228]. Plus récemment, il a été démontré qu'une population de T_{FH} aux propriétés régulatrices, appelée T_{FH} régulatrice, contribue également au réservoir viral sous traitement [229].
De plus, la compréhension de la fonctionnalité et de la différenciation des T_{FH} est essentielle au développement de nouvelles immunothérapies et stratégies vaccinales. Quelques sujets VIH+ sont capables de générer des anticorps anti-VIH hautement neutralisants dont la production nécessite une maturation longue et complexe des lymphocytes B et pourrait être en lien direct avec la fonctionnalité des cellules T_{FH} chez ces sujets.

Quant aux cellules Th17 ($CCR4^+CCR6^+$) et Th1Th17 ($CXCR3^+CCR6^+$), elles sont préférentiellement infectées *in vitro* [156]. *Ex vivo*, les cellules $CCR6^+$ (Th17 et Th1Th17) du sang périphérique et du colon sigmoïde sont enrichies en ADN viral total chez les sujets sous TAR [156]. De plus, une portion des lymphocytes Th17 à longue durée de vie et infectés par un

virus compétent pour la réplication contribue de façon majeure à la persistance du VIH [230] (*voir section 1.2. Les cellules Th17 dans l'infection à VIH-1*)

Dans nos études au laboratoire, nous utilisons CCR6 comme un marqueur de cellules Th17. Hors, si toutes les cellules Th17 expriment CCR6, toutes les cellules exprimant CCR6 ne sont pas des Th17. Une étude d'une importance particulière a démontré qu'une fraction des cellules T $CD4^+CCR6^+$ exprime le facteur de transcription des Th17, RORC, mais ne produisent pas d'IL-17A ($CCR6^+IL\text{-}17A^-$) après stimulation via CD3/CD28 ou PMA/iono (*phorbol 12-myristate 13-acetate/ionomycine*). Néanmoins, une fraction non négligeable (20-40%) de ces cellules $CCR6^+$ initialement $IL17A^-$, produisent de l'IL-17A après avoir été cultivées pendant quatre jours en présence d'IL-2. Ces travaux démontrent que les cellules T $CCR6^+$ sont particulièrement bien disposées à exprimer l'IL-17 en réponse à la stimulation par de l'IL-2, quel que soit leur expression d'IL-17A *ex vivo* et suggèrent que les analyses des cellules Th17 sur la base de leur expression d'IL-17A *ex vivo* sous-estiment la fréquence des cellules T capables de produire de l'IL-17A dans des contextes inflammatoires [231].

Dans le contexte du VIH, l'altération des fonctions effectrices des cellules T causée par l'infection et l'épuisement du système immunitaire en général est à prendre également en considération. En effet, il a été montré *in vitro* que la réplication active du VIH altère la capacité des cellules Th17 à produire de l'IL-17A [232]. Il est à noter que même *in vitro*, l'exposition aux ARVs après l'établissement de l'infection ne permet pas une complète restauration des réponses Th17 [233] [234]. Chez les sujets non infectés par le VIH, une corrélation positive existe entre la fréquence des cellules T $CD4^+$ à profil de polarisation Th17 ($CD161^+CCR6^+$) et les cellules T $CD4^+$ produisant de l'IL-17A. Cette corrélation n'est pas retrouvée chez les sujets infectés suggérant une altération des fonctions effectrices des Th17 chez les sujets VIH+ [234]. Ces données suggèrent que l'identification des sous-populations de cellules T $CD4^+$ sur la base des cytokines qu'elles sécrètent n'est pas idéale pour étudier, par exemple, leur contribution au réservoir du VIH. Une étude a d'ailleurs identifié un enrichissement en ADN viral avec un génome intact dans les cellules produisant de l'IFN-γ suggérant que les cellules T $CD4^+$ à profil de polarisation Th1 sont les principaux composants du réservoir compétent pour la réplication [235]. Dans cette étude l'altération des fonctions effectrices des sous-

populations de cellules T CD4$^+$ n'est pas prise en compte, ni les cellules Th1Th17 (IL-17$^+$IFN-γ$^+$) et les cellules T folliculaire (T_{FH}) pourtant décrites comme des principaux contributeurs du réservoir viral. [236] [237] [230].

Par ailleurs, les cellules exprimant la combinaison de trois marqueurs d'exhaustion (TIGIT (*T cell immunoreceptor with Ig and ITIM domains*), PD-1 et *LAG3 (lymphocyte-activation gene 3)* sont largement enrichies en ADN proviral [238]. Le CD32a a été récemment identifié comme étant un marqueur de cellules T CD4$^+$ quiescentes portant de l'ADN proviral compétent pour la réplication [239]. Ces travaux sont aujourd'hui controversés par manque de reproductibilité [240] [241, 242] [243]. Les cellules du sang périphérique et des organes lymphoïdes associés à l'intestin exprimant CD30 sont enrichies en ARN viral chez les sujets sous TAR. Il s'agit donc probablement d'un marqueur de réservoirs actifs [244].

1.1.9.5. Réservoirs anatomiques du VIH

La majorité des études visant à caractériser les cellules réservoirs sont réalisées à partir des cellules du sang périphérique de par leur facile accessibilité. Cependant, les cellules T CD4$^+$ du sang périphérique représentent moins de 5% des cellules T CD4$^+$ totales [245] et celles qui y sont infectées ne représentent donc qu'une partie infime du réservoir viral. En fait, une majeure fraction des cellules infectées est retrouvée dans différents organes du corps que l'on nomme les tissus réservoirs. Ces tissus sont des sanctuaires où les médicaments, comme les antirétroviraux, pénètrent faiblement, ce qui permet une propagation virale résiduelle [181].

Chez les sujets sous TAR, la présence de cellules contenant de l'ADN viral peut-être détectée dans la moelle osseuse, le cerveau, les poumons, les parties reproductrices masculines et féminines et plus particulièrement dans les ganglions lymphatiques et l'intestin [246] [247, 248]. Au niveau de l'intestin, le nombre de cellules infectées est au moins deux fois plus élevé que dans le sang [249, 250]. Le nombre de copies d'ADN proviral par cellules T CD4$^+$ est nettement plus importante dans le duodenum, l'ileum, le colon et le rectum comparativement au sang périphérique, avec une transcription virale davantage active dans le duodenum et

l'ileum [251]. Une étude plus approfondie en 2018 a trouvé un niveau de transcrits précoces de VIH par provirus plus faible dans les cellules T $CD4^+$ isolé du rectum *versus* du sang périphérique suggérant que les cellules infectées dans le rectum sont dans un état de profonde latence [252].

Les mécanismes maintenant la latence dans les cellules T $CD4^+$ du sang périphérique et dans celle de l'intestin, aussi bien que dans les différentes sous-populations de cellules T, sont probablement différentes. L'ensemble des réservoirs viraux cellulaires et anatomiques représentent un obstacle majeur à l'éradication du VIH. Les différences au sein des sous-populations de cellules T $CD4^+$ et ainsi que les différences en les cellules provenant du sang périphérique et des tissus sont à prendre en considération dans la mise en place de nouvelles stratégies thérapeutique.

1.2. LES CELLULES TH17 DANS L'INFECTION À VIH-1

Revue – ***Publié dans Current Opinion in HIV and AIDS***

New Th17-Specific Therapeutic Strategies for HIV Remission

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Contribution des auteurs :

DP a conçu et écrit le manuscrit.
JPR a révisé le manuscrit.
PA a contribué à la conception du manuscrit et a effectué les dernières révisions.
Tous les auteurs ont examiné et accepté le manuscrit.

New Th17-Specific Therapeutic Strategies for HIV Remission

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ABSTRACT

Purpose of the review: This review highlights current knowledge on the dichotomous role played by Th17-polarized $CD4^+$ T-cells in maintaining mucosal immunity homeostasis *versus* fueling HIV/SIV replication/persistence during antiretroviral therapy (ART), with a focus on molecular mechanisms underlying these processes.

Recent finding: Th17 cells bridge innate and adaptive immunity against pathogens at mucosal barrier surfaces. Th17 cells are located at portal sites of HIV/SIV entry, express a unique transcriptional/metabolic status compatible with viral replication, and represent the first targets of infection. The paucity of Th17 cells during HIV/SIV infection is caused by infection itself, but also by an altered Th17 differentiation, survival and trafficking into mucosal sites. This causes major alterations of mucosal barrier integrity, microbial translocation and disease progression. Unless initiated during the early acute infection phases, ART fails to restore the frequency/functionality of mucosal Th17 cells. A fraction of Th17 cells are long-lived and carry HIV reservoir during ART. Recent studies identified Th17-specific host factors controlling HIV transcription, a step untargeted by current ART.

Summary: The identification of molecular mechanisms contributing to HIV replication/persistence in mucosal Th17 cells paves the way toward the design of new Th17-specific therapeutic strategies aimed at improving mucosal immunity in HIV-infected individuals.

Key words: Th17 cells, mucosal immunity, HIV, microbial translocation, ART

Key points:

Th17 cells are located at mucosal sites of HIV transmission

Th17 cell frequency/function is not restored with ART

Th17 cells are major sites for HIV replication and viral reservoir persistence

Novel Th17-specific therapies are needed for HIV remission/cure

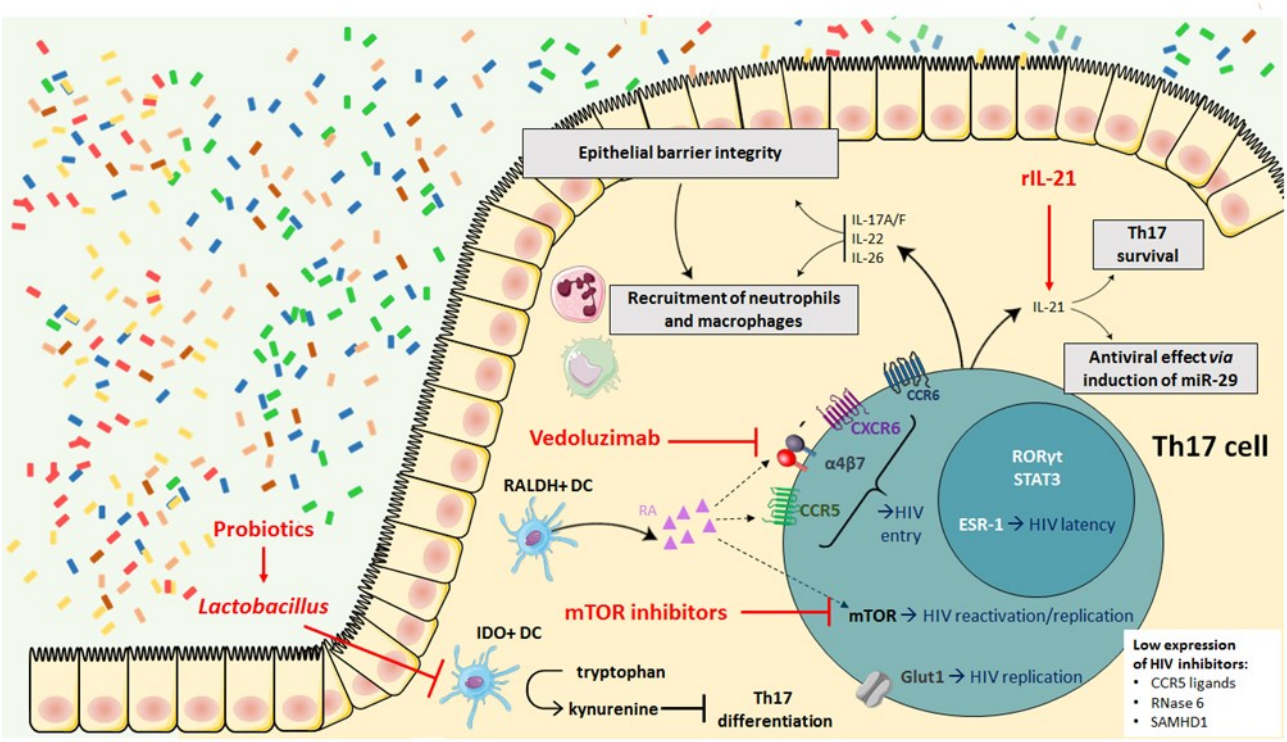

Figure 8. **<u>Novel Th17-specific strategies for HIV remission/cure.</u>**
Mucosal Th17 cells express HIV permissiveness factors (*e.g.,* CCR5, CXCR6, integrin α4β7, GLUT1, mTOR) as well molecular determinants critical for the maintenance of HIV-1 latency (*e.g.,* ESR-1), and low levels of HIV inhibitors (CCR5 ligands, SAMHD1 and RNAse 6). Intestinal Th17 cells reside in an environment rich in retinoic acid (RA), produced by RALDH^{+} dendritic cells (RALDH^{+} DC). RA acts on Th17 cells to up-regulate CCR5 and integrin α4β7 expression, promote mTOR activation, and subsequently increase HIV replication and viral reservoir reactivation in Th17 cells. Current therapeutic strategies designed to restore Th17 frequency/function include ART supplementation with: ***i)*** recombinant IL-21, a survival factor for Th17 cells and promoter of antiviral immunity *via* the induction of MiR-29; ***ii)*** probiotics to restore healthy microbiota; and ***iii)*** vedoluzimab (anti-α4β7 antibodies) for virological and immunological restoration. Finally, considering the role of mTOR in regulating Th17 polarization and HIV replication/transcription, future clinical trials should test the benefits of ART supplementation with mTOR inhibitors in reducing residual HIV transcription/replication in Th17 cells and subsequently in restoring Th17 functions in mucosal immunity.

1.2.1. DICHOTOMOUS FEATURES OF TH17 CELLS IN MUCOSAL IMMUNITY *VERSUS* DISEASE PATHOGENESIS

The discovery of T helper 17 cells (Th17) revealed a previously unrecognized phenotypic/functional heterogeneity of $CD4^+$ T-cells (1, 2). Th17 cells are unique in their ability to bridge innate and adaptive immunity at skin/mucosal barrier surfaces (1, 2). Th17 cells produce lineage-specific cytokines (*i.e.,* IL-17A/F, IL-21, IL-22, IL-26) with pivotal roles in maintaining epithelial cell (EC) physical/biological barrier functions (1, 2). The Th17-EC interplay leads to the subsequent recruitment of other effector cells (*e.g.,* neutrophils, macrophages) that further contribute to mucosal defense, mainly in response to bacterial and fungal pathogens (1, 2), but also to human immunodeficiency virus type 1 (HIV-1) (2, 3). The Th17 specification and acquisition of effector functions are under the control of specific components of the microbiota and cytokines, mainly produced by antigen presenting cells (*e.g.,* IL-23, IL-1β, IL-6, TGF-β, IL-21) (2). In contrast to other Th lineages that are more fixed in their differentiation program, Th17 cells exhibit a tremendous developmental flexibility/plasticity (1, 2). Noteworthy, Th17 ability to dedifferentiate into regulatory T-cells (Tregs) is essential for the resolution of inflammation and tissue homeostasis (4). Th17 functions are under the control of a complex network of signaling molecules and transcription factors, with retinoic acid (RA)-related orphan receptor (ROR) gamma t (RORγt) representing the master regulator (1, 2). While mutations in RORγt were linked to impaired Th17-mediated immunity in humans (5), exacerbated Th17 functions contribute to autoimmunity (1, 2). Mice/human studies identified molecular determinants of pathogenic (*e.g.,* T-bet, multidrug resistance 1 (MDR1), IFN-γ, GM-CSF) and non-pathogenic (*e.g.,* AhR, MAF, IL-22) Th17 cells (6).

This review focuses on the beneficial *versus* deleterious roles played by Th17 cells during HIV-1 infection and highlights recent discoveries that orient the design of Th17-specific therapeutic strategies toward HIV remission.

1.2.2. PAUCITY OF INTESTINAL TH17 CELLS DURING HIV/SIV INFECTION

Mucosal Th17 cells are lost during the acute HIV infection and their frequency/function are not restored by antiretroviral therapy (ART) (2). Of note, compared to rapid progressors, HIV-infected slow progressors exhibit a higher frequency HIV-specific Th17 cells that are functionally competent (7). Th17 depletion is associated with impaired mucosal immune responses, EC barrier disruption, and microbial translocation, leading to chronic immune activation in ART-treated HIV-infected individuals (2). Intestinal EC dysfunction precedes Th17 depletion during acute SIV infection (8). In contrast, Th17 cells are preserved in the lungs (9, 10), supporting the idea of tissue-specific functional Th17 heterogeneity.

Several mechanisms were reported to explain Th17 depletion/dysfunction during HIV/SIV infections and their non-restoration with ART, including: ***i)*** altered CCL25/CCL20-mediated trafficking into the gut (11, 12); ***ii)*** polarization artefacts due to overexpression of Th17 negative regulators (13); ***iii)*** paucity of naive Th17 (14) and $CCR6^+$ stem cell-like memory T (T_{SCM}) Th17 precursors (15); ***iv)*** increased activity of indoleamine 2,3-dioxygenase (IDO), an enzyme catabolizing tryptophan into kynurenin and promoting Treg expansion to the detriment of Th17 cells (16); ***v)*** loss of intestinal $CD103^+$ dendritic cells (DC) involved in Th17 differentiation (17); ***vi)*** deficit in IL-21 (18, 19), a major Th17 survival factor; and ***vii)*** HIV-mediated depletion (2). Of particular importance, Th17 cells are located at portal sites of HIV/SIV entry (20, 21). In a model of SIV vaginal transmission, $CCR6^+RORgt^+IL\text{-}17A^+$Th17 cells were identified as the first infection targets (21). Consistently, an increased Th17 frequency in the rectal mucosa of high risk human populations was associated with an increased HIV acquisition risk (22, 23).

1.2.3. THE ANTIGENIC SPECIFICITY OF HIV-PERMISSIVE TH17 CELLS

HIV infects preferentially HIV-specific $CD4^+$ T-cells and also T-cells specific to pathogens that cause HIV-associated opportunistic infection (*e.g., Mycobacterium tuberculosis* (MTb)) (24, 25). HIV- and MTb-specific T-cells, as well as T-cells specific to pathogens predominant in the microbiota of HIV-infected individuals (*e.g., Candida albicans*, *Staphylococcus aureus*, and

Prevotella stercorea) (22, 26, 27) exhibit Th17 features (2). Considering the fact that Th17 cells integrate signals from the microbiota to increase their HIV permissiveness (27, 28), future studies are needed to document the antigenic specificity of Th17 cells carrying HIV reservoirs during ART.

1.2.4. TH17 CELLS CONTRIBUTE TO HIV RESERVOIR PERSISTENCE DURING ART

Subsets of Th17 cells exhibiting a stem-cell like molecular signature are present in the blood, lymph nodes and colon (reviewed in (2)) and contribute to HIV reservoirs persistence during ART (29-32). In this context, finding new strategies to prevent Th17 infection, depletion and/or dysfunction in ART-treated HIV+ individuals remains a topic of active investigations.

1.2.5. HIV ENTRY IN TH17 CELLS

HIV infects cells expressing its receptor CD4 and coreceptors CCR5 and CXCR4 (33). Th17 *versus* Th1 cells highly express CCR5 and secrete low level of CCR5-binding chemokines (2). Th17 cells also express CCR6 and CXCR6, two documented minor HIV/SIV co-receptor (2, 34, 35). Furthermore, Th17 cells express the gut-homing molecule integrin α4β7 (36, 37), an adhesion molecule up-regulated by RA (38), a derivative of vitamin A metabolized by intestinal DC (39). In addition to its role in cell trafficking, integrin α4β7 was identified as an HIV binding receptor incorporated into virions (40). Finally, mucosal vascular addressin cell adhesion molecule 1 (MadCAM-1), expressed by endothelial cells and DC, mediates signaling through integrin α4β7 and promotes HIV infection of naive T-cells (41). Of note, our group demonstrated the presence of memory T-cells with a $CCR6^{+}CCR5^{+}\alpha4\beta7^{+}$ signature in the colon of ART-treated HIV-infected individuals (42), consistent with the idea that Th17 cells are present at portal sites of HIV entry.

1.2.6. HIV POST-ENTRY MECHANISMS IN TH17 CELLS

Multiple mechanisms restrict HIV reverse transcription and integration (33). Single-round infection using VSV-G-pseudotyped-HIV to bypass receptor/co-receptor-mediated entry

demonstrated still a superior ability of Th17 and Th1Th17 cells to support HIV transcription/integration (43-45). To identify post-entry HIV permissiveness factors, our group performed genome-wide transcriptional profiling that led to the identification of a unique molecular signature associated with HIV permissiveness in Th17 and Th1Th17 cells (*e.g.,* ZAP-70, Lck, NF-kB, MAP3K3, PTPN13, SERPINB6) (45). Similar transcriptional studies linked HIV permissiveness in Tetanus toxoid- and Candida albicans-specific T-cells to a Th17 signature and to limited antiviral responses (46). Another transcriptional study revealed a decreased expression of the RNase A superfamily proteins 2, 3 and 6 in Th17 *versus* Th1 cells and reported on the ability of RNase 6 to inhibit HIV replication (47). In line, circulating Th17 cells express low levels of SAMHD1 (48), an HIV restriction factor acting at the level of reverse transcription (33) and transcription (49). Noteworthy, a HIV latency model was established in Th17 cells, and allowed the discovery of the estrogen receptor-1 (ESR-1) as a host factor maintaining HIV-1 latency in a gender-dependent manner (50). Although the precise molecular mechanism used by ESR1 to promote latency remains unknown (50), it is important to highlight the ability of estradiol to repress RORγt transcription (51). The latter mechanism, may explain sex hormone-mediated modification of the female genital tract (FGT) microenvironment (*i.e.,* mucosal dysbiosis, HIV-target cells) with impact on the HIV acquisition risk (52).

1.2.7. THE UNIQUE METABOLIC STATUS OF TH17 CELLS

Glucose metabolism plays a central role in HIV replication (53). The glucose transporter 1 (GLUT1) is identified as a marker of T-cells highly permissive to HIV infection (54). High glucose metabolism in $GLUT1^{+}CD4^{+}$ T-cells is associated with immune activation, CD4 loss during chronic HIV infection (55), and Akt/PI3K activation (56). Th17 cells express the hypoxia-inducible factor-1 (HIF-1α), a key regulator of glucose metabolism (57). Th17 differentiation depends on the Akt/PI3K pathway (58), with high glucose levels promoting the activation of the mammalian target of rapamycin (mTOR) (57). mTOR, a sensor for intracellular nutrients, controls protein synthesis, cell growth and metabolism (59). In our search for HIV permissiveness factors in gut-homing Th17 cells, we identified mTOR phosphorylation as a signature for $CCR6^{+}$T-cells exposed to RA *in vitro* and those infiltrating the colon of ART-treated

HIV+ individuals (42). mTOR phosphorylation in CCR6^{+}T-cells coincided with high CCR5 and integrin β7 co-expression and HIV permissiveness (32, 42). Finally, in a viral outgrowth assay (VOA), the mTOR inhibitors rapamycin and INK128 inhibited HIV reactivation from CCR6^{+}T-cells of ART-treated individuals (42). Other groups identified mTOR as a positive regulator of HIV replication by acting at the level of viral entry (60) and transcription (61, 62), or *via* the modulation of autophagy (63, 64). In line, ART-treated HIV+ individuals receiving kidney transplants and treated with sirolimus (mTORC1 inhibitor) exhibit reduced HIV-DNA reservoirs (65). This evidence provides a molecular explanation for the preferential HIV persistence in colon CCR6^{+}Th17 cells during ART (30, 32, 42), and point to mTOR as a new antiviral drug target.

1.2.8. GUT-HOMING TH17 CELLS, DRUG PENETRATION AND RESIDUAL HIV REPLICATION DURING ART

Gut-associated lymphoid tissues (GALT) are enriched in HIV-DNA reservoirs during ART (66). Th17 cells express the gut-homing molecules CCR6, CCR9 and integrin α4β7 (2). The expression of integrin α4β7 and CCR9 is induced by RA (38). Of note, RA-mediated imprinting for gut-homing promotes HIV replication in CCR6^{+}Th17 cells (42, 43). By using the next-generation HIV/SIV DNA/RNAscope *in situ* hybridization technology, Estes *et al.* documented viral RNA persistence in multiple lymphoid/non-lymphoid tissues, including the GALT, of SIV-infected Rhesus macaques (RM) and HIV-infected individuals receiving ART (67). Of note, antiretroviral drug levels in lymph nodes, gut and rectum were lower compared to blood cells (67), consistent with the possibility that poor drug penetration in specific tissues fuels viral replication during ART (68, 69). Drug transporters are key regulators of drug penetration in tissues/cells (70). Pathogenic Th1Th17 cells express MDR1/P-glycoprotein, a drug efflux pump that renders cells refractory to glucocorticoids used to treat autoimmune diseases (6). Since CCR6^{+} T-cells with a Th17 and Th1Th17 phenotype are enriched in the colon and carry HIV reservoirs during ART (30, 32, 42), their expression of drug transporters in relationship with intracellular drug concentration and residual HIV transcription deserves investigations.

1.2.9. NOVEL TH17-SPECIFIC THERAPIES FOR HIV REMISSION/CURE

Considering the dichotomous role played by Th17 cells in maintaining mucosal immunity homeostasis and fueling HIV replication/persistence, multiple groups including ours focus their efforts on the identification of new strategies to prevent infection, limit residual viral replication, and restore Th17 frequency/function in HIV+ individuals (Figure 1).

Early ART initiation. Studies in high risk HIV acquisition cohorts revealed that early ART initiation is beneficial in preserving the frequency/poly-functionality of intestinal Th17 cells (71-73) and promotes a gradual Th17-Tscm precursors reconstitution (15). Nevertheless, early ART initiation is strictly dependent on early HIV diagnosis that still remains a challenge even in developed countries. Also, ART does not block HIV transcription in reservoir cells (33), and residual viral transcription/replication during ART causes chronic immune activation (74). This justifies the need for finding alternative strategies to target HIV transcription in reservoir cells, such as mTOR inhibitors (see above).

IL-21 supplementation. IL-21 acts on Th17 cells to facilitate their survival (1, 2). IL-21 is produced by follicular helper cells (Tfh) and by specific Th17 subsets with long-lived features (2, 31). The paucity of IL-21 was reported in the plasma of HIV-infected individuals (18). Depletion of IL-21-producing $CD4^+$ T-cells was observed in both peripheral blood and intestinal mucosa during pathogenic but not non-pathogenic SIV infection (19). Administration of recombinant IL-21 (rIL-21) during acute SIV infection restored intestinal Th17 cells, decreased microbial translocation, reduced immune activation, and diminished viral reservoirs (75, 76). In combination with probiotics, rIL-21 improved poly-functional Th17 expansion in ART-treated SIV+ RM and restored mucosal homeostasis (77). Of note, a beneficial effect of rIL-21 on the antiviral functions of $CD8^+$ T-cells was also observed (78). Finally, IL-21 is known to promote anti-HIV immunity *via* the induction of MiR-29 (79). Thus, human clinical trials are needed to explore this intervention for HIV remission/cure.

***Probiotics*.** Considering the link between mucosal dysbiosis and chronic inflammation in ART-treated HIV+ individuals (80, 81), as well as reported interferences between microbiota components and tenofovir (82), a new therapeutic concept emerged in which restoring a healthy microbiota will contribute to HIV remission. Indeed, prebiotic/probiotic supplementation led to $CD4^+$ T-cell reconstitution and the reduction of inflammation in the GALT of ART-treated SIV^+ RM (83). Similarly, probiotics reduced immune activation/inflammation and decreased microbial translocation in ART-treated HIV-infected individuals (84-86). Components of the microbiota, such as *Lactobacillus,* which are under-represented during HIV/SIV infection (87), facilitate Th17 differentiation by limiting the indoleamine 2,3-dioxygenase 1 (IDO-1) activity in the intestine (88). Probiotics reduced plasma levels of IDO metabolites in HIV-infected individuals (89). Finally, probiotics increased the frequencies of mucosal/systemic Th17 cells and decreased T-cell activation in both compartments (90). Nevertheless, the link between mucosal dysbiosis and SIV/HIV disease progression still remains a matter of investigation (91).

Vedoluzimab. The monoclonal anti-integrin α4β7 antibodies (Abs), vedoluzimab, is effective in treating inflammatory bowel disease (IBD) (92, 93) and is currently tested for HIV cure/remission (40). In the SIVmac251 model, blood memory $CD4^+$ T-cells expressing high levels of integrin α4β7 were reported to be preferentially infected during the very early phase of infection (36) and selectivelly depleted from gut tissues as early as the first 2 weeks following infection in humans (94). Consistent with these observations, blocking integrin α4β7 before SIV infection prevented/delayed SIV acquisition, decreased gut viral load, and reduced gut damages (95, 96). This protective effect of anti-α4β7 Abs may provide long term benefits, as demonstrated in a study in which the combination of anti-α4β7 Abs with ART promoted sustained virological control upon ART interruption (97). Surprisingly, the later results were not reproduced in another study using the same RM model, experimental design and SIVmac239nefstop virus (98). In humans, vedolizumab administration in a cohort of HIV-infected IBD patients led to the attenuation of lymphoid aggregates without HIV reservoir size changes (99). Accordingly, preliminary results from a clinical trial performed in HIV^+ individuals

treated with ART >2 years demonstrated no differences between vedolizumab-treated and placebo groups in viral rebound upon ART interruption (100). Additional clinical trials are warranted to elucidate potential benefits of vedoluzimab in HIV prevention/treatment.

Other strategies to explore

The recruitment into tissues of Th17 cells is also regulated by CCR6, a chemokine receptor identified as a stable Th17 surface marker (2). Of note, the inhibition of CCR6-CCL20 migration reduces experimental autoimmune encephalopathy and multiple sclerosis (101). Similarly, the microbicide glycerol monolaurate (GM) blocks SIV dissemination upon vaginal challenge by interfering with the CCL20-mediated recruitment of $CCR6^+$ DC at the portal site of SIV entry (102).Whether GM interferes with the recruitment of $CCR6^+$Th17 cells in the vaginal mucosa remains to be investigated. Of note, CCR6 blocking strategies were conceived for autoimmunity treatment and their use in ART-treated HIV+ humans remains to be investigated. Finally, non-steroidal anti-inflammatory drugs may be used to reduce HIV acquisition by decreasing the recruitment of HIV targets ($CCR5^+$Th17) into the FGT mucosa (103).

1.2.10. CONCLUSIONS

The preservation of mucosal Th17 pool is key for maintaining intestinal homeostasis in the context of complex interactions with the commensal/pathogenic microbiota. HIV-1 infection is associated with a profound alteration of Th17 frequency/functions and such alterations are not restored with viral-suppressive ART, unless treatment is initiated during the early phases of acute infection. Understanding common molecular mechanisms that govern lineage commitment/specification *versus* HIV replication/persistence in Th17 cells (*e.g.,* signaling molecules, metabolic regulators, transcription factors) will open the path toward the intelligent design of novel Th17-specific therapeutic strategies for HIV remission/cure.

Acknowledgments: This study was supported by grants from the Canadian Institutes of Health Research (CIHR) to PA (#MOP-82849; #MOP-114957; #TCO125276; #PJT-153052) and JPR (MOP-103230; MOP-154051), grants from the CIHR Canadian HIV Trials Network (CTN #247), the *Fonds de Recherche du Québec-Santé* (FRQ-S)/AIDS and Infectious Diseases Network, Québec, Canada to JPR, and by The Canadian HIV Cure Enterprise Team Grant HIG-133050 (to PA and JPR) from the CIHR in partnership with CANFAR and IAS. DP received a Doctoral awards from the Université de Montréal and the FRQ-S. JPR holds a Louis Lowenstein Chair in Hematology and Oncology, McGill University. The authors thank Laurence Raymond Marchand and Dr. Nicolas Chomont for the critical reading of the manuscrit.

Author Contributions: DP conceived and wrote the manuscript. JPR revised the manuscript. PA contributed to the conception of the manuscript and performed final revisions.

Conflicts of Interest: The authors declare no conflict of interest. The founding sponsors had no role in the writing of the manuscript and in the decision to publish.

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1.3. TRAITEMENTS ACTUELS ET NOUVELLES STRATÉGIES THÉRAPEUTIQUES

1.3.1. TRAITEMENTS ANTIRÉTROVIRAUX

En 1987 est mis sur le marché le premier ARV, le zidovudine (AZT; *azidothymidine*); un inhibiteur de la transcriptase inverse du VIH. L'apparition rapide de résistance virale à l'AZT a accéléré la mise en marché d'autres inhibiteurs nucléosidiques de la transcriptase inverse (NRTIs) dès 1991. Cependant, l'efficacité des traitements donnés en monothérapie était seulement de courte durée en raison de l'émergence automatique de résistance virale. Des avancées fondamentales dans la compréhension du cycle de réplication virale ont permis la mise sur le marché d'une nouvelle classe d'ARV, les inhibiteurs de protéase virale [253]. La combinaison de deux classes de drogues ARVs, un inhibiteur de RT et un inhibiteur de protéase, fut adoptée comme stratégie de traitement en 1996 [254]. Cette thérapie antirétrovirale hautement active (HAART; *highly active anti-retroviral therapy*) permet, chez les sujets vivant avec le VIH, de maintenir une charge virale indétectable, d'augmenter le compte de lymphocytes T $CD4^+$ en périphérie et d'empêcher la progression de la maladie vers la phase SIDA [255, 256]. En 2000, Daria Hazuda et al., mettent en évidence une nouvelle stratégie thérapeutique anti-VIH qui est dirigée contre l'enzyme virale responsable de l'intégration du génome virale dans la cellule hôte. [257]. Ce n'est que quelques années plus tard que les inhibiteurs d'intégrase s'intègrent aux méthodes de traitement du VIH avec la mise en marché du raltégravir en 2007. Ces molécules pharmacologiques sont préconisées comme médicaments de première ligne contre l'infection au VIH [258].

Il existe plusieurs classes d'ARVs ciblant différentes étapes du cycle viral [2, 259](Tableau 1).

Tableau I. **Liste non exhaustive des médicaments antirétroviraux de chaque classe.**

Inhibiteurs du site de l'entrée (EI; entry inhibitors)
Inhibiteurs de fusion : Enfuvirtide
Antagoniste du récepteur CCR5 : Maraviroc
Inhibiteur direct post-attachement à CD4 : Ibalizumab
Inhibiteurs nucléosidiques et nucléotidiques de la transcriptase inverse (NRTIs; Nucleoside reverse transcriptase inhibitors)
AZT, 3TC, Tenofovir, Zidovudine, Abacavir, Lamivudine, Stavudine
Inhibiteurs non nucléosidiques de la transcriptase inverse (NNRTIs; Nonnucleoside reverse transcriptase inhibitors)
Efavirenz, Névirapine, Delavirdine, Etravirine, Rilpivirine
Inhibiteurs de l'intégrase (INSTIs; Integrase inhibitors)
Raltegravir, Dolutegravir, Elvitegravir
Inhibiteurs de la protéase (Pis; Protease inhibitors)
Atazanavir, Nelfinavir, Saquinavir, Indinavir, Darunavir, Fosamprénavir, Ritonavir

Le traitement actuel combiner le plus souvent plusieurs médicaments. Le choix de ces combinaisons suit les lignes directrices thérapeutiques et doit prendre en compte l'historique médical du sujet (résistance préalable, interactions médicamenteuses, effets secondaires) [260].

Les ARVs sont aussi utilisés aujourd'hui en prévention pour les personnes non-infectées, mais à haut risque d'être exposées au VIH. Ce concept de prophylaxie préexposition (PrEP) est une combinaison de deux NRTIs (Tenofovir et Emtricitabine) [261]. Plusieurs essais cliniques réalisés dans différentes villes à travers le monde ont montré que les individus qui sont sous

PrEP avant un rapport sexuel à risque sont protégés de l'infection à plus de 80% [262-266]. Cependant, en plus de sa toxicité à long terme, la PrEP ne permet pas d'éviter la transmission des autres infections bactériennes (*e.g.,* gonorrhée, chlamydia, syphilis) et virales (*e.g.,* VHC, VPH) transmises sexuellement; notamment l'émergence et la transmission des souches bactériennes résistantes aux antibiotiques représente un problème de santé grave à considérer. Une information correcte de la population s'impose sur les avantages et les désavantages de la PrEP. De façon importante, des études ont mis en évidence l'induction des réponses immunologiques spécifiques au VIH chez les utilisateurs de la PrEP non-infectés [267]. Ceci soulève le besoin d'études futures sur la possibilité de vaccination naturelle anti-VIH des personnes exposées au VIH sous PrEP.

1.3.2. NOUVELLES STRATÉGIES THÉRAPEUTIQUES

La recherche sur le VIH, depuis 1983, a permis une caractérisation approfondie du virus ([2]. L'accumulation des connaissances moléculaires et cellulaires a rendu cette infection autrefois létale en une maladie chronique gérable. Malgré cette avancée, la transmission du VIH est toujours élevée dans certaines populations (*e.g.,* communauté homosexuelle, utilisateurs de drogues, travailleurs du sexe, transmission accidentelle iatrogène dans les pays en voie de développement). Des progrès politiques et éducatifs sont nécessaires pour délivrer les traitements ou proposer de nouvelles options thérapeutiques et pour prévenir les nouvelles infections afin d'envisager une éradication du VIH au niveau de la population. Dans ce sens les objectifs de l'OMS consistent à encourager chacun à connaître, par le dépistage, son état sérologique vis-à-vis du VIH afin d'accéder aux services de traitement et de soins; et à encourager les responsables politiques à promouvoir un programme de « santé pour tous » pour le VIH et l'accès aux services connexes, comme ceux pour la tuberculose, l'hépatite et les maladies non transmissibles [268]. Ces stratégies sont essentielles pour atteindre la cible fixée par l'ONUSIDA qui vise à atteindre, d'ici 2020, le « 90-90-90 ». Cela statue que 90% des personnes séropositives pour le VIH connaissent leur statut sérologique que 90% de ces individus reçoivent un traitement antirétroviral et que 90% des personnes sous traitement antirétroviral voient leur charge virale disparaître. L'objectif second de l'ONUSIDA consiste à

lutter contre les inégalités d'accès aux services sanitaires et sociaux de base et à mettre un terme à l'épidémie du VIH comme menace de santé publique d'ici 2030 [269].

Au niveau individuel, un effort scientifique majeur est nécessaire pour mettre en place des nouvelles stratégies thérapeutiques visant soit une éradication totale du VIH de l'organisme des personnes vivant avec le virus soit une guérison fonctionnelle ou hybride, comme décrit ci-dessous en plus de détails [270].

1.3.2.1. Stratégies vaccinale préventive

La conception de vaccins empiriques a conduit à la génération de vaccins approuvés cliniquement contre 26 agents pathogènes dont certains virus tels que le virus de la polio, le virus de fièvre jaune et autres. Cependant aucunes approches similaires n'ont permis de générer un vaccin préventif anti-VIH efficace. Cet échec est en partie dû au taux de mutation particulièrement élevé du VIH, à la faible densité de protéine d'enveloppe (Env) sur la surface virale ainsi qu'au nombre limité de sites de vulnérabilité neutralisante présentes sur la protéine Env qui nécessite des empreintes de fixation et des angles d'attaque hautement spécialisés pour la neutralisation à l'aide d'anticorps. Ceci limite considérablement l'évolution des anticorps neutralisants.

Seulement 6 essais vaccinaux peu fructueux ont vu le jour jusqu'à présent [271]. L'un d'eux, l'essai thaïlandais RV144, a cependant montré une efficacité modeste mais statistiquement significative. Cet essai incluant plus de 16 000 individus VIH- mais à risque d'infection a montré une diminution du taux d'infection de 31.2 % dans le groupe vacciné par rapport au groupe placebo [272].

1.3.2.2. Stratégies d'éradication du VIH

L'éradication du VIH consiste à éliminer définitivement la totalité des cellules infectées. Jusqu'à très récemment, le seul exemple d'éradication du VIH est Timothy Ray Brown, le patient de Berlin [273]. Les circonstances de sa guérison complète sont très particulières, car infecté par le VIH depuis 1995, il apprend, en 2006, qu'il est atteint d'une leucémie aigüe

myéloïde. Il subit alors deux transplantations allogéniques de moelle osseuse pour le traitement de sa leucémie. Chacune de ces transplantations était précédée d'une irradiation corporelle totale afin de détruire le système immunitaire du receveur. Le donneur était homozygote pour la mutation Δ32 sur le gène CCR5 et donc les cellules du donneur étaient « résistantes au VIH » [273].

À la suite du cas de Timothy Ray Brown, une transplantation de moelle osseuse allogénique a été réalisée chez deux patients atteints d'un lymphome, dits les patients de Boston. Dans ces cas, les donneurs n'avaient pas de mutation sur le gène CCR5 et les conditions de chimiothérapie étaient moins invasive que pour le patient de Berlin. Ainsi, malgré un remplacement quasi-total de leur système immunitaire, un rebond viral a été observé chez ces deux participants après 12 semaines et 8 mois après l'arrêt du traitement [274]. Ces travaux, d'une importance particulière, mettent la lumière sur la présence, chez les patients de Boston, de cellules infectées ayant persisté après une chimiothérapie intensive et une transplantation de moelle osseuse [274]. Ceci soulève des questions sur la dynamique du réservoir du VIH, surtout sur le « re-seeding » des nouvelles cellules réservoirs sous ART.

En mars dernier, des chercheurs européens ont annoncé un deuxième cas de rémission [275]. Ce sujet, appelé le patient de Londres, a subi une transplantation de moelle osseuse afin de traité un lymphome de Hodgkin. Contrairement à Thimothy Ray Bown, le sujet de Londres n'a pas subi d'irradiation corporelle complète. Par contre le donneur était aussi homozygote pour la mutation Δ32 sur le gène CCR5. Après 18 mois d'interruption de traitement, aucun rebond viral n'a été observé. Ce cas démontre que la rémission du VIH-1 peut être possible avec une approche moins agressive et toxique que celle utilisé pour Timothy Ray Brown. Cela souligne surtout l'importance des cellules $CCR5^{+}$ permissives au VIH dans la persistance des réservoirs du VIH et le rebond viral après arrêt du traitement.

Il est important de souligner qu'à grand échelle, une telle stratégie de guérison n'est pas envisageable. Le risque de mortalité d'une transplantation de moelle osseuse étant très élevé,

cette procédure n'est pas justifiable chez des sujets infectés par le VIH mais n'ayant pas de cancers et qui se portent relativement bien sous TAR. Des stratégies d'éradications alternatives doivent donc être considérées.

1.3.2.3. Stratégies de guérison fonctionnelle

La guérison fonctionnelle du VIH consiste à atteindre un contrôle, par le système immunitaire, de la réplication virale sans pour autant éliminer les cellules réservoirs.

Moins de 1% de la population infectée par le VIH est capable de contrôler la réplication virale en absence de traitement. Ceci laisse penser que la modulation et la restauration du système immunitaire pourraient être une piste intéressante pouvant mener à la guérison fonctionnelle. Ces rares individus, appelés les « contrôleurs élites », sont caractérisés par une infection connue depuis plus de dix ans et une mesure de la charge virale < 400 copies/ml dans 90 % des mesures effectuées en l'absence de TAR [276] [277] [278]. L'étude des contrôleurs élites a permis de mettre en évidence plusieurs caractéristiques immunologiques communes. Ils sont, pour la plupart, porteurs des allèles protecteurs HLAB57 et B27 et maintiennent une réponse des cellules T $CD8^+$ anti-VIH efficace et polyfonctionnelle [279] [280] [281] [282] [283]. Les contrôleurs élites présentent également une réponse immunitaire humorale efficace contre le VIH via la génération d'anticorps neutralisants à large spectre (bNAbs; *broadly neutralizing antibody*) [284] [285] [286].

Plusieurs études ont mis en évidence l'intérêt d'initier le traitement antirétroviral très tôt après la séroconversion en vue d'une rémission à long terme. Les études ACTG A5217 et SPARTAC ont montré que l'initiation du traitement dans les six mois post-infection augmentait les chances que le système immunitaire puisse, par la suite, contrôler la réplication virale lors d'interruptions de traitement [287] [288]. Pour sa part, l'étude VISCONTI menée en France a inclus 14 sujets qui ont initié les ARVs au cours de la phase de primo-infection (autour de la dixième semaine suivant l'infection) et qui ont atteint un contrôle viral durable durant plusieurs années (médiane de 89 mois) après interruption de traitement [289]. Ces sujets

appelés contrôleurs post-traitement (« post-treatment controlers ») ne sont pas porteurs des allèles protecteurs HLAB57 et B27. Les mécanismes impliqués dans le contrôle du virus chez les contrôleurs post-traitement semblent être différents des ceux retrouvés chez les contrôleurs élites. La compréhension de la capacité des contrôleurs élites et post-traitement à maitriser la réplication virale est un enjeu majeur dans le développement des stratégies thérapeutiques et préventives.

L'une de ces stratégies vise à modifier génétiquement les cellules souches hématopoïétiques ou les cellules T $CD4^+$ des sujets infectés afin de les rendre résistante à l'infection par le VIH. La perfusion de cellules T $CD4^+$ autologues génétiquement modifiées *ex vivo* pour rendre le gène CCR5 dysfonctionnel a été testée chez 12 sujets infectés par le VIH [290]. Malgré des changements immuno-virologiques, la procédure n'a pas permis d'éviter un rebond viral après interruption de traitement, probablement en raison de la faible proportion de cellules génétiquement modifiées chez le receveur infecté [290]. Une stratégie alternative consiste à modifier les cellules autologues génétiquement afin qu'elles expriment l'endonucléase CRISPRCas9 (*Clustered Regularly Interspaced Short Palindromic Repeats Cas associated protein 9*). L'élimination du gène CCR5 en utilisant cette technologie a montré un profil de résistance à l'infection *in vitro* et *in vivo* dans un modèle de souris humanisées [291] [292]. L'utilisation de CRISPRCas9 pour éliminer l'ADN proviral a également été testé avec succès *in vitro* [293]. Cependant, le recours à cette technique *in vivo* est compliquée à imaginer notamment à cause de la grande diversité des séquences des provirus.

La découverte et la caractérisation des anticorps neutralisant à large spectre, les bNAbs, a relancé l'espoir d'un vaccin anti-VIH curatif [294-296] [297]. Environ 15% des sujets VIH développe ces anticorps 2 à 5 ans après avoir été infecté. Ces bNAbs ont un pouvoir neutralisant supérieure et couvrent davantage de souches virales qu'un anticorps neutralisant classique [298].

La guérison fonctionnelle peut également consister à réduire la taille du réservoir ainsi qu'à induire le contrôle par le système immunitaire du réservoir résiduel. L'une de ces stratégies est appelée le « kick and kill » ou « shock and kill ». Dans ce contexte, la réactivation du réservoir latent est d'abord induite par des agents chimiques (LRAs; *latency reversing agents*) et il s'en suit la mort des cellules réactivées et infectées grâce à l'effet cytopathique des protéines du VIH. Les LRAs testés inclus, entre autres, des inhibiteurs des histones déacétylase (HDACs), des inhibiteurs de l'ADN méthyltransférase, des inhibiteurs de la bromodomaine et des agonistes de la protéine kinase C (PKC) [299]. Les HDACs sont, à ce jour, les plus étudiés et agissent en conservant les histones acétylées au niveau du LTR du VIH, ce qui est associé à une transcription active du virus [300] [301] [302].

Malgré leur efficacité *in vitro* [303], les LRAs testés *in vivo* ne permettent pas de diminuer la taille du réservoir [300] [304] [302] [305]. Ceci est, en grande partie, dû à l'incapacité du système immunitaire à éliminer efficacement les cellules infectées, mais aussi à l'efficacité différentielle des LRAs en fonction du statut de différenciation des lymphocytes T $CD4^+$ [306].

Dans une approche similaire, les travaux de ma thèse visent à l'utilisation d'un antagoniste de PPARγ (régulateur négatif de la transcription du VIH et de RORC) comme nouvelle stratégie de « shock and kill » visant à réactiver le virus latent tout en induisant les fonctions effectrices des cellules Th17 *(Manuscrit #3, Chapitre 5)*.

Une stratégie opposée, le « lock and block », consiste à plonger les cellules dans une profonde latence en utilisant des inhibiteurs de la protéine virale Tat [307] [308]. Alternativement, d'autres stratégies « block and lock » pourraient cibler des facteurs moléculaires des cellules hôtes. Ceci est, entre autres, la stratégie que nous utilisons au laboratoire. Effectivement, les travaux de ma thèse ont identifié mTOR, un régulateur positif de la transcription du VIH, comme étant hautement exprimé par les cellules Th17 intestinaux. L'utilisation d'inhibiteurs de mTOR vise à un « block and lock » comme stratégie thérapeutique ciblant

préférentiellement des cellules infectées, c'est-à-dire, les lymphocytes Th17 intestinaux *(Manuscrits #1-2, Chapitres 3-4).*

Bien que des essais cliniques sont encore nécessaires pour évaluer le potentiel d'utiliser ces stratégies de guérison fonctionnelle à large échelle chez les personnes vivant avec le VIH, les résultats inclus dans cette thèse soulignent l'importance de rétablir le système immunitaire et l'homéostasie de la muqueuse intestinale dans le développement d'approches thérapeutiques.

CHAPITRE 2 : CAPTATIO BENEVOLENTIAE

2.1. ÉCARTS DES SAVOIRS

Comme discuté plus tôt, les cellules T $CD4^+$ polarisées Th17 jouent un rôle important dans l'immunité des muqueuses et le maintien de l'intégrité de la barrière intestinale. Elles sont aussi les cibles principales du VIH et sont rapidement épuisées au niveau intestinal après l'infection. La TAR ne permettant pas la restauration de la fréquence et des fonctions de ces cellules au niveau intestinal [309] [77] [310] [311], il est indispensable de mettre en évidence de nouvelles stratégies thérapeutiques qui permettraient de réduire les réservoirs viraux en ciblant spécifiquement les cellules Th17 contenant le VIH dormant afin de restaurer l'immunité mucosale.

Le récepteur aux chimiokines CCR6, qui est un marqueur bien établi pour les cellules Th17 humaines [312] [313], est important pour la migration de ces cellules dans les Plaques de Peyer [314]. Le tropisme intestinal des cellules T $CD4^+$ est induit par l'acide rétinoïque (AR) qui induit l'expression de gènes codant pour de molécules clefs pour la migration vers cet organe telles que l'intégrine α4β7 et le récepteur de chimiokine CCR9 [315].

L'AR est un dérivé de la vitamine A produit majoritairement par les cellules dendritiques $CD103^+$ intestinales. L'AR se lie au récepteur RAR (RAR isoforme α, β et γ) et au récepteur X des rétinoïdes (RXR isoforme α, β et γ). Ce complexe interagit avec des régions spécifiques de l'ADN, les éléments de réponse à l'AR (RAREs). Lorsque les cellules T naïves sont activées par des cellules dendritiques en présence d'AR, celles-ci acquièrent un potentiel de migration au niveau de l'intestin.

Des travaux antérieurs du laboratoire ont démontré que l'ATRA (*all-trans retinoic acid*) augmente de façon préférentielle la permissivité à l'infection par le VIH des cellules T $CCR6^+$ *vs.* $CCR6^-$ [316] (Figure 9). En accord, le laboratoire a récemment démontré la persistance du VIH dans les cellules T $CCR6^+$ du colon chez les sujets avirémiques sous TAR [317] (Figure 10).

Identifier les mécanismes impliqués dans l'infection préférentielle et la persistance du VIH dans les cellules CCR6$^+$ intestinales représente l'objectif du chapitre 3 de ma thèse.

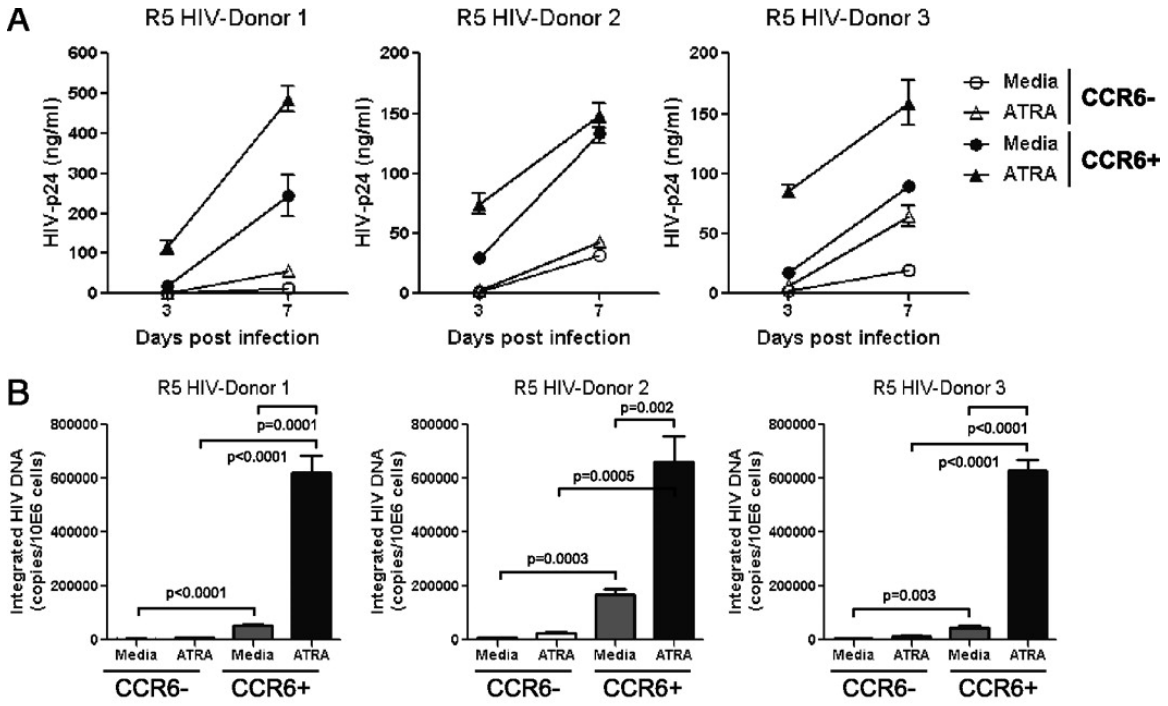

Figure 9. L'ATRA augmente la permissivité au VIH des cellules T CCR6+ mémoires.
Les cellules T CD4$^+$ mémoires CCR6$^+$ et CCR6- ont été triées par MACS et FACS à partir de PBMCs de sujets VIH-. Les cellules ont été stimulées via CD3/CD28 en présence ou absence d'ATRA (10 nM) pendant 4 jours. Les cellules ont ensuite été exposées à un virus R5 NL4.3BaL (compétant pour la réplication). **(A)** Le niveau de HIV-p24 dans le surnageant de culture a été mesuré par ELISA à jours 3 et 7 post-infection. B. Le niveau d'ADN proviral a été quantifié par PCR nichée en temps réel à jours 3 post-infection. **[316]**

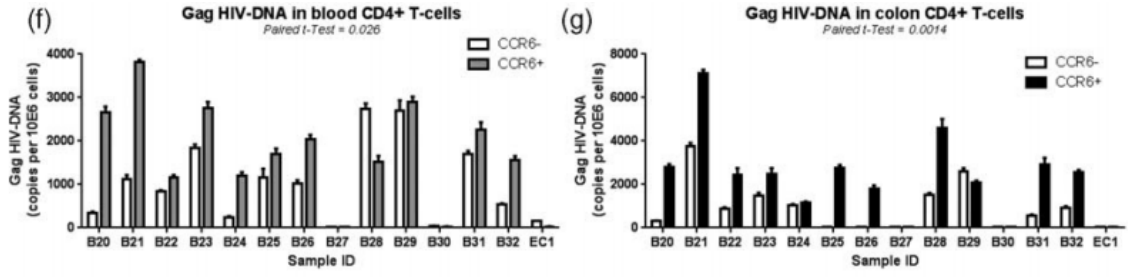

Figure 10. CCR6 est un marqueur de cellules enrichis en ADN viral chez les personnes recevant la TAR.
Le sang et les biopsies du colon sigmoïde ont été collectés de façon appariée chez de sujets VIH+ sous TAR avec une charge viral indétectable (<40 VIH-ARN copies/ml). Les cellules ont été

extraites des biopsies par digestion enzymatique (Libérase) et marquées avec un cocktail d'anticorps afin de trier les cellules T CD3+CD4+CD45- CCR6+ et CCR6- par cytométrie en flux (BDAria III). (A-B) Niveaux d'ADN viral total dans les cellules CCR6- (bars blanche) et CCR6+ (barres grises et noires) isolées du sang périphérique (A) et des biopsies du colon (B). (moyenne±SD des triplicatas). [317]

2.2. HYPOTHÈSES ET OBJECTIFS

2.2.1. MANUSCRIT #1

Les cellules T $CD4^+$ $CCR6^+$ à tropisme intestinal sont les cibles majeures de l'infection et de la réplication virale *via* des mécanismes moléculaires qui restent à élucider pour l'identification de nouvelles cibles thérapeutiques antirétrovirales. Nous nous sommes basés sur l'hypothèse selon laquelle le programme transcriptionnel induit par l'ATRA dans les cellules T $CD4^+$ $CCR6^+$ est favorable à la réplication virale. À l'opposé, l'ATRA agit sur la sous-population $CCR6^-$ tout en préservant leur résistance à l'infection. L'objectif 1 de mon travail de doctorat consistait à identifier ces déterminants moléculaires induits par l'ATRA et associés à la permissivité dans les cellules T $CD4^+$ $CCR6^+$ via des analyses transcriptionnelles au niveau du génome entier.

2.2.2. MANUSCRIT #2

Les travaux du projet 1 [171], ont permis d'identifier la voie Akt/PI3K/mTORC1 comme étant modulée par l'AR dans les cellules T $CD4^+$ $CCR6^+$. Nous avons montré que les inhibiteurs du complexe mTORC1 (rapamycin) ou mTORC1/2 (INK128) abrogent les effets de l'AR sur la réplication/réactivation du VIH dans cette sous-population. De plus, de hauts niveaux d'expression de CCR5 et de phospho-mTOR ont été observés dans les cellules T $CD4^+CCR6^+$ infiltrant le colon des individus traités avec des ARVs, ce qui fournit une explication à la permissivité et à la persistance préférentielle du VIH dans ces lymphocytes. Sur la base de ces travaux démontrant que le VIH cible sélectivement les cellules Th17 $CCR6^+$ à tropisme intestinal via des mécanismes mTOR-dépendants, nous avons testé **l'hypothèse** selon laquelle l'administration clinique de la metformine, un inhibiteur indirect de mTOR [318], diminuerait la réplication/persistance du VIH dans les cellules Th17 du colon et favoriserait la restauration de l'immunité mucosale pendant la TAR (**Objectif 2**).

2.2.3. MANUSCRIT #3

La déplétion des cellules Th17 au niveau de la muqueuse intestinale conduit à des altérations dramatiques de l'homéostasie du GALT responsables d'une inflammation persistante et des comorbidités non-SIDA chez les personnes infectées [319] [77] [310] [74]. La préservation de la fréquence des cellules Th17 intestinales est associée à une progression lente de la maladie. Des études réalisées chez le singe montrent que la survie et la restauration de la fréquence/fonction effectrice des cellules Th17 intestinales est associées à une diminution de la translocation microbienne, une restauration de l'homéostasie intestinale et une diminution de la taille des réservoirs viraux [320] [321]. Dans ce contexte, la mise en évidence de nouvelles stratégies thérapeutiques anti-VIH pouvant restaurer les cellules Th17 chez les sujets VIH+ représente un enjeu majeur des recherches actuelles. Des études précédentes au laboratoire ont permis d'identifier le *Peroxisome Proliferator-activated Receptor-gamma* (PPARγ) comme facteur de résistance au VIH hautement exprimé dans les cellules Th17 et Th1Th17 $CCR6^+$ du sang périphérique [322] [237]. PPARγ appartient à la famille de récepteurs nucléaires agissant comme senseurs lipidiques et contrôlant l'expression de gènes impliqués dans le métabolisme lipidique et de glucidique. Une fois PPARγ activé par son ligand, il forme un complexe avec RXR et se lie spécifiquement aux éléments de réponse des *peroxisome proliferator* (PPRE; *peroxisome proliferator responsive element*) afin de réguler l'expression des gènes cibles [323]. [318]

Il a été rapporté que PPARγ inhibe la différenciation des cellules Th17 en régulant négativement l'expression de RORγt (*RAR-related orphan receptor gamma*) [324]. Nous avons testé ***l'hypothèse selon laquelle*** un antagoniste de PPARγ pourrait être utilisé comme stratégie de réactivation du VIH et en même temps comme stratégie de restaurer les fonctions effectrices des cellules Th17 chez les sujet VIH+ sous TAR (**Objectif 3**).

L'**objectif majeur** de mes travaux de doctorat consiste ainsi à identifier de nouveaux déterminants moléculaires de permissivité/résistance au VIH dans les cellules Th17 infiltrant l'intestin,

déterminants qui représentent des nouvelles cibles de traitement lors des stratégies d'éradication de type « block and lock » et « shock and kill » et de guérison fonctionnelle

CHAPITRE 3 : HIV-1 SELECTIVELY TARGETS GUT-HOMING CCR6^{+}CD4^{+} T-CELLS VIA MTOR-DEPENDENT MECHANISMS (MANUSCRIT #1)

Publié dans JCI Insight, août 2017

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Contribution des auteurs :

DP a effectué la majorité des expériences, analysé des données et rédigé le manuscrit.
YZ et PM ont effectué des expériences.
JPG a effectué les analyses des données de microarray et les figures associés.
AG a effectué des expériences.
AF a contribué à la conception de la recherche et à la rédaction du manuscrit.
NG et TJH ont fourni des protocoles et des réactifs et ont contribué à la conception de la recherche.
JPR a fourni l'accès aux échantillons/informations cliniques, mis en place les protocoles de recherche clinique.
PA a conçu le projet de recherche, analysé les données et rédigé le manuscrit.
Tous les auteurs ont examiné et accepté le manuscrit.

HIV-1 selectively targets gut-homing CCR6^{+}CD4^{+} T-cells *via* mTOR-dependent mechanisms

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Running title: mTOR regulates HIV in gut-homing Th17 cells
Key words: human, Th17, CCR6, HIV, ATRA, gut-homing, ART

ABSTRACT

Gut-associated lymphoid tissues (GALT) are enriched in $CCR6^+$ Th17-polarized $CD4^+$ T-cells that contribute to HIV-1 persistence during antiretroviral therapy (ART). This raises the need for Th17-targeted immunotherapies. In an effort to identify mechanisms governing HIV-1 permissiveness/persistence in gut-homing Th17 cells, we analyzed the transcriptome of $CCR6^+$ *versus* $CCR6^-$ T-cells exposed to the gut-homing inducer retinoic acid (RA) and performed functional validations in colon biopsies of HIV-infected individuals receiving ART (HIV+ART). Although both $CCR6^+$ and $CCR6^-$ T-cells acquired gut-homing markers upon RA exposure, the modulation of unique sets of genes coincided with preferential HIV-1 replication in RA-treated $CCR6^+$ T-cells. This molecular signature included the up-regulation of HIV-dependency factors acting at entry/post-entry levels, such as CCR5 and PI3K-Akt-mTORC1 signaling pathway. Of note, mTOR expression/phosphorylation was distinctively induced by RA in $CCR6^+$ T-cells. Consistently, mTOR inhibitors counteracted the effect of RA on HIV replication *in vitro* and viral reactivation in $CD4^+$ T-cells from HIV+ART individuals, *via* post-entry mechanisms independent of CCR5. Finally, $CCR6^+$ *versus* $CCR6^-$ T-cells infiltrating the colon of HIV+ART individuals expressed unique molecular signature including higher levels of CCR5, integrin β7, and mTOR phosphorylation. Together, our results identify mTOR as a druggable key regulator of HIV permissiveness in gut-homing $CCR6^+$ T-cells.

INTRODUCTION

Human immunodeficiency virus type 1 (HIV-1) causes the progressive depletion of $CD4^+$ T-cells thus impairing the ability of the immune system to fight pathogens. Although current antiretroviral therapies (ART) efficiently control viral replication and restore CD4 counts, the HIV eradication is not achieved (1-3). Major $CD4^+$ T-cell alterations occur in gut-associated lymphoid tissues (GALT) rapidly upon HIV transmission (4, 5) and these alterations persist despite viral suppressive ART (3, 6), even in patients treated during acute HIV infection (7). The GALT is considered an important site of HIV replication and viral reservoir persistence during ART (8). GALT-infiltrating $CD4^+$ T-cells represent optimal HIV targets likely due to their high expression of CCR5 (4, 5, 8), a major co-receptor for HIV entry (9-11), and integrin α4β7, a gut-homing molecule (12) identified as an HIV-binding molecule (13). Very recently, integrin α4β7 blocking Abs proved efficacy in controlling viral replication upon ART interruption in a SIV infection model (14), indicative that interfering with HIV replication in gut-homing $\alpha4\beta7^+CD4^+$ T-cells represents a potential curative strategy.

Among GALT-infiltrating $CD4^+$ T-cells, Th17 cells play a beneficial role in maintaining epithelial cell integrity at barrier surfaces *via* the production of effector cytokines under the control of lineage-specific transcription factors (15). Studies by our group and others demonstrated that Th17-polarized $CCR6^+CD4^+$ T-cells are highly permissive to infection (16-18), as a consequence of their expression of HIV dependency factors (HDFs) acting at entry and post-entry levels (19, 20), combined with the lack of HIV restriction mechanisms (21-23). Most recently, $CD4^+$ T-cells expressing the Th17 markers CCR6/RORγt were identified as the first targets of SIV during vaginal transmission (24); also, the detrimental role of viral permissive $CCR6^+$ T-cell infiltration into the gut was documented (25). The depletion of Th17 cells from the GALT upon HIV infection persists despite viral-suppressive ART (26-28), even when ART is intensified with integrase and CCR5 inhibitors (29). Other promising strategies tested for mucosal Th17 restoration include an ideal ART initiation at very early stages of infection (Fiebig I/II but not III) (7), the use of probiotics, and IL-21 supplementation (30-32). The non-

restoration of GALT Th17 cells leads to significant damages of the intestinal barrier thus allowing the microbial translocation, a cause for chronic immune activation and occurrence of non-AIDS co-morbidities during ART (3, 6, 33). Despite their dramatic depletion, long-lived Th17 cells contribute to HIV reservoir persistence during ART (34, 35). Consistently, our group identified a subset of $CCR6^{+}CCR4^{-}CXCR3^{-}$ Th17 cells predominant in blood and lymph nodes and carrying replication-competent HIV reservoirs (36) and further demonstrated preferential HIV-DNA persistence in colon infiltrating $CCR6^{+}CD4^{+}$ T-cells during ART (37). These advances orient our search for immunotherapeutic strategies to limit HIV infection/persistence in mucosal Th17 cells in the ART era.

The homing of $CCR6^{+}$ Th17 cells into the GALT is mediated *via* the CCR6/CCL20 axis (28, 38), as well as the adhesion molecule integrin α4β7 (12) and chemokine receptor CCR9 (39). The imprinting for gut-homing is induced by *all-trans* retinoic acid (ATRA) (40), a vitamin A metabolite produced by GALT dendritic cells (41). Our previous studies demonstrated that ATRA increases HIV-1 permissiveness preferentially in $CCR6^{+}$ compared to $CCR6^{-}$ T-cells (42). This effect coincided with the preferential ability of ATRA to up-regulate the expression of the HIV co-receptor CCR5 on $CCR6^{+}$ T-cells, indicative of a most efficient HIV entry. Nevertheless, exposure to single-round VSV-G-pseudotyped HIV entering cells by endocytosis provided evidence that ATRA also promotes HIV replication in gut-homing $CCR6^{+}$ T-cells *via* post-entry mechanisms (42). These findings led to the current hypothesis that CCR6 is a marker for Th17-polarized $CD4^{+}$ T-cells transcriptionally programmed to become HIV targets in the gut, where ATRA promotes the expression of unidentified HIV permissiveness factors, which may be manipulated to protect gut-homing Th17 cells from HIV.

In our search for new druggable molecular targets to prevent HIV replication/persistence in gut-homing Th17 cells, herein we performed a genome-wide transcriptional profiling to identify HDFs modulated by ATRA in memory $CCR6^{+}$ T-cells. Together, our studies *i)* provide an in-depth characterization of molecular mechanisms contributing to HIV replication/persistence in ATRA-exposed $CCR6^{+}$ Th17 cells; *ii)* identified

mTOR as a druggable target modulated by ATRA in these cells; and *iii)* support the beneficial use of mTOR inhibitors in clinics to preserve mucosal Th17 cells from HIV infection/persistence during ART.

RESULTS

Transmitted/founder (T/F) HIV preferentially infects retinoic-acid treated CCR6$^+$CD4$^+$ T-cells. We previously reported that *all-trans* retinoic acid (ATRA) mediated-imprinting for gut-homing increases replication of the laboratory-adapted R5 NL4.3BaL HIV preferentially in CCR6$^+$ *versus* CCR6$^-$ T-cells (42). T/F HIV strains are isolated early upon seroconversion and are unique in their virulence/sensitivity to antiviral mechanisms (43). To determine whether T/F HIV also target gut-homing CCR6$^+$ T-cells for preferential replication, FACS-sorted memory CCR6$^+$ and CCR6$^-$ T-cells stimulated *via* CD3/CD28 in the presence/absence of ATRA were exposed in parallel to T/F THRO (44) and NL4.3BaL HIV (Figure 1A). The relative frequency of CCR6$^+$ and CCR6$^-$ T-cells in peripheral blood mononuclear cells (PBMCs) before sort is depicted in Figure 1B-C. The dose of ATRA used (10 nM) (42) is consistent with physiological plasma levels (40) and had no influence on cell viability (data not shown). ATRA robustly increases replication of both NL4.3BaL and T/F THRO HIV in CCR6$^+$ T-cells, while the effects on CCR6$^-$ T-cells were only minor, as reflected by the quantification of early (RU5), late (Gag), and integrated (Alu/HIV) HIV reverse transcripts (Figure 1 D-E). Thus, T/F similar to NL4.3BaL HIV also target ATRA-treated CCR6$^+$ T-cells for preferential replication, suggesting a critical role played by gut-homing CCR6$^+$ T-cells during the early steps of mucosal HIV transmission.

CCR6$^+$ *versus* CCR6$^-$ T-cells exhibit higher proliferation, TNF-α production, and NF-κB DNA-binding activity in the presence/absence of ATRA. In search of post-entry permissiveness mechanisms, we first investigated whether ATRA modulated proliferation and IL-2 production. In the absence of ATRA, CCR6$^+$ compared to CCR6$^-$ T-cells exhibited higher proliferation upon TCR triggering but a markedly lower production of IL-2 (Suppl. Figure 1A-C), consistent with previous studies (16), likely as a consequence of RORγt-mediated IL-2 inhibition (45). ATRA further decreased IL-2 production in CCR6$^+$ T-cells but had no significant effect on cell proliferation (Suppl. Figure 1A-C). Levels of CD25 (IL-2Rα) were similarly high on CCR6$^+$ and CCR6$^-$ T-cells exposed or not to ATRA (Suppl. Figure 1D-G), indicative that differences in IL-2 production are not related to IL-2 auto-consumption. Thus, increased HIV replication in CCR6$^+$

T-cells upon exposure to ATRA cannot be explained by enhanced cell proliferation and/or IL-2 production.

HIV permissiveness in CCR6$^+$Th17 *versus* CCR6$^-$Th1 cells is associated with higher NF-κB nuclear translocation (20). The pro-inflammatory cytokine TNF-α enhances HIV replication, in part by triggering the NF-κB pathway (46). This prompted us to investigate differences in TNF-α production and NF-κB-p65 activity. Upon TCR triggering, CCR6$^+$ *versus* CCR6$^-$ T-cells produced significantly higher levels of TNF-α in the absence of ATRA; although ATRA slightly decreased TNF-α production in CCR6$^+$ T-cells, levels remained significantly higher in ATRA-treated CCR6$^+$ *versus* CCR6$^-$ T-cells (Suppl. Figure 2A). The NF-κB-p65 DNA-binding activity was higher in CCR6$^+$ *versus* CCR6$^-$ T-cell nuclei when cells were cultured in the presence/absence of ATRA; exposure to ATRA slightly decreased NF-κB-p65 DNA-binding activity in CCR6$^+$ T-cells (Suppl. Figure 2B), consistent with previous reports (47). Nevertheless, NF-κB-p65 DNA-binding activity remained significantly higher in ATRA-treated CCR6$^+$ *versus* CCR6$^-$ T-cells (Suppl. Figure 2B). Together these results demonstrate that TNF-α production and NF-κB-p65 nuclear translocation/DNA-binding activity are significantly higher in memory CCR6$^+$ *versus* CCR6$^-$ T-cells upon TCR triggering, but ATRA has no positive impact on these differences.

ATRA promotes a unique transcriptional program associated with HIV permissiveness in CCR6$^+$ T-cells. To gain insights into mechanisms underlying the differential effects of ATRA on CCR6$^+$ *versus* CCR6$^-$ T-cells, their ability to integrate ATRA-mediated signals was investigated. CCR6$^+$ and CCR6$^-$ T-cells expressed similar levels of RA receptor alpha (RARα) mRNA *ex vivo* (Figure 2A) and ATRA up-regulated the expression of RA-inducible gene I (RIG-I)**/**Retinoic acid receptor responder protein 3 (RARRES3), a direct target of RA (48), in both cell subsets (Figure 2B). We hypothesized that CCR6$^+$ and CCR6$^-$ T-cell subsets respond to ATRA in distinct fashions, with the expression of HIV dependency factors (HDFs) being promoted preferentially in CCR6$^+$ T-cells. To test this hypothesis, a genome-wide transcriptional profiling was performed in

CCR6$^+$ and CCR6$^-$ T-cells stimulated *via* the TCR in the presence/absence of ATRA for four days; this time point coincided with the time of HIV exposure in Figure 1.

Differentially expressed genes were identified based on p-values (p) or adjusted p-values (adj. p) and fold change (FC) of gene expression. Results in Figure 2C and Suppl. Tables 1-4 illustrate transcriptional differences between CCR6$^+$ and CCR6$^-$ T-cells upon TCR triggering in the presence/absence of ATRA. In the absence of ATRA, up-regulated transcripts (n=348; adj. p-values<0.05, FC cut-off 1.3) in CCR6$^+$ *versus* CCR6$^-$ T-cells included Th17 markers such as CCL20, IL-22, IL-17F, CTSH, KLRB1/CD161, IL-8, RORA, CSF2/GM-CSF, IL-26, RORC, CCR6, SGK1, PPARG, IL-23R, ARNTL, and PTPN13 (Suppl. Table 1). Transcripts up-regulated by ATRA in CCR6$^+$ T-cells (n=95; adj. p-values<0.05, FC cut-off 1.3) included RARRES3, CD38, DHRS3, UBD, LGMN, EVI5, LZTFL1, NAPSA, GBP4, HIC1, CCR9, CCR2, CXCR6, and ITGB7 (Suppl. Table 2). Transcripts up-regulated by ATRA in CCR6$^-$ T-cells (n=119; adj. p-values<0.05, FC cut-off 1.3) included NAPSA, CD38, EVI5, SAMD9, GALR2, DHRS3, ANKRD37, PTGER2, SLC26A4, and RARRES3, ARG2, APOBEC3B, RIPK2, and NFIL3 (Suppl. Table 3). The analysis of the n=319 transcripts over expressed in ATRA-treated CCR6$^+$ *versus* CCR6$^-$ T-cells revealed the preservation of a Th17 signature (*e.g.,* CCL20, IL-17F, CTSH, IL-22, KLRB1, CSF2, ANTXR2, GPR56, CXCR6, KLF2, RORC, RORA, S100A4, CCR6, MAP3K4, CD96, IL-26, FURIN, ABCB1, ARNTL, and PTPN13) (Suppl. Table 4). Finally, in the presence/absence of ATRA, CCR6$^+$ *versus* CCR6$^-$ T-cells show superior expression of the Th17 markers RORC, ARNTL, and CCR6, and inferior expression of the RORC nuclear repressor NFIL3 (49) (Suppl. Figure 3). Together these results demonstrate that CCR6$^+$ and CCR6$^-$ T-cells respond to ATRA in a unique transcriptional fashion and that ATRA exposure does not modify the Th17-polarisation profile of CCR6$^+$ T-cells.

An extended intelligent search within top modulated transcripts revealed that ATRA induced expression of gut-homing markers integrin α4, integrin β7 and CCR9 (12) in both CCR6$^+$ and CCR6$^-$ T-cells. Of note, CXCR6, a minor HIV co-receptor (50, 51), was significantly up-regulated by ATRA in CCR6$^+$ but not CCR6$^-$ T-cells (Suppl. Tables 2-4; Suppl. Figure 3). FACS analysis validated the microarray results for integrin β7 and CXCR6 expression **(**Suppl. Figure 4A-C).

Although CCR5 transcripts were not differentially expressed in microarrays (data not shown), we confirm (42) that ATRA significantly up-regulates CCR5 preferentially on $CCR6^+$ T-cells **(**Suppl. Figure 4A and D). This coincided with the up-regulation of KLF2 (Suppl. Figure 3), a positive regulator of CCR5 transcription (52). The down-regulation of CCR5 ligands CCL3 and CCL3L1 by ATRA was mainly observed in $CCR6^+$ T-cells, indicative of a more efficient CCR5-mediated HIV entry (53). Although the T-cell activation marker CD38 was similarly up-regulated by ATRA in both subsets **(**Suppl. Figure 3), transcripts for MAP3K4, a Th17-specific kinase (54) we previously identified as a HDF (20), were expressed at superior levels and up-regulated by ATRA in $CCR6^+$ *versus* $CCR6^-$ T-cells (Suppl. Figure 3). Moreover, the transcripts for PPARγ, a transcriptional repressor of RORγt (55) and Th17-intrinsec negative regulator of HIV replication (19), was down-regulated by ATRA specifically in $CCR6^+$ T-cells (Suppl. Figure 3). Furthermore, $CCR6^+$ *versus* $CCR6^-$ T-cells stimulated *via* the TCR in the presence/absence of ATRA expressed decreased levels of interferon (IFN) stimulated genes, such as IFITM1, 2, and 3, and IRF8 (Suppl. Tables 1-4), consistent with the low Th17 ability to respond to IFN (23). Finally, although differences in the expression of SAMHD1, a key IFN-induced HIV restriction factor (22, 56, 57), were not observed in the microarrays, in the presence/absence of ATRA, levels of SAMHD1 mRNA measured by RT-PCR were significantly lower in $CCR6^+$ *versus* $CCR6^-$ T-cells (Suppl. Figure 5). This stresses the existence of false negatives in high-throughput transcriptional screenings. Together, these findings reveal multiple HDFs acting at entry and post-entry levels to facilitate HIV replication in ATRA-treated $CCR6^+$ T-cells, as well as a state of antiviral immunity acting at different levels in $CCR6^-$ T-cells.

Among probe sets differentially modulated by ATRA in $CCR6^+$ and $CCR6^-$ T-cells, ($p\text{-value}<0.05$), only 466 transcripts were shared, while 1,285 and 1,538 transcripts were unique to $CCR6^+$ and $CCR6^-$ T-cells, respectively (Figure 2D). A meta-analysis using the NCBI HIV-1 interactions data-base allowed the identification of human genes previously described to enhance HIV-1 protein expression that were differentially regulated by ATRA in $CCR6^+$ *versus* $CCR6^-$ T-cells (Suppl. Figure 6). Among those transcripts, LGALS3, ADAM15, TRIM8, IL-1α, FOXO3, and ARNTL were up-regulated by ATRA in $CCR6^+$ *versus* $CCR6^-$ T-cells (Suppl. Figure 3 and 6).

Finally, Gene Set Variation Analysis (GSVA) identified pathways modulated by ATRA in CCR6$^+$ T-cells, including the Akt and PI3K signaling pathways (Figure 3), critical for the Th17 polarization (58-60). A custom pathway was created using the genes from BIOCARTA_AKT_PATHWAY and PID_PI3K_PLC_TRK_PATHWAY, genes that were differentially modulated by ATRA in CCR6$^+$ T-cells (Figure 4A). Of note, the mammalian target of rapamycin (mTOR) appeared up-regulated by ATRA in CCR6$^+$ T-cells in this gene interaction network representation (Figure 4A). Ingenuity Pathway Analysis (IPA) was further used to illustrate genes associated with the mTORC1 pathway and regulated by ATRA in CCR6$^+$ T-cells (Figure 4B).

Together, this genome-wide transcriptional analysis reveals unique molecular signatures associated with HIV permissiveness in Th17-polarized CCR6$^+$ T-cells upon exposure to ATRA and point to a critical role played by mTOR in this process.

ATRA induces mTOR expression and phosphorylation selectively in CCR6$^+$ T-cells. To explore the potential contribution of mTOR to HIV permissiveness in ATRA-treated CCR6$^+$ T-cells, the expression of total and phosphorylated mTOR was first investigated by western blotting in sorted CCR6$^+$ and CCR6$^-$ T-cells stimulated *via* the TCR in the presence/absence of ATRA. Levels of total/phosphorylated mTOR were low to undetectable in TCR-activated CCR6$^+$ T-cells but significantly increased by ATRA treatment. In contrast, TCR-activated CCR6$^-$ T-cells expressed high levels of total/phosphorylated mTOR and ATRA did not modify this expression (Figure 5A-B). The molecular weight of the main mTOR band was 80 kDa, which corresponds to the β isoform of mTOR typically expressed in T-cells (61). To further validate these results, we performed semi-quantitative RT-PCR and visualized on gel the expression of mTOR mRNA corresponding to isoform β (2,118 Kb), using established primers (61). Levels of mTOR isoform β mRNA were significantly higher in CCR6$^+$ T-cells upon exposure to ATRA (Suppl. Figure 7). The findings that mTOR mRNA expression and mTOR phosphorylation are induced by ATRA preferentially in CCR6$^+$ T-cells point to the existence of mTOR-dependent HIV permissiveness mechanisms in gut-homing CCR6$^+$ T-cells.

mTORC1/2 inhibitors counteract the effect of ATRA on HIV replication. To determine the functional role of ATRA-mediated mTOR activity, two commercially available mTOR inhibitors were tested for their ability to counteract the effect of ATRA on HIV permissiveness: rapamycin, an inhibitor of mTORC1, and INK128, an inhibitor of mTORC1/2 (62). Memory $CD4^+$ T-cells were stimulated *via* the TCR and further exposed to HIV in the presence/absence of ATRA, rapamycin or INK128 (Figure 6A). Rapamycin and INK128 significantly reduced levels of early/late reverse transcripts and integrated HIV-DNA in ATRA-treated T-cells from five different donors, with INK128 having the most robust inhibitory effects, especially when added before and after infection (Figure 6B-D). Similarly, rapamycin and most significantly INK128 had a robust inhibitory effect on HIV replication measured by HIV-p24 ELISA at days 3, 6 and 9 post-infection (Figure 6E). Of note, the treatment with mTOR inhibitors in combination with ATRA significantly reduced the ability of cells to produce IL-17A but did not alter IFN-γ production, cell viability and proliferation (Suppl. Figure 8A-D). Similar experiments were further performed with FACS-sorted memory $CCR6^+/CCR6^-$ T-cells. INK128 significantly reduced HIV integration/replication in ATRA-treated $CCR6^+$ T-cells. Although levels of HIV integration/replication were lower in $CCR6^-$ compared to $CCR6^+$ T-cells, INK128 similarly acted on $CCR6^-$ T-cells (Figure 7A-B), consistent with the mTOR expression in these cells (Figure 5; Suppl. Figure 7). Together, these results support the role of ATRA-induced mTOR signaling pathway as a positive regulator of HIV permissiveness in gut-homing $CCR6^+$ T-cells.

mTORC1/2 inhibitors limit HIV replication at post-entry level in ATRA-treated T-cells. Studies in humanized mice demonstrated the ability of rapamycin and INK128 to inhibit HIV replication *via* the regulation of CCR5 expression (62). To investigate this possibility, we tested the effects of rapamycin and INK128 on CCR5 expression in memory $CD4^+$ T-cells stimulated *via* TCR in the presence/absence of ATRA. Although rapamycin and INK128 significantly reduced CCR5 expression in the absence of ATRA, no significant changes were observed in the presence of ATRA (Figure 8A-B). Similarly, rapamycin and INK128 had no significant effects on the expression of integrin β7 (Figure 8C-D), a gut-homing molecule induced by ATRA (12) that was

also identified as an HIV-gp120 binding molecule (13). To further distinguish between HIV entry and post-entry mTOR-mediated mechanisms in the context of ATRA, we performed a single-round infection using replication-defective VSV-G-pseudotyped HIV and tested the effects of rapamycin or INK128 on HIV reverse transcription and integration. Both rapamycin and INK128 induced a partial decrease in levels of early (59±3 and 65±7% inhibition) and late (88±2 and 90±5% inhibition) HIV reverse transcript and an almost complete abrogation of HIV-DNA integration (98±0.1 and 99±0.1% inhibition, n=3) (Figure 8E-G). Together these results demonstrate that mTORC1/2 inhibitors limit HIV replication in gut-homing $CCR6^{+}CD4^{+}$ T-cells *via* post-entry mechanisms, during reverse transcription and prior to integration, without impact on CCR5- and/or integrin β7-mediated viral entry or cell homing into the gut.

ATRA promotes HIV reactivation in $CD4^{+}$ T-cells of ART-treated individuals in an mTOR-dependent manner. In a very recent study, we demonstrated that ATRA facilitates HIV reactivation from latency in $CCR6^{+}CD4^{+}$ T-cells from ART-treated individuals (37). To determine whether HIV reservoir reactivation was dependent on mTOR activity, an optimized viral outgrowth assay (VOA) was performed in the presence/absence of ATRA and mTORC1/2 inhibitor INK128 (Figure 9A). Consistent with previous findings (37), ATRA significantly improved the efficacy of HIV reactivation in memory $CD4^{+}$ T-cells isolated from ART-treated individuals (Suppl. Table 5), as measured by intracellular HIV-p24 staining (Figure 9B-C) and soluble HIV-p24 in cell-culture supernatants at day 12 post-reactivation (Figure 9D). Of particular importance, INK128 inhibited HIV reactivation mediated by TCR triggering in the presence of ATRA (Figure 9B-D**).** Further, we tested the effects of INK128 on HIV reservoir reactivation in memory $CCR6^{+}$ and $CCR6^{-}$ T-cells isolated by FACS from ART-treated individuals (Suppl. Table 5). In the absence of ATRA, HIV reactivation was observed in $CCR6^{+}$ but not $CCR6^{-}$ T-cells in 1/3 donors (ART #3), while in the presence of ATRA HIV reactivation occurred in 3/3 donors preferentially (ART #3) or only (ART#4 and ART #6) in $CCR6^{+}$ T-cells. Of particular importance, INK128 dramatically reduced HIV reactivation in ATRA-treated $CCR6^{+}$ T-cells, as measured by intracellular HIV-p24 expression (Figure 9E-F) and soluble HIV-p24 production in cell-culture supernatants at day 12 post-reactivation (data not shown). Together, these results

reveal mTOR as a key player in ATRA-mediated HIV reactivation in CCR6$^+$ T-cells of ART-treated individuals.

Colon-infiltrating CCR6$^+$CD4$^+$ T-cells express high levels of CCR5, integrin β7, and phosphorylated mTOR. The GALT is enriched in DCs metabolizing vitamin A into ATRA (41). We further explored whether the molecular "*signature*" induced by ATRA *in vitro* including the up-regulation of CCR5, integrin β7 and mTOR phosphorylation on CCR6$^+$ T-cells can be observed as well in the GALT. To test this possibility, we had access to matched blood and sigmoid biopsies from n=10 HIV-infected individuals receiving ART (Suppl. Table 5; COL #1-10). Memory CD4$^+$ T-cells were identified using the gating strategy depicted in Figure 10A and further analyzed for the expression of CCR5, integrin β7, and phosphorylated mTOR in CCR6$^+$ *versus* CCR6$^-$ T-cells. The expression of CCR5 and integrin β7 was higher on cells from the colon *versus* the blood; with colon-infiltrating CCR6$^+$ *versus* CCR6$^-$ T-cells expressing significantly higher levels of CCR5 and integrin β7 (Figure 10B-E). Similarly, mTOR phosphorylation was mainly observed in the colon, with colon-infiltrating CCR6$^+$ *versus* CCR6$^-$ T-cells expressing the highest levels of phosphorylated mTOR in all individuals tested (Figure 10F-G). Thus, the activation of the mTOR pathway occurs almost exclusively in colon-infiltrating CCR6$^+$ T-cells and coincides with the highest levels of CCR5 and integrin β7 expression. These results provide a molecular explanation for the preferential HIV replication in colon CCR6$^+$ T-cells and point to mTOR as a new antiviral drug target in these cells (Figure 11).

DISCUSSION

The role of *all-trans* retinoic acid (ATRA) in regulating tolerance and immunity *via* the modulation of regulatory and effector functions of $CD4^+$ T-cells, respectively, is well-established (41). Our current results support the idea that in the intestinal environment HIV takes advantage of ATRA-mediated signaling pathways for replication/persistence in $CCR6^+CD4^+$ T-cells. Briefly, we *i)* identified mTOR as a major regulator of HIV permissiveness in $CCR6^+CD4^+$ T-cells with gut-homing tropism mimicked *in vitro* by exposure to ATRA; *ii)* demonstrated the ability of mTOR inhibitors to counteract the effects of ATRA on HIV replication *in vitro* and viral reservoir reactivation in T-cells from ART-treated individuals; and *iii)* revealed that $CCR6^+CD4^+$ T-cells infiltrating the colon of HIV-infected individuals on ART express a unique signature comprising high levels of CCR5, integrin β7, and phosphorylated mTOR, signature likely reflecting exposure to ATRA *in vivo* (Figure 11). Considering the strategic location of Th17 cells at portal sites of HIV/SIV entry (6, 24, 27, 28, 63), as well as their important role in maintaining mucosal immunity homeostasis in the context of a complex microbiota (6, 64, 65), our results point to the potential beneficial use of mTOR inhibitors in preventing HIV infection/persistence in gut-homing Th17 cells.

In this manuscript, we used the Illumina bead array technology to unveil post-entry HIV regulatory mechanisms modulated by ATRA in $CCR6^+$ *versus* $CCR6^-$ T-cells. Among genes commonly modulated by ATRA in $CCR6^+$ and $CCR6^-$ T-cells, the RA-inducible gene-I (RIG-I) is essential for sensing of viral nucleic acids and promoting antiviral immunity (66). HIV is known to subvert RIG-I-mediated antiviral mechanisms (67); this may explain robust HIV replication in RIG-I-expressing ATRA-treated $CCR6^+$ T-cells. Of particular importance, Li *et al*. recently demonstrated that acitretin, a RA derivative, increases HIV transcription in $CD4^+$ T-cells from ART-treated HIV-infected individuals and enhances RIG-I signaling thus leading to an antiviral response and the apoptosis of infected cells (68). Similarly, in a previous report (37) and in this manuscript, we demonstrated that ATRA significantly increases HIV reactivation in a viral outgrowth assay (VOA) without significant changes observed in T-cell survival/proliferation.

Differences between our results and those published by Li *et al.* (68), may be explained by differences in the experimental design. Nevertheless, these findings commonly support the fact that ATRA promotes HIV reservoir reactivation.

A meta-analysis using the NCBI HIV interaction database revealed HDFs preferentially expressed by ATRA-exposed CCR6$^+$ T-cells. These HDFs include LGALS3, promoting HIV-1 budding *via* association with Alix and Gag p6 (69); ADAM15, involved in the nuclear import of the HIV-1 pre-integration complex (70); TRIM8, a regulator of NF-κB and STAT3-dependent signaling cascades (20, 71); and ARNTL (72), a component of the circadian clock machinery and regulator of Th17 development (49). All these HDFs contribute to robust HIV replication in gut-homing CCR6$^+$ T-cells.

Gene Set Variation Analysis (GSVA) of transcripts uniquely modulated by ATRA in CCR6$^+$ T-cells identified unique pathways, including Akt and PI3K. The network representation of Akt/PI3K pathways, previously linked to Th17 lineage polarization (58-60) and reported to be up-regulated in human lamina propria T-cells (73), pointed to the up-regulation of mTOR, a metabolic sensor involved in the regulation of numerous cellular functions *via* the formation of two different signaling complexes, mTORC1 and mTORC2 (74-77). Considering the documented role of mTOR-mediated processes in the positive regulation of HIV replication (62, 78), we proceeded to the validation of mTOR expression at protein and mRNA levels, as well as functional validations, in cells from the blood and/or colon of HIV-infected individuals receiving ART and uninfected study participants.

The regulation of HIV permissiveness *via* mTOR-dependent mechanisms was demonstrated by the use of mTORC1 (rapamycin) and/or mTORC1/2 (INK128) inhibitors, that counteracted the effects of ATRA on HIV replication in CCR6$^+$ T-cells *in vitro* and inhibited HIV reservoir reactivation in an ATRA-based VOA performed with CCR6$^+$ T-cells from ART-treated individuals. Nevertheless, mTOR expression/phosphorylation is not always associated with HIV permissiveness. Indeed, CCR6$^-$ T-cells are relatively resistant to HIV despite high levels of mTOR

expression, likely due to the existence of HIV restriction mechanisms acting at different levels including entry (low/undetectable CCR5 expression), reverse transcription (high SAMHD1 expression), and/or transcription (limited TNF-α production and NF-κB activity). Thus, mTOR favors HIV replication/reactivation only within the unique transcriptional context of ATRA-exposed $CCR6^+$ T-cells.

Evidence exists in the literature that mTOR regulates HIV replication *via* multiple mechanisms (78, 79). One possible mechanism is *via* the regulation of CCR5 expression (62). However, CCR5 expression on ATRA-treated T-cells was not affected by mTOR inhibitors. This suggests that ATRA induces CCR5 expression *via* mTOR-independent mechanisms and that mTOR regulation of HIV permissiveness in gut-homing $CD4^+$ T-cells likely occurs at post-entry levels. Consistent with the evidence that mTOR regulates nucleotide biosynthesis essential for transcription (76, 80, 81), mTOR inhibitors may limit HIV reverse transcription by reducing the pool of nucleotides. Indeed, our single-round infection using VSV-G-pseudotyped HIV revealed restriction mechanisms acting during reverse transcription and prior to integration. Moreover, mTOR is a well-established regulator of autophagy (79, 82), a process that may be involved in the degradation of the incoming HIV upon fusion/uncoating (83, 84). By promoting the mTOR activity, ATRA may impair the autophagy process in $CCR5^+CCR6^+$ T-cells at mucosal sites. Furthermore, considering the fact that ATRA-treated $CCR6^+$ T-cells maintain their Th17 features (42) and that mTORC1 *via* the induction of the kinase S6K2 is involved in the nuclear translocation of RORC (60, 85), one other possibility is that mTOR inhibitors interfere with the RORC-mediated transcriptional program in Th17 cells, which is favorable to HIV replication (20). Consistent with this hypothesis, we demonstrated that decreased HIV replication in the presence of rapamycin and INK128 coincided with a significant reduction in IL-17A production by ATRA-treated $CD4^+$ T-cells.

Of particular importance, we found that $CCR6^+$ but not $CCR6^-$ T-cells infiltrating the colon of HIV-infected individuals receiving ART express a unique molecular signature including superior levels of CCR5, integrin β7 and phosphorylated mTOR expression, likely as a consequence of

ATRA exposure in the intestinal environment (12, 41). In line with our recent report that HIV-DNA persists in colon-infiltrating CCR6$^+$ T-cells during ART (37), it remains to be determined whether mTOR expression identifies a fraction of CCR6$^+$ T-cells enriched in HIV-DNA and whether mTOR activation contributes to residual HIV replication in colon-infiltrating CCR6$^+$ T-cells during ART.

While this manuscript was in preparation for submission, Besnard *et al.* reported the results of a shRNA screen that revealed mTOR as a regulator of HIV latency (86) via mechanisms involving CDK9 and NF-κB activation that controls Tat-dependent HIV transcription (86). Also, Martin *et al.* reported on the potential beneficial use of rapamycin in the context of HIV "*shock-an-kill*" strategies (87). These two reports (86, 87) further support the conclusions of our current study revealing the key role played by mTOR in regulating multiple post-entry and post-integration HIV replication steps in gut-homing Th17-polarized CCR6$^+$ T-cells.

In conclusion, our findings point to CCR6 as a "zip code" molecule expressed on the surface of CD4$^+$ T-cells transcriptionally programmed to become HIV targets upon recruitment into the intestine and provide the first detailed molecular explanation for preferential HIV/SIV replication and persistence in gut-homing CCR6$^+$CD4$^+$ T-cells (24, 25, 37, 42). Most significantly, we reveal the role of the mTOR pathway in regulating effector functions as well as HIV permissiveness in gut-homing CCR6$^+$ Th17 cells at multiple post-entry levels. Additionally, we demonstrate the ability of mTOR inhibitors to counteract the effects of ATRA on HIV replication/reactivation in CCR6$^+$ T-cells. Our findings raise new questions on the potential beneficial effects of mTOR inhibitors in decreasing HIV reservoirs and restoring the Th17-mediated immunity at the intestinal level during ART. This knowledge will open the path for new studies toward the implementation of new therapeutic strategies aimed at HIV functional cure.

EXPERIMENTAL PROCEDURES

Study subjects

Study subjects were recruited at the Montreal Chest Institute, McGill University Health Centre, and CHUM-Hôpital Saint-Luc (Montreal, Quebec). PBMCs were collected by leukapheresis (88) and cryopreserved until use. Sigmoid biopsies (≈32 biopsies/donor) were collected from HIV-infected individuals receiving antiretroviral therapy (ART) (Suppl. Table 5) during colonoscopy and processed using Liberase DL (Roche Diagnostics), as previously described (37, 89, 90). Matched peripheral blood (20 ml/donor) was collected the same day from biopsy donors.

Flow cytometry staining

Surface staining was performed with fluorochrome-conjugated Abs (Suppl. Table 6), as previously described (16). Cells extracted from sigmoid biopsies and matched PBMCs were stained on the surface with a cocktail of CD3-Alexa700, CD4-PE-Cy7, CD45RA-APC-eF780, CCR5-FITC, integrin β7-FITC, and CCR6-PE Abs, as well as non-T cell lineage Abs: CD326-BV650, CD8-PerCP-Cy5.5, CD19-PerCP-Cy5.5, CD66b-PerCP-Cy5.5 (37). Intracellular staining was subsequently performed with phosphorylated-mTOR (S2448)-eFluor-450 Abs (eBiosciences). The viability dye LIVE/DEAD® Fixable Aqua Dead Cell Stain Kit (Invitrogen) was used to exclude dead cells. Cells were analyzed using a LSRII cytometer, Diva version 6 (BD Biosciences, San Jose, CA), and FlowJo version 10.0.6 (Tree Star, Inc). Flow cytometry gates were defined using the *fluorescence minus one* (FMO) strategy (16, 91).

Magnetic (MACS) and fluorescence activated cell sorting (FACS)

Total or memory $CD4^+$ T-cells were enriched from PBMCs by negative selection using magnetic beads (MACS, Miltenyi), with a purity >95%, as previously described (42). Cells were then stained with CD45RA-APC-Cy7 and CCR6-PE Abs and a cocktail of FITC-conjugated Abs to exclude $CD8^+$ T-cells (CD8), NK (CD56), and B cells (CD19). The sorting gates were set on FITCneg memory (CD45RAneg) $CCR6^+/CCR6^-$ T-cells that were subsequently sorted by flow

cytometry (BDAria III), with dead cells being excluded. Post-sort quality control analysis demonstrated >97% $CCR6^+/CCR6^-$ T-cell purity, as previously reported (42).

HIV-1 infection *in vitro*

The following HIV-1 molecular clones were used in this study: *(i)* replication-competent CCR5-using (R5) NL4.3BAL; *(ii)* single-round VSVG-HIV-GFP, an *env*-deficient NL4.3 provirus pseudotyped with the VSV-G envelope and encoding for *gfp* in place of *nef* (42, 92); and *(iii)* replication-competent Transmitted/Founder (T/F) THRO (NIH AIDS Reagent Program) (44). HIV stocks were produced, titrated, and used to infect cells (25-50 ng HIV-p24/10^6 cells), as previously described (16, 19, 20, 36, 42).

Quantification of integrated and Gag HIV-DNA

Levels of integrated and Gag HIV-DNA were quantified in cell lysates by ultrasensitive nested real-time PCR (10^5 cells/test in triplicates; detection limit, 3 HIV-DNA copies), as previously described (16, 19, 20, 36, 37, 42, 93).

Quantification of early HIV reverse transcripts

Early HIV reverse transcripts (RU5) were quantified using a SYBR™ Green master mix (Qiagen). The external primers were: forward AA55 5'-CGTCTAGAGATTTTCCACAC-3' and reverse M667 5'-CTAACTAGGGAACCCACTG-3'. The internal primers were: forward SK29 5'-ACTAGGGAACCCACTGCT-3' and reverse SK30 5'-GGTCTGAGGGATCTCTAG-3' (94). The HIV and CD3 DNA were amplified together in the first PCR and separately during the second PCR (LightCycler; Roche). The first PCR products (diluted 1/10) were used as template for the second PCR: 5 min at 94°C, 35 cycles of 1 min at 94°C, 1 min at 50°C, and 30 sec at 72°C; and final elongation of 15 min at 72°C. The ACH2 cells carrying one copy of integrated HIV DNA per cell (NIH AIDS reagent program) were used as a standard curve, as previously described (16, 19, 20, 36, 37, 42, 93).

Genome-wide transcriptional profiling

Genome-wide transcriptional profiling was performed in collaboration with Génome Québec (Montreal, Quebec, Canada) using the Illumina bead array technology, as previously described (20). Briefly, total RNA was isolated using the RNeasy kit (Qiagen) and quantified using the Pearl nanophotometer (Implen, Germany). The RNA quality was determined using the Agilent 2100 Bioanalyzer chip. High quality RNA was reverse transcribed and hybridized on the Illumina HumanHT-12 v4 Expression BeadChip providing coverage for more than 47,000 transcripts and known spliced variants across the human transcriptome. The expression of differentially expressed genes was analyzed as previously described (19, 20, 36). The entire microarray dataset and technical information requested by Minimum Information About a Microarray Experiment (MIAME) are available at the Gene Expression Omnibus (GEO) database under accession number GSE93660. Differentially expressed genes (cut-off 1.3-fold; $p < 0.05$) were classified through Gene Ontology using the NetAffx web-based application (Affymetrix), while differentially expressed pathways were identified using *Ingenuity Pathway Analysis* (IPA) and *Gene Set Variation Analysis* (GSVA). Corresponding heat maps for biological function categories were generated using programming language R (19, 20, 36).

Real-time RT-PCR

One step SYBR Green real-time RT-PCR (Qiagen) was carried out in a LightCycler 480 II (Roche) according to manufacturer's recommendations, as we previously reported (16, 19, 20, 36, 42). Briefly, a standard curve was prepared with cDNA obtained upon reverse transcription of total RNA using specific QuantiTect Primers (Qiagen). The expression of each gene was normalized to the internal control 28S rRNA (forward 5'-CGAGATTCCTGTCCCCACTA-3'; reverse 5'-GGGGCCACCTCCTTATTCTA-3, IDT). Melting curve analysis performed after real-time amplification revealed the uniformity of thermal dissociation profile for each amplification product. Samples without template or without reverse transcriptase were used as negative controls. Each RT-PCR reaction was performed in triplicate.

mTOR protein and mRNA quantification

Cells were lysed with RIPA buffer 1x (Cell Signaling) containing phosphatase inhibitors (PhosSTOP) and protease inhibitors (Complete Mini EDTA-free, Roche), for 10 minutes at 4°C and centrifuged at 14,000g for 10 min. Proteins were quantified in supernatants by Bradford assay (Biorad). Samples (15 µg protein/well) were loaded onto 7% acrylamide gels and SDS-PAGE migration was performed (90 min, 100 V). Proteins were transferred to PVDF membranes and further blotted with phosphorylated (D9C2) or total mTOR (7C10) Abs (Cell signaling). Proteins were revealed with ECL prime western blotting detection reagent (Amersham, GE Healthcare). In parallel, the expression of the mTOR mRNA was measured by semi-quantitative RT-PCR performed using the superscript III one-step RT-PCR system (Invitrogen, USA) and specific primers (forward: S1, ATGCTTGGAACCGGACCTGCCG ; reverse: AS1, TACCAGAAAGGGCACCAGCCAAT) (Integrated DNA Technologies, USA) that could amplify both mTOR isoforms α (7.647Kb Kb) and β (2.118 Kb) (61) (Suppl. Figure 7A). However, the RT-PCR conditions were set for the optimal amplification of the short β mTOR isoform, as follows: reverse transcription (30 min at 55 °C), 40 cycles of PCR amplification (denaturation, 15 secs at 94 °C; annealing, 30 secs at 63 °C; elongation, 130 secs at 68 °C) and final elongation (15 min at 68 °C). mTOR expression was normalized relative to 28S rRNA. Total RNA was extracted using the RNeasy kit (Qiagen) and used for RT-PCR amplification of mTOR (250 ng/test) and 28S rRNA (5 ng/test).

Nuclear NF-κB quantification

The NF-κB DNA-binding activity was quantified using an activity-based ELISA kit (Assay Designs & Stressgen, Ann Arbor, Mi, USA) according to the manufacturer's protocol, as described (20). Briefly, nuclear extracts were obtained using the BD transfactor extraction kit (Clonetech Laboratories). Nuclear protein content was quantified by Nanodrop (Thermo Scientific). The active form of NF-κB-p65 was quantified in nuclear extracts (1 µg protein/test). The assay used streptavidin-coated plates with bound NF-κB biotinylated-consensus sequence to capture only the active form of NF-κB. The captured NF-κB was then incubated with a specific NF-κB-p65 Abs further detected using an HRP conjugated Abs. The assay was developed with a chemiluminescent substrate and the signal was detected using a luminometer. Results were

expressed as Light Units. The specificity of the binding was demonstrated by the use of a wild type and mutated NF-κB Competitor Duplex included in the kit.

ELISA quantification

HIV-p24 levels were quantified in cell culture supernatants using a homemade ELISA, as described (16, 19, 20, 36, 37, 42). Cytokine levels in cell culture supernatants were quantified by ELISA assays specific for TNF-α, CCL20 (R&D Systems), IL-17, IFN-γ and IL-2 (eBioscience) according to the manufacturers' protocols.

Viral outgrowth assay

This viral outgrowth assay (VOA) was designed to allow optimal HIV reservoir reactivation from small numbers of primary $CD4^+$ T-cells (36, 37). Briefly, memory $CD4^+$ T-cells ($2x10^6$ cells/ml/well) or FACS sorted memory $CCR6^+/CCR6^-$ T-cells (10^6 cells/ml/well) were cultured in 48-well plates in the presence of immobilized CD3 and soluble CD28 Abs (1 µg/ml) for 3 days. Cells were further cultured in the presence of IL-2 (5 ng/ml) up to day 12. Every 3 days, cells were split and media was refreshed. The VOA was performed in the presence/absence of ATRA (10 nM) and/or INK128 (50 nM). HIV-p24 levels were quantified by ELISA in cell culture supernatants, while intracellular HIV-p24 expression was quantified by flow cytometry at day 12 post-reactivation.

Statistics

Statistical analyses were performed using the GraphPad Prism 6. RM One-way ANOVA or ordinary ANOVA with Tukey's or Dunnetts's post-test were used for multiple comparisons. Two-tailed paired t-test was used for matched pairwise analyses. A P-value less than 0.05 was considered significant. (*, p-value<0.05; **, p-value<0.01; ***p-value<0.001; ****p-value<0.0001). Details are included in Figure legends.

Microarrays accession number

The entire microarray dataset and technical information requested by Minimum Information About a Microarray Experiment (MIAME) are available at the Gene Expression Omnibus database under accession number GSE93660.

Study approval

This study used biological samples (blood and sigmoid biopsies) from HIV-infected and uninfected participants and 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. All human participants provided written informed consent for their participation in the study and agreed with the publication of the scientific results.

Competing interests

The authors declare no financial and non-financial competing interests.

Authors' contributions

DP performed research, analyzed data, and wrote the manuscript. YZ and PM performed research and contributed to manuscript writing. JPG performed microarray data analysis and generated figures. AG performed research. AF contributed to research design and manuscript writing. NG and TJH provided protocols and reagents and contributed to research design. JPR provided access to clinical samples/information, set up clinical research protocols, and contributed to manuscript writing**.** PA designed research, analyzed data, and wrote the manuscript. All authors reviewed and accepted the manuscript.

Funding

This study was supported by grants from the Canadian Institutes of Health Research (CIHR) (#MOP-82849; #MOP-114957 to PA), the CIHR Canadian HIV Trials Network (CTN #247 to JPR), the *Fonds de Recherche du Québec-Santé* (FRQ-S)/AIDS and Infectious Diseases Network, Québec, Canada (to PA and JPR), and by The Canadian HIV Cure Enterprise Team Grant HIG-

133050 from the CIHR in partnership with CANFAR and IAS (to PA and JPR). JPR holds a Louis Lowenstein Chair in Hematology and Oncology, McGill University. The funding institutions played no role in the design, collection, analysis, and interpretation of data.

Acknowledgements

The full-length transmitted/founder (T/F) THRO HIV-1 Infectious Molecular Clone (Cat #11919) was obtained from Dr. John Kappes through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH. The authors acknowledge the contribution of Dr. Dominique Gauchat (Flow Cytometry Core Facility, CHUM-Research Centre, Montreal, QC, Canada) for expert technical support with flow cytometry analysis and sorting, Mr. Mario Legault for help with ethical approvals and informed consents, and Josée Girouard and Angie Massicotte for their critical contribution to sigmoid biopsies, blood, and leukapheresis collection from HIV-infected and uninfected donors. The authors thank Drs. Rosalie Ponte, Franck Dupuy, and Vikram Meraj for their contribution to colon biopsies access and processing, and Dr. Andrew Mouland for the critical reading of the manuscript. The authors also thank Natalia Zamorano Cuervo and Sandra L. Cervantes-Ortiz for their technical assistance with western blotting. Finally, the authors acknowledge study participants for their gift of biological samples essential for this study.

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FIGURES

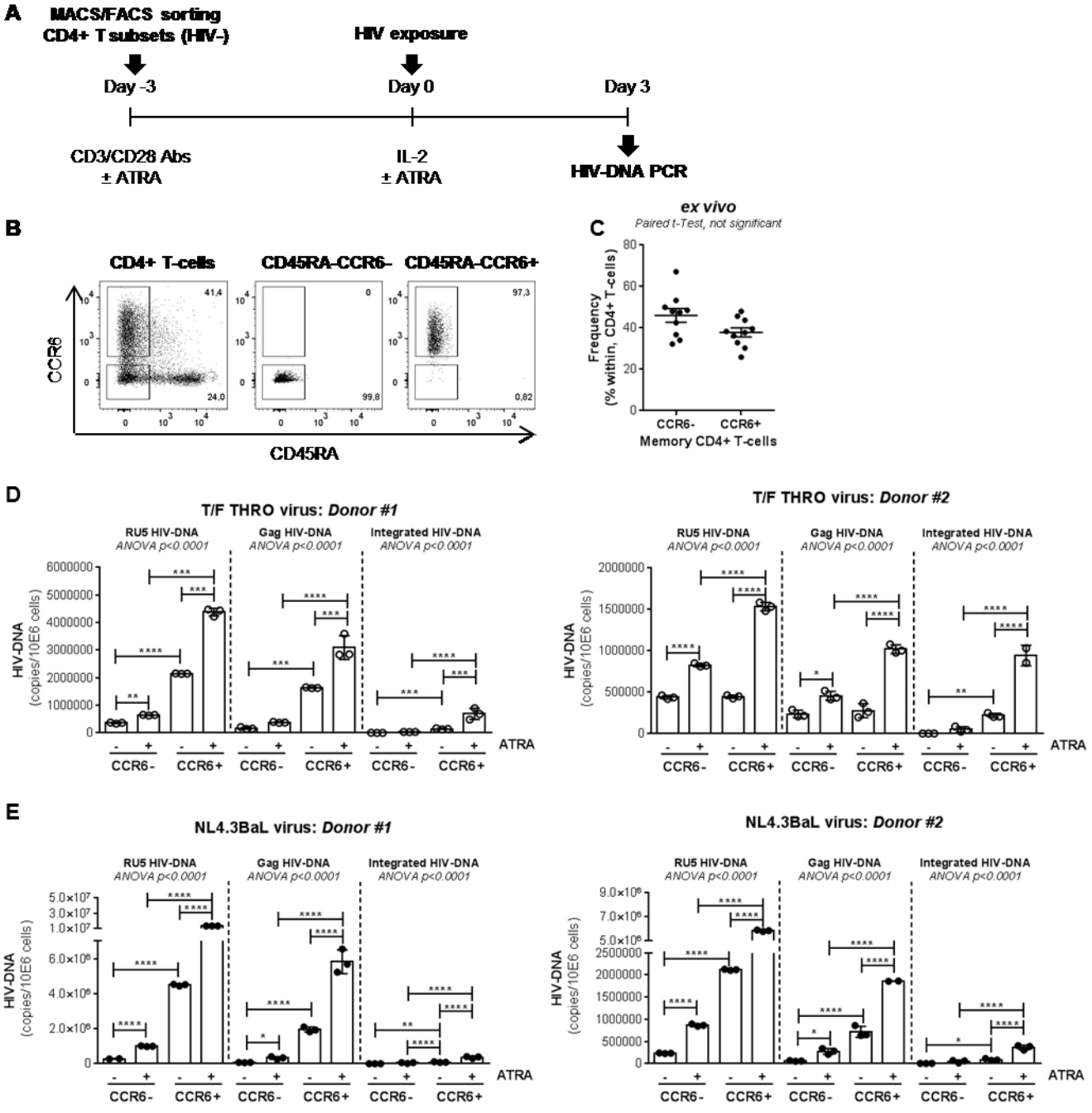


Figure 1: Replication advantage of transmitted founder HIV in CCR6^{+} *versus* CCR6^{-} T-cells upon exposure to ATRA. Memory CCR6+ and CCR6- T-cells were isolated by MACS (magnetic-activated cell sorting) and FACS from the PBMCs (peripheral blood mononuclear cells) of HIV-uninfected individuals

and tested for their ability to replicate HIV *in vitro*. Shown is (A) the experimental flow chart, (B) the expression of CCR6 and CD45RA on total $CD4^+$ T-cells isolated using magnetic beads and on memory ($CD45RA^-$) $CCR6^+$ and $CCR6^-$ T-cells isolated by FACS, and (C) the frequency of $CCR6^-$ and $CCR6^+$ T-cells within memory $CD4^+$ T-cells in n=10 donors. Paired t-Test p-values are indicated on the graphs (D-E). Sorted cells were stimulated *via* CD3/CD28 in presence/absence of ATRA (*all-trans retinoic acid*) (10 nM) for 4 days and exposed to (D) transmitted founder (T/F) THRO HIV or (E) laboratory-adapted NL4.3BaL HIV (25 ng HIV-p24/10^6 cells). Early (RU5 primers) and late (Gag primers) HIV-DNA reverse transcripts, as well as integrated HIV-DNA levels (Alu/HIV-LTR primers) were quantified by nested real-time PCR in matched subsets isolated from n=2 different donors at day 3 post-infection (mean±SD of triplicate wells). (D-E) One-way ANOVA p-values with the significance of the Tukey's multiple comparisons test are indicated on the figures. Each symbol represents one triplicate value.

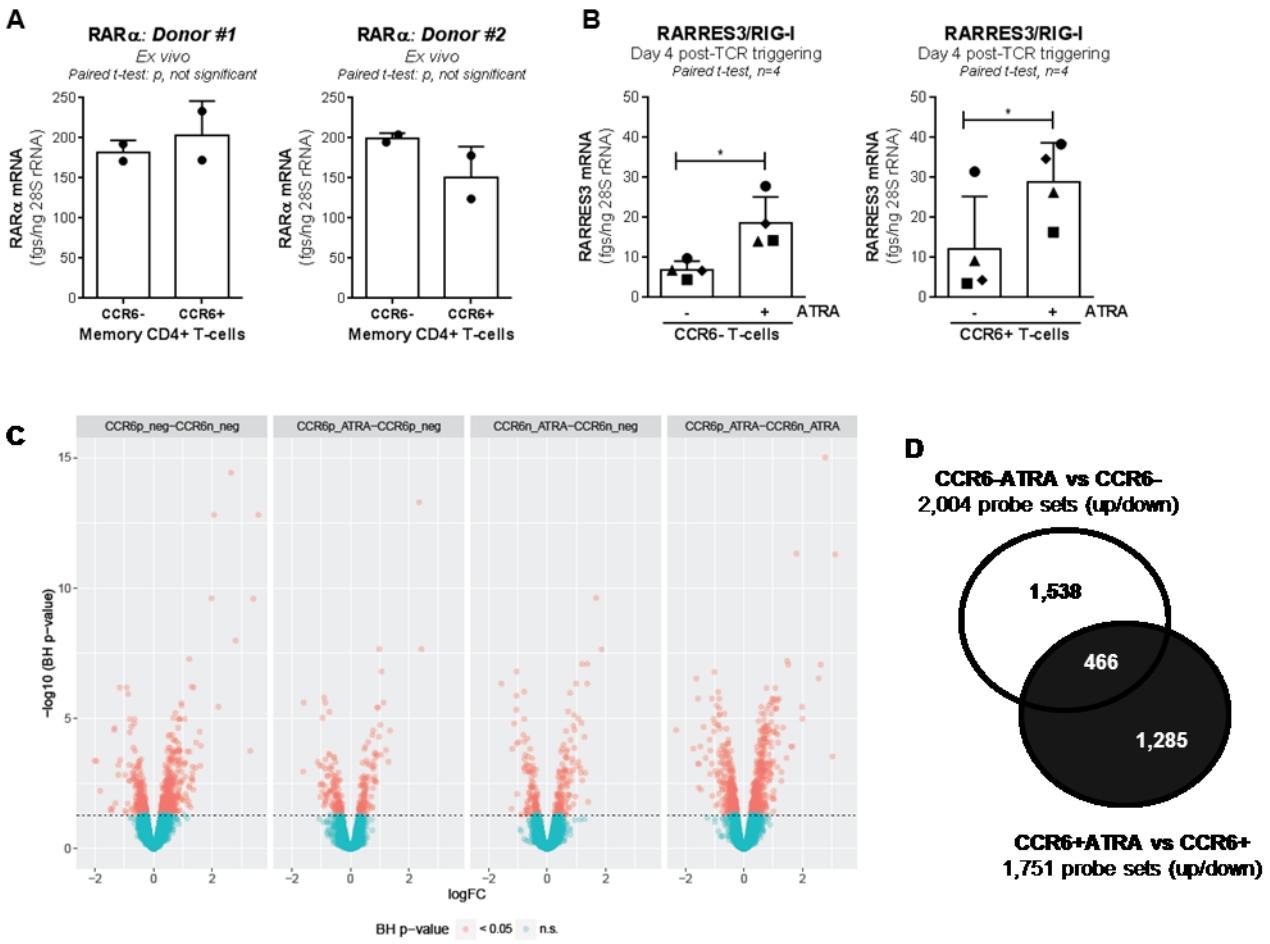


Figure 2: ATRA differentially modulates gene expression in CCR6$^-$ and CCR6$^+$ T-cells. Memory CCR6$^+$/CCR6$^-$ T-cells were sorted and stimulated as described in Figure 1. Shown is the SYBR green real-time RT-PCR quantification of RARα (retinoic acid receptor α) mRNA *ex vivo* (A; n=2, mean±SD) and RIG-I/RARRES3 (retinoic acid induced gene I) mRNA upon CD3/CD28 triggering in the presence/absence of ATRA (B; n=4, mean±SEM). (A-B) Paired t-Test p-values are indicated on the graphs. Each symbol represents (A) one duplicate value or (B) one different donor. (C-E) Total RNA from matched T-cell subsets of n=6 different HIV-uninfected donors was used for genome-wide transcriptional profiling. One-way ANOVA analysis identified differentially expressed genes based on p-values or adjusted p-values (<0.05) and/or fold-change (FC, cut-off 1.3). (C) Shown are volcano plots for all probes in each linear model with the log2 FC on the x axis and the negative logarithm of the adjusted p-values for false discovery rate (FDR) on the y axis. The red/green color code is based on the 5% FDR threshold. (D) The Venn diagram depicts the number of probe-sets modulated by ATRA uniquely in CCR6$^+$ (grey) and CCR6$^-$ T-cells (white) (p-values<0.05), as well as 466 probe sets commonly modulated.

Figure 3: Canonical pathway modulated by ATRA in CCR6$^+$ T-cells. Memory CCR6$^+$/CCR6$^-$ T-cells were sorted and stimulated as described in Figure 1. Total RNA from matched T-cell subsets of n=6 different HIV-uninfected donors was used for genome-wide transcriptional profiling as described in Figure 2. One-way ANOVA analysis identified differentially expressed genes based on p-values or adjusted p-values (<0.05) and/or fold-change (FC, cut-off 1.3). The heat map depicts top 34 pathways up (red) and down (blue) modulated by ATRA in CCR6$^+$ T-cells, identified using gene-set variation analysis (GSVA; p-value<0.01;enrichment z-score).

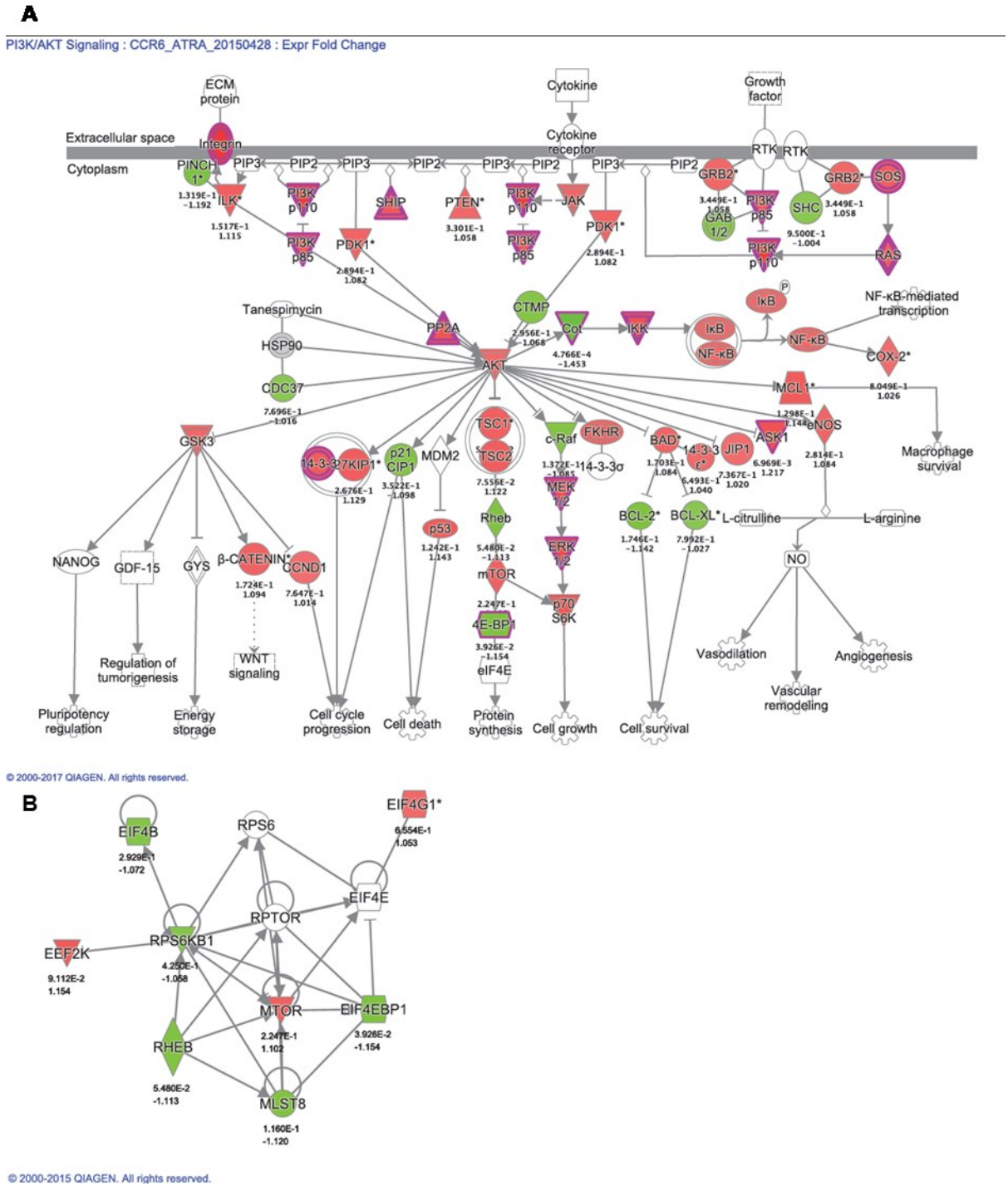


Figure 4: Components of the Akt/PI3K pathways modulated by ATRA in CCR6$^+$ T-cells. Memory CCR6$^+$/CCR6$^-$ T-cells were sorted and stimulated as described in Figure 1. Total RNA from matched T-cell subsets of n=6 different HIV-uninfected donors was used for genome-wide transcriptional profiling as described in Figure 2. One-way ANOVA analysis identified differentially expressed genes based on p-values or adjusted p-values (<0.05) and/or fold-change (FC, cut-off 1.3). Shown are (A) combined

BIOCARTA_AKT_PATHWAY and PID_PI3K_PLC_TRK_PATHWAY Canonical pathways significantly modulated by ATRA in $CCR6^+$ T-cells, identified using Ingenuity Pathway Analysis®; and (B) Gene expression network generated using individual genes representing REACTOME_MTORC1_MEDIATED_SIGNALING, significantly modulated by ATRA in $CCR6^+$ T-cells identified through GSVA. The color code is based on expression FC (red and green for up-regulated and down-regulated transcripts, respectively). Nodes representing individual genes are annotated with their corresponding p-values and FC.

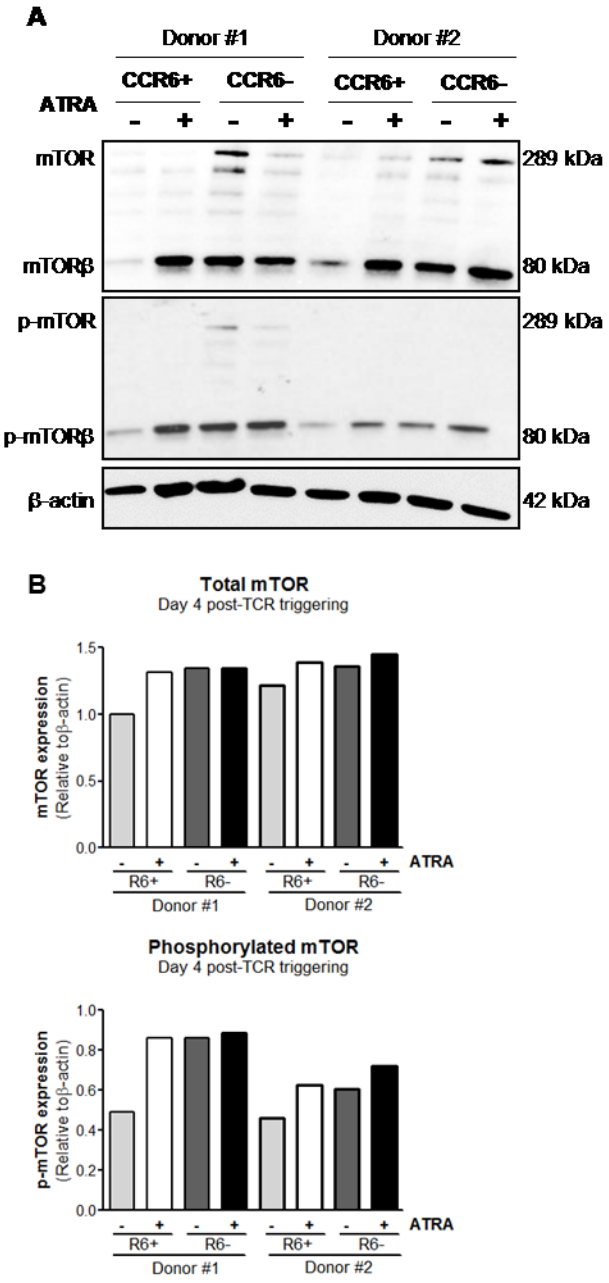


Figure 5: ATRA induces mTOR expression and phosphorylation selectively in CCR6$^+$ T-cells. Memory CCR6$^+$ and CCR6$^-$ T-cell subsets were sorted and stimulated as described in Figure 1. (A) Cell lysates were used to visualize total and phosphorylated mTOR expression by Western Blotting. (B) Levels of total and phosphorylated mTOR expression were quantified by densitometry and normalized relative to β-actin levels. Of note, the molecular weight of the predominant mTOR band was 80 kDa, corresponding to the β isoform of mTOR (1). Shown are results obtained with cells from two different donors.

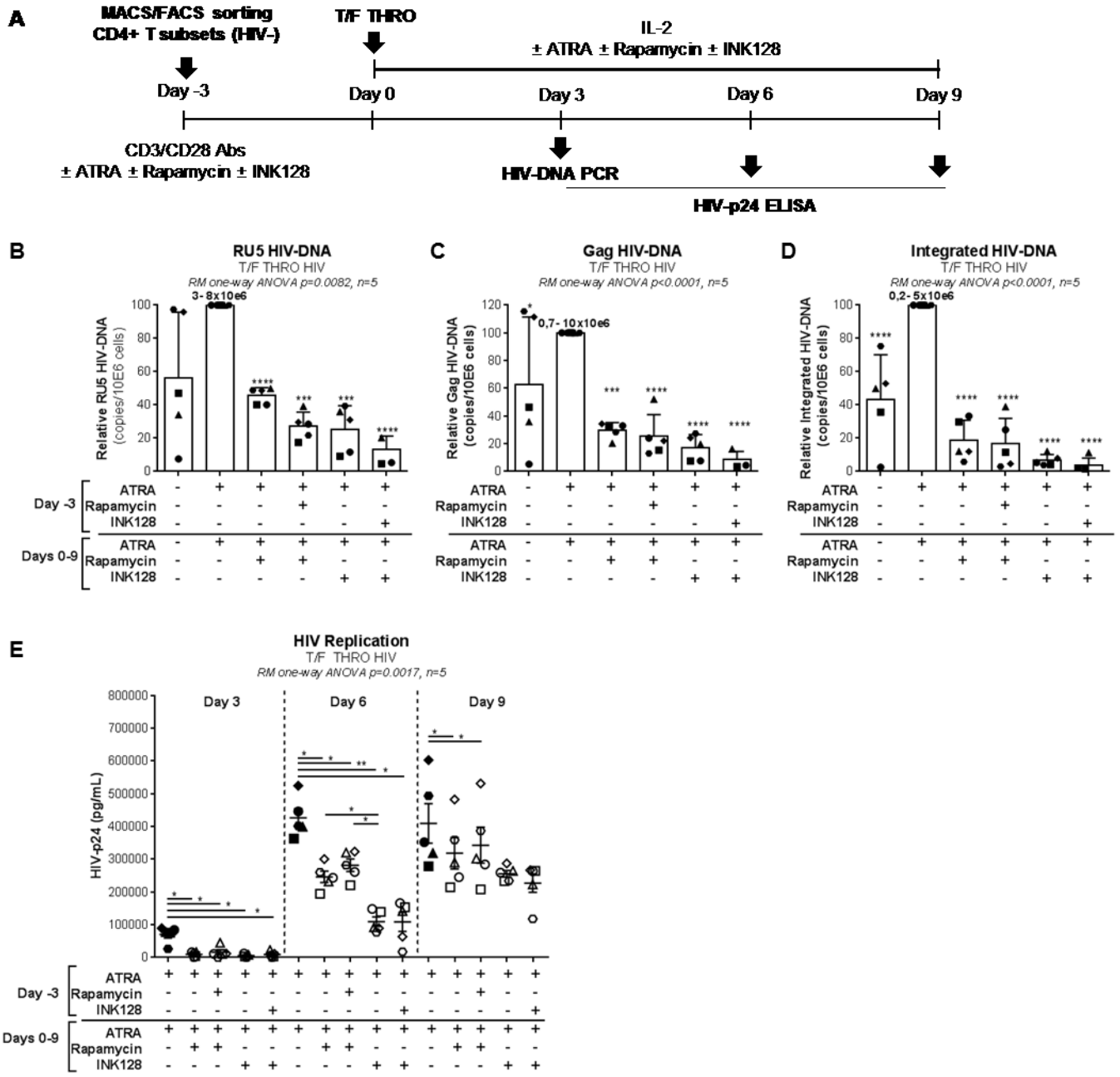


Figure 6: mTOR inhibitors counteract the effect of ATRA on HIV permissiveness in memory CD4+ T-cells. The ability of mTOR inhibitors to counteract the effects of ATRA on HIV replication was tested in total memory $CD4^+$ T-cells (A). Briefly, total memory $CD4^+$ T-cells were stimulated *via* CD3/CD28 in presence/absence of ATRA and/or rapamycin (10 nM) or INK128 (50 nM) for 4 days. Then, cells were exposed to replication competent T/F HIV THRO (25 ng HIV-p24/10^6 cells). Further, cells were cultured for 9 additional days in presence/absence of ATRA and/or rapamycin or INK128. Shown are the relative RU5, Gag, and integrated HIV-DNA levels quantified by real-time nested PCR at day 3 post-infection (with the range of absolute HIV-DNA copies/10^6 cells in ATRA-treated cells, considered 100%, being indicated on the figures) (B-D) and HIV-p24 levels quantified by ELISA in cell culture supernatants at

days, 3, 6 and 9 post-infection (E) (n=5, mean±SEM) . The RM one-way ANOVA p-values (B-E), with the significance of the Dunnetts's (relative to cells stimulated *via* the TCR in the presence of ATRA) (B-C-D) or Tukey's (E) multiple comparisons test are indicated on the figures. Each symbol represents one different donor.

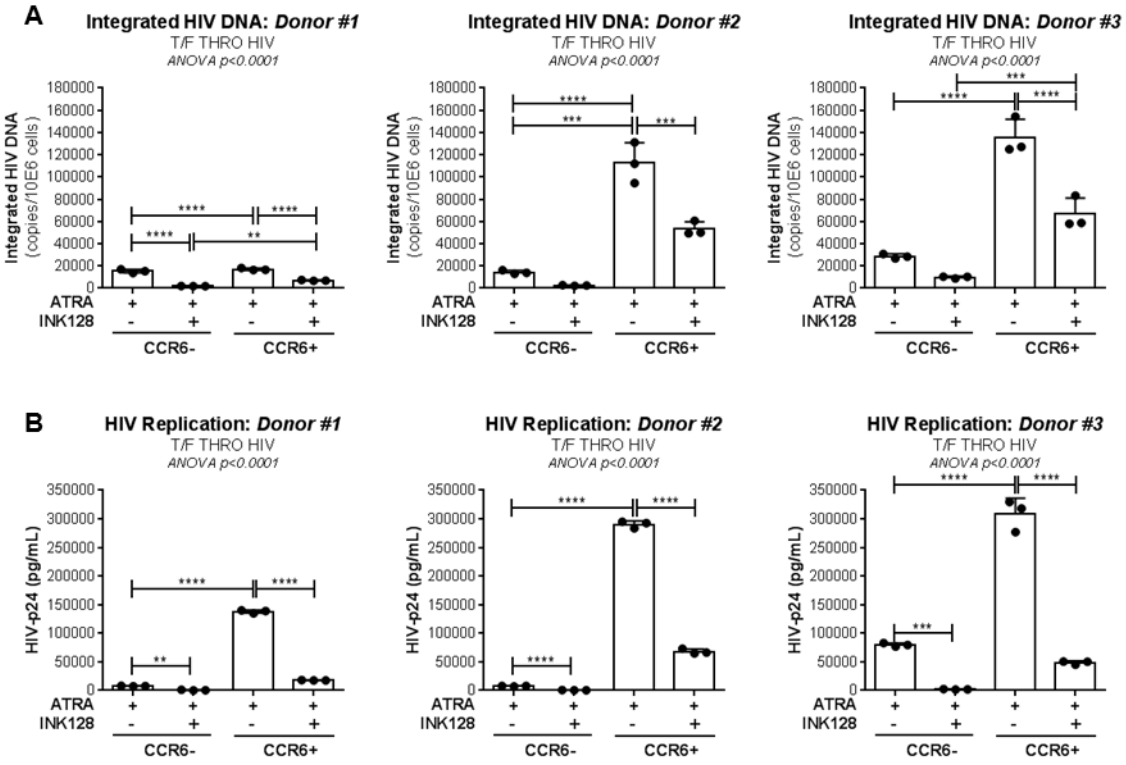


Figure 7: mTOR inhibitors counteract the effect of ATRA on HIV permissiveness in sorted CCR6+ and CCR6- T-cells. Memory CCR6+ and CCR6- T-cells were sorted as depicted in Figure 1 and stimulated *via* CD3/CD28 in presence of ATRA or ATRA and INK128 (50 nM) for 4 days. Then, cells were exposed to replication competent T/F HIV THRO (25 ng HIV-p24/10^6 cells). Further, cells were cultured for 9 additional days in presence of ATRA or ATRA and INK128. (A-B) Shown are absolute levels of integrated HIV-DNA at day 3 post-infection (A) and HIV-p24 levels in cell culture supernatants at day 6 post-infection (B) (n=3, mean±SD of triplicate wells), Ordinary one-way ANOVA with the significance of the Tukey's multiple comparisons test are indicated on the figures. Each symbol represents one experimental triplicate value in individual donors.

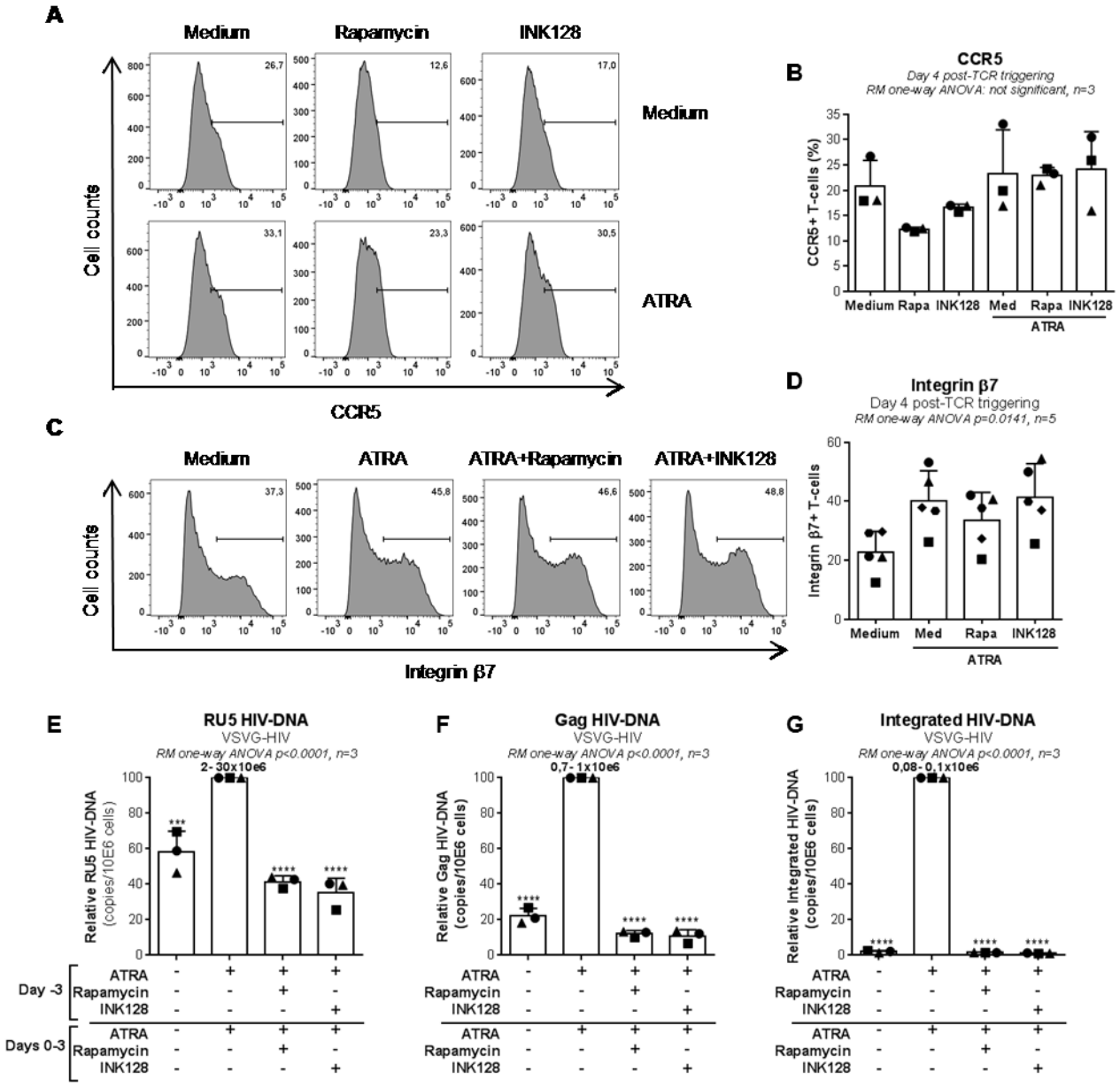


Figure 8: mTOR inhibitors limit HIV replication at post-entry levels in ATRA-treated CD4⁺ T-cells. (A-D) Memory CD4⁺ T-cells were first analyzed for the surface expression of CCR5 and integrin β7 at day 4 post-TCR triggering in the presence/absence of ATRA (10 nM), rapamycin (10nM), and/or INK128 (50nM). Shown are levels of CCR5 (A) and integrin β7 (C) expression in one representative donor and statistical analysis of CCR5 (B; n=3) and integrin β7 (D; n=5) expression in different donors (mean±SEM). (E-G) Then, stimulated cells were exposed to VSV-G-pseudotyped HIV (25 ng HIV-p24/10^6 cells) (single round infection) and cultured in presence/absence of ATRA and/or rapamycin or INK128 for 3 additional days. Levels of RU5 (E), Gag (F) and integrated HIV-DNA (G) were quantified by nested real-time PCR in cells harvested at day 3 post-infection. (E-G) Shown are relative HIV-DNA levels (mean±SEM; n=3); the range of absolute HIV-DNA copies/10^6 cells in ATRA-treated CCR6⁺ T-cells (considered 100%) is indicated on the figure. The RM one-way ANOVA p-values with the significance of

the Tukey's (B-D) or Dunnetts's (relative to cells stimulated *via* the TCR the presence of ATRA) (E, F, G) multiple comparisons tests are indicated on the graphs. Each symbol represents one different donor.

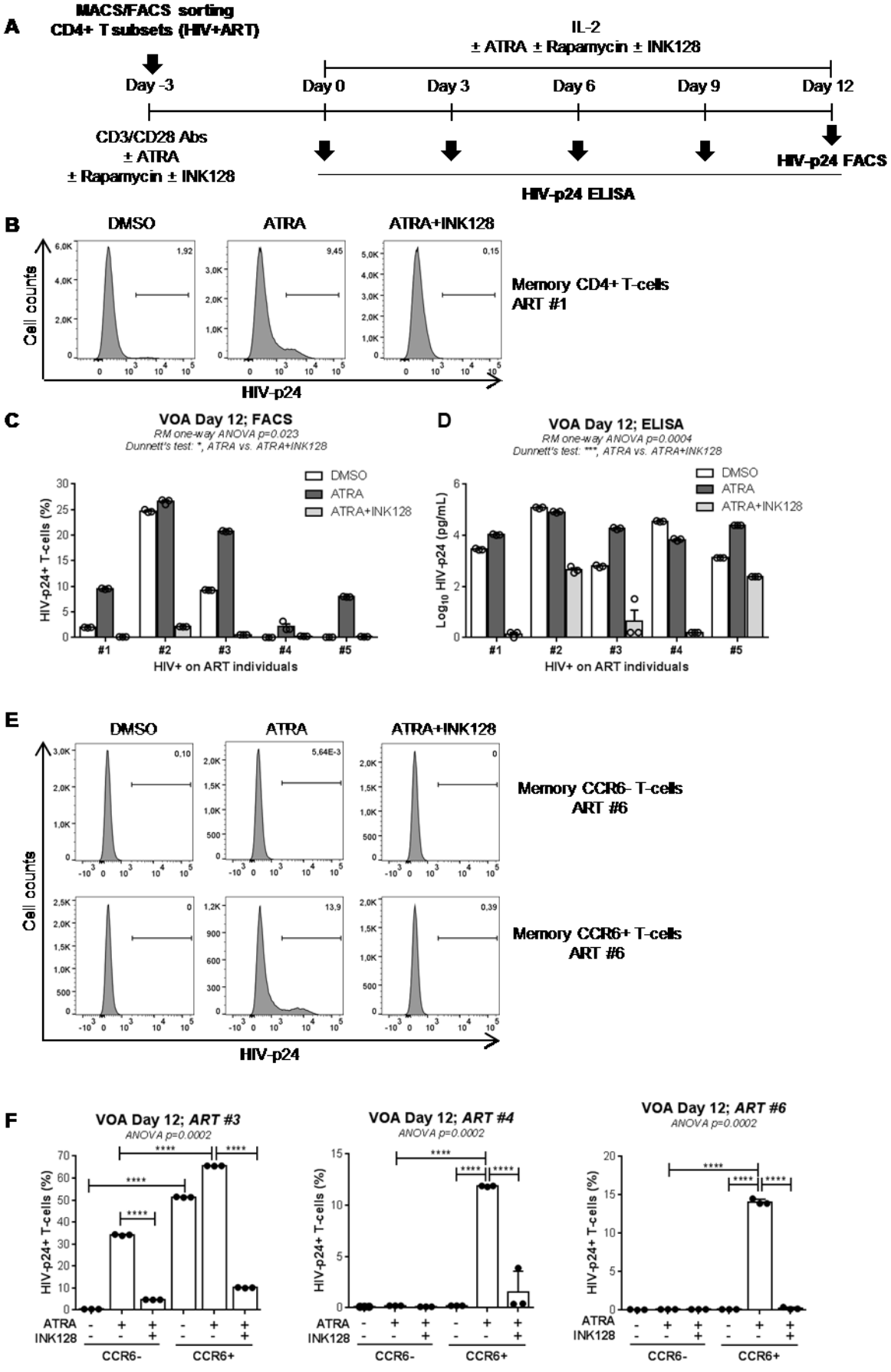

A
MACS/FACS sorting
CD4+ T subsets (HIV+ART)
Day -3
CD3/CD28 Abs
± ATRA
± Rapamycin ± INK128
IL-2
± ATRA ± Rapamycin ± INK128
Day 0
Day 3
Day 6
Day 9
Day 12
HIV-p24 FACS
HIV-p24 ELISA
B
DMSO
ATRA
ATRA+INK128
Cell counts
HIV-p24
Memory CD4+ T-cells
ART #1
C
VOA Day 12; FACS
RM one-way ANOVA p=0.023
Dunnett's test: *, ATRA vs. ATRA+INK128
HIV-p24+ T-cells (%)
HIV+ on ART individuals
D
VOA Day 12; ELISA
RM one-way ANOVA p=0.0004
Dunnett's test: ***, ATRA vs. ATRA+INK128
Log10 HIV-p24 (pg/mL)
HIV+ on ART individuals
E
Memory CCR6- T-cells
ART #6
Memory CCR6+ T-cells
ART #6
F
VOA Day 12; ART #3
ANOVA p=0.0002
VOA Day 12; ART #4
ANOVA p=0.0002
VOA Day 12; ART #6
ANOVA p=0.0002
ATRA
INK128
CCR6-
CCR6+

Figure 9: Drug-mediated mTOR blockade counteracts the enhancing effects of ATRA on HIV reactivation in a viral outgrowth assay. HIV reactivation was measured in a viral outgrowth assay (VOA), as summarized in the flowchart (A). Briefly, total memory $CD4^+$ T-cells (B-D; n=5) and FACS-sorted memory CCR6+ and CCR6- T-cells (E-F; n=3) were isolated from PBMCs of HIV+ART individuals (Table S5; ART#1-6). Cells were activated *via* CD3/CD28 and cultured in presence/absence of ATRA (10 nM) and/or INK128 (50 nM). Shown is intracellular HIV-p24 expression in total memory CD4+ T-cells (B-C) and sorted memory CCR6+ and CCR6- T-cells (E-F) quantified by FACS, as well as HIV-p24 levels quantified by ELISA in cell culture supernatants of total memory CD4+ T-cells (D) harvested at day 12 post-activation. Results are depicted in individual donors. Each point represents one experimental triplicate (mean±SD of triplicate wells). The RM one-way ANOVA p-values with the significance of the Dunnetts's multiple comparisons test (relative to cells stimulated *via* the TCR the presence of ATRA) (C-D) and the ordinary one-way ANOVA with the significance of the Tukey's multiple comparisons test (F) are indicated in the figures.

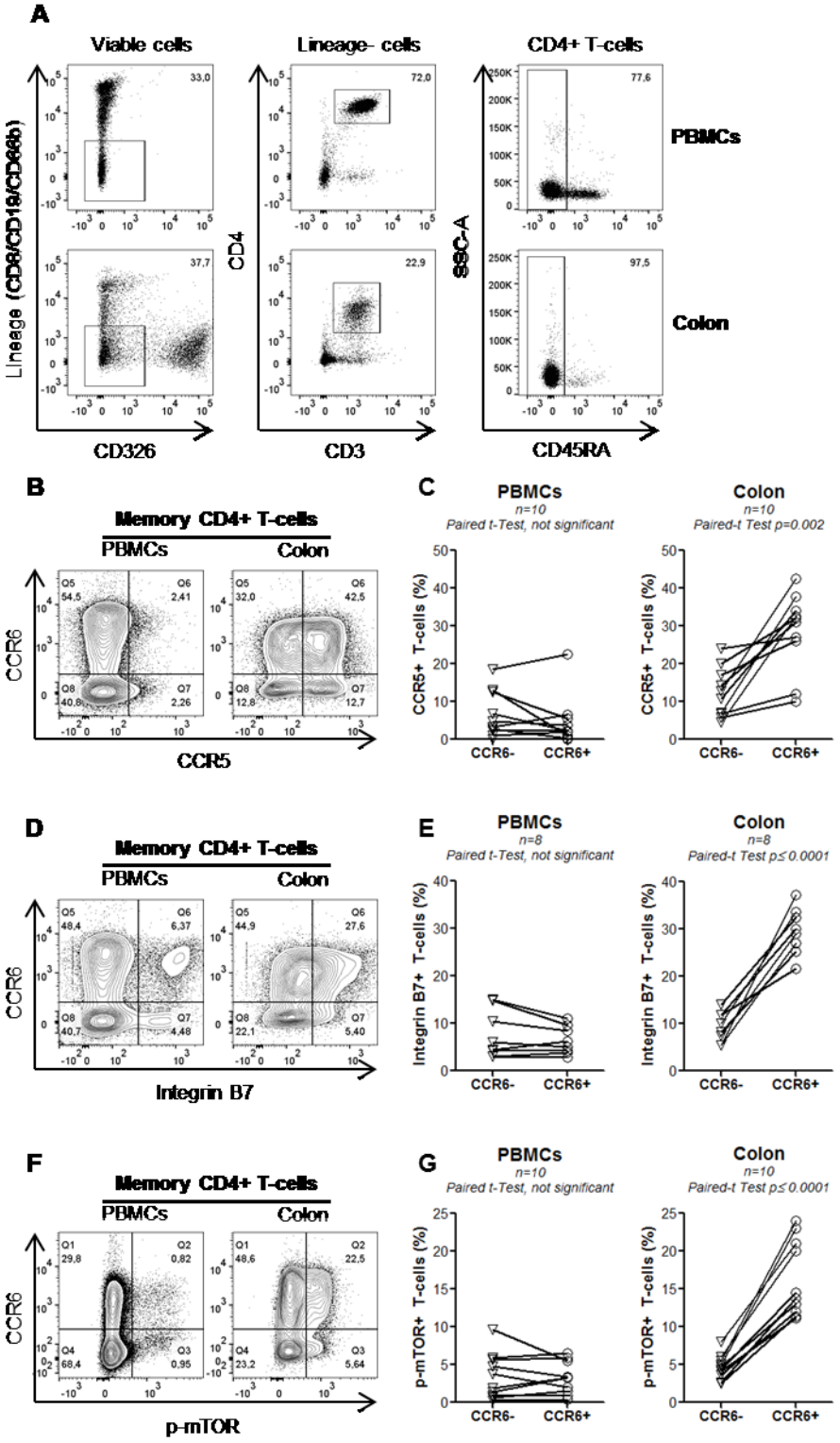


Figure 10: Memory CCR6^{+}CD4^{+} T-cells infiltrating the colon of HIV+ART individuals express high levels of CCR5, integrin β7, and phosphorylated mTOR. Matched blood and sigmoid biopsies were collected from HIV-infected individuals with undetectable plasma viral load (<40 HIV-RNA copies/ml) under ART

(Table S5; COL#1-10). Cells were extracted and stained with a cocktail of specific Abs. Shown is the gating strategy for the flow cytometry identification of $CD3^{+}CD4^{+}CD45RA^{-}$ T-cells (A), as well as the surface expression of CCR5 (B-C) and integrin β7 (D-E) and the intracellular expression of phosphorylated mTOR (F-G) on $CCR6^{+}$ and $CCR6^{-}$ subsets from sigmoid biopsies *versus* PBMC in one representative donor (B, D, and F) and statistical analysis in all donors (C, E, and G). Paired t-Test values are indicated on the graphs.

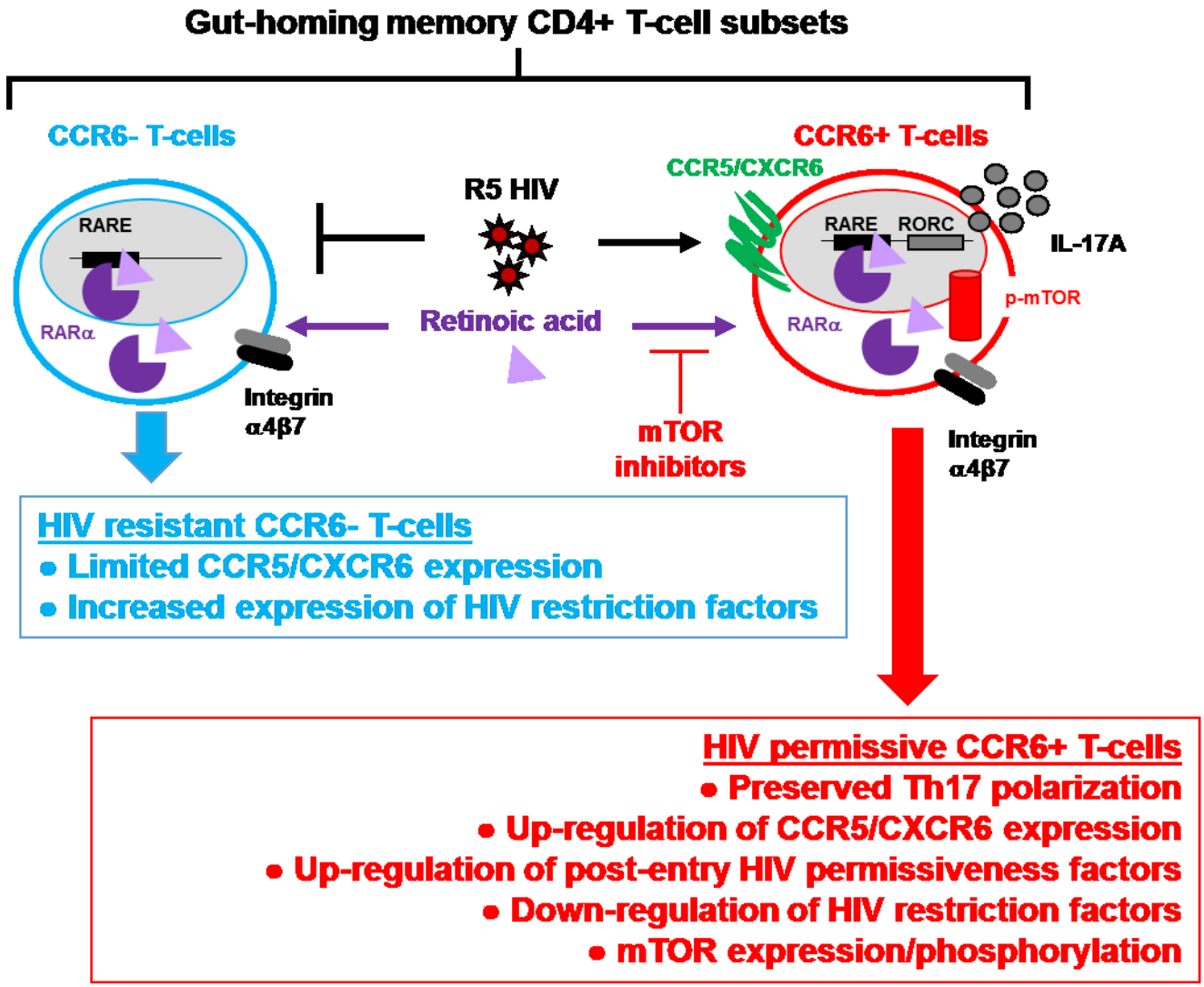
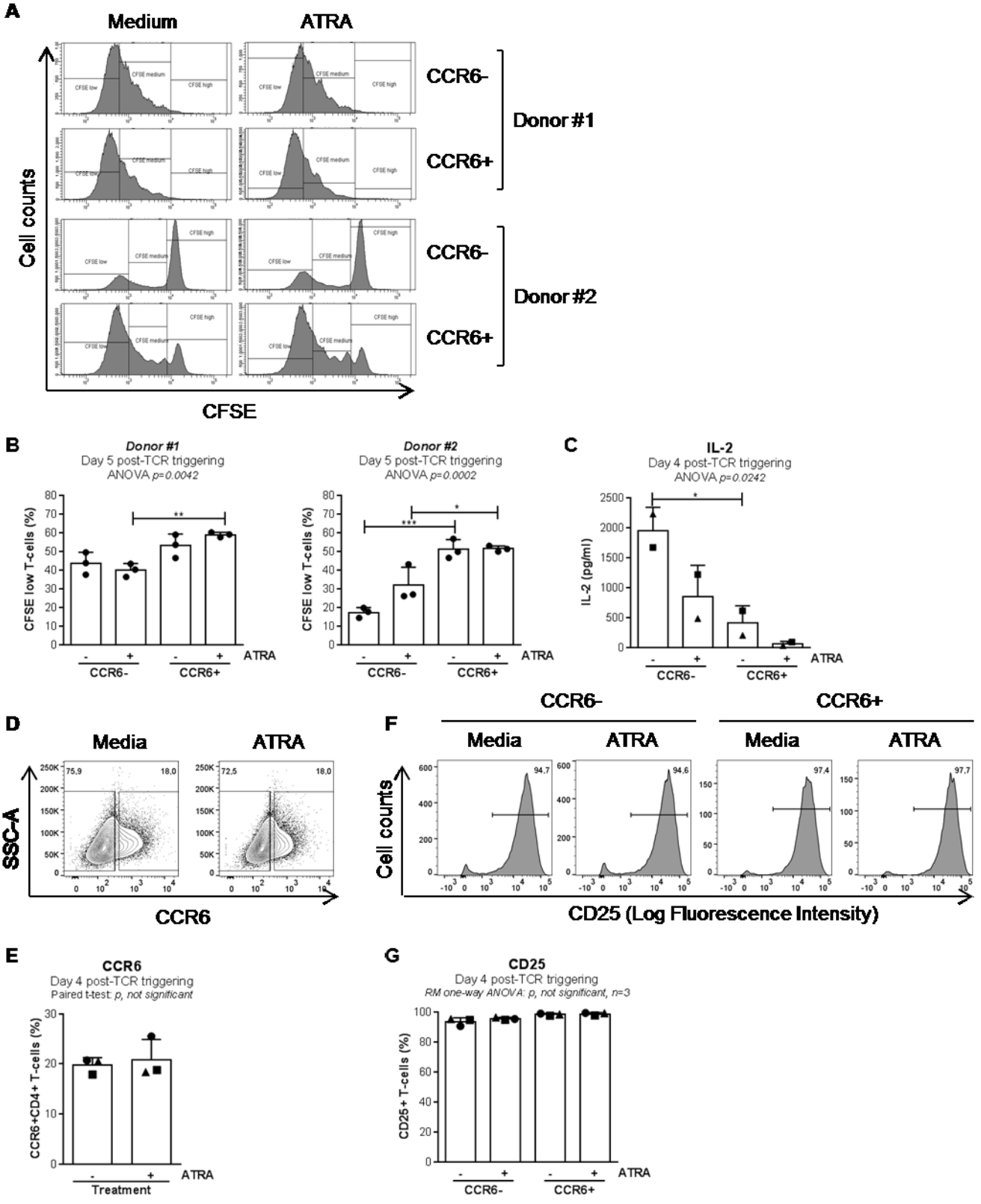
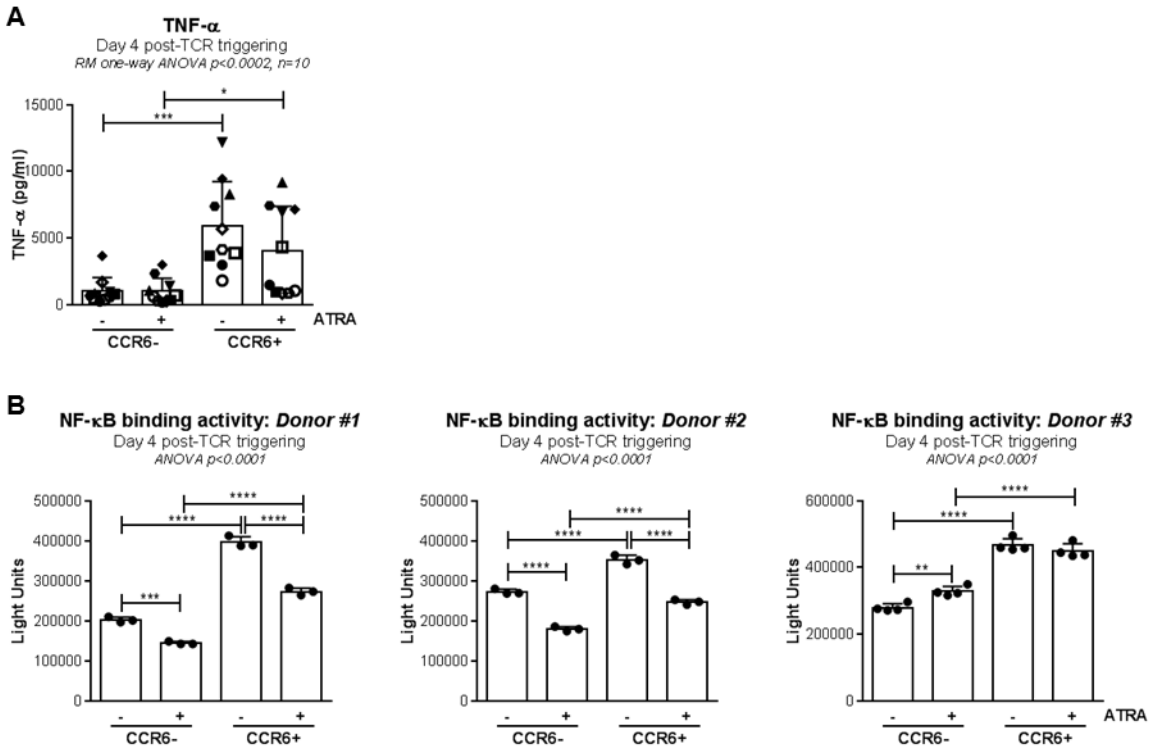


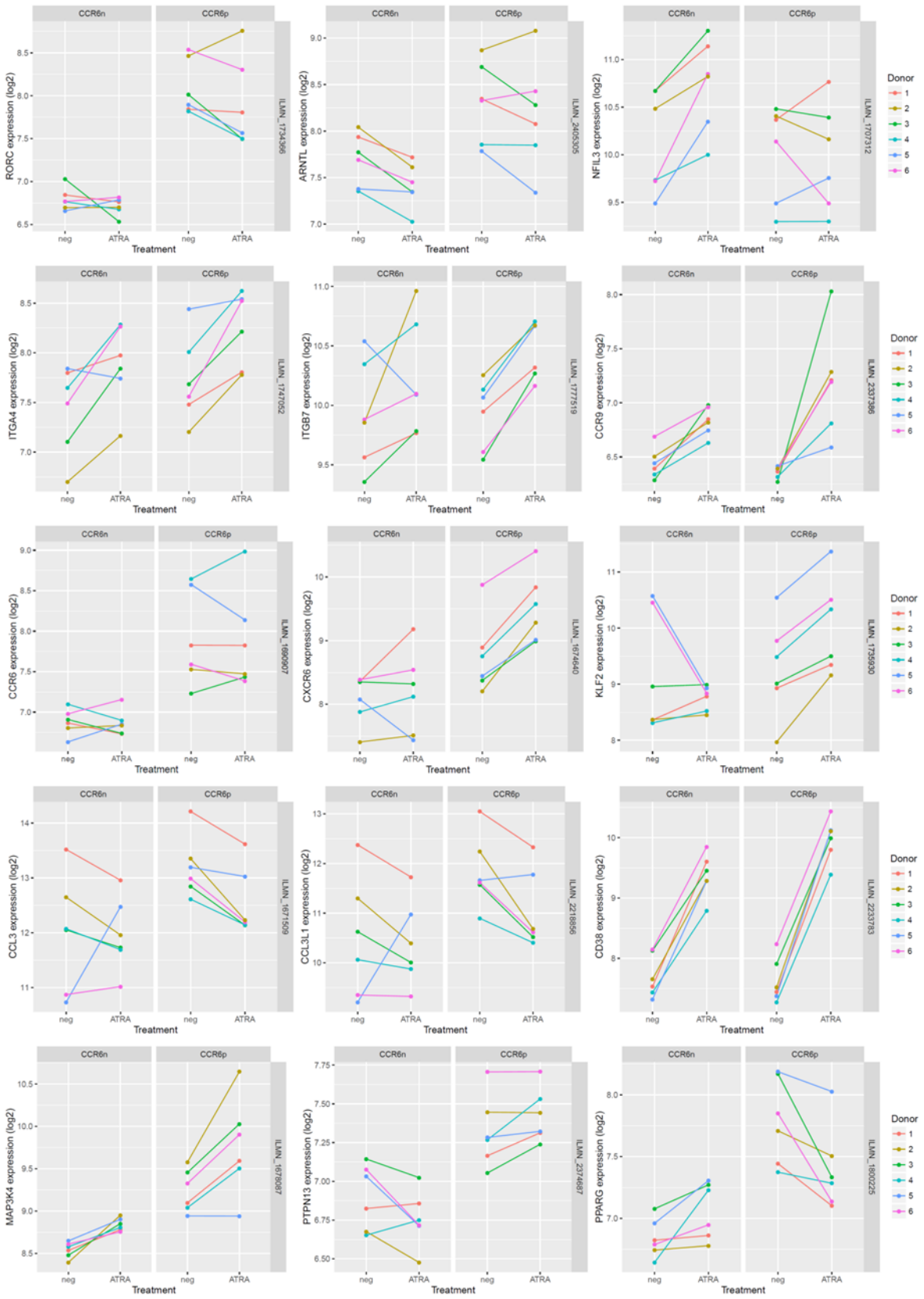
Figure 11: Mechanisms contributing to HIV replication in gut-homing CCR6$^+$ T-cells. In this manuscript, we provide evidence that CCR6 is a marker for memory CD4$^+$ T-cells programmed to become HIV targets, especially in the intestinal environment rich in retinoic acid, due to their expression of a unique Th17-polarized transcriptional program and a mTOR-governed metabolic state. We propose the use of mTOR inhibitors together with ART to protect gut-homing Th17 cells from HIV infection/persistence.


A
Medium
ATRA
CCR6-
CCR6+
Donor #1
Donor #2
Cell counts
CFSE
B
Donor #1
Day 5 post-TCR triggering
ANOVA p=0.0042
Donor #2
Day 5 post-TCR triggering
ANOVA p=0.0002
CFSE low T-cells (%)
ATRA
C
IL-2
Day 4 post-TCR triggering
ANOVA p=0.0242
IL-2 (pg/ml)
D
Media
ATRA
SSC-A
CCR6
E
CCR6
Day 4 post-TCR triggering
Paired t-test: p, not significant
CCR6+CD4+ T-cells (%)
Treatment
F
CCR6-
CCR6+
Media
ATRA
Cell counts
CD25 (Log Fluorescence Intensity)
G
CD25
Day 4 post-TCR triggering
RM one-way ANOVA: p, not significant, n=3
CD25+ T-cells (%)

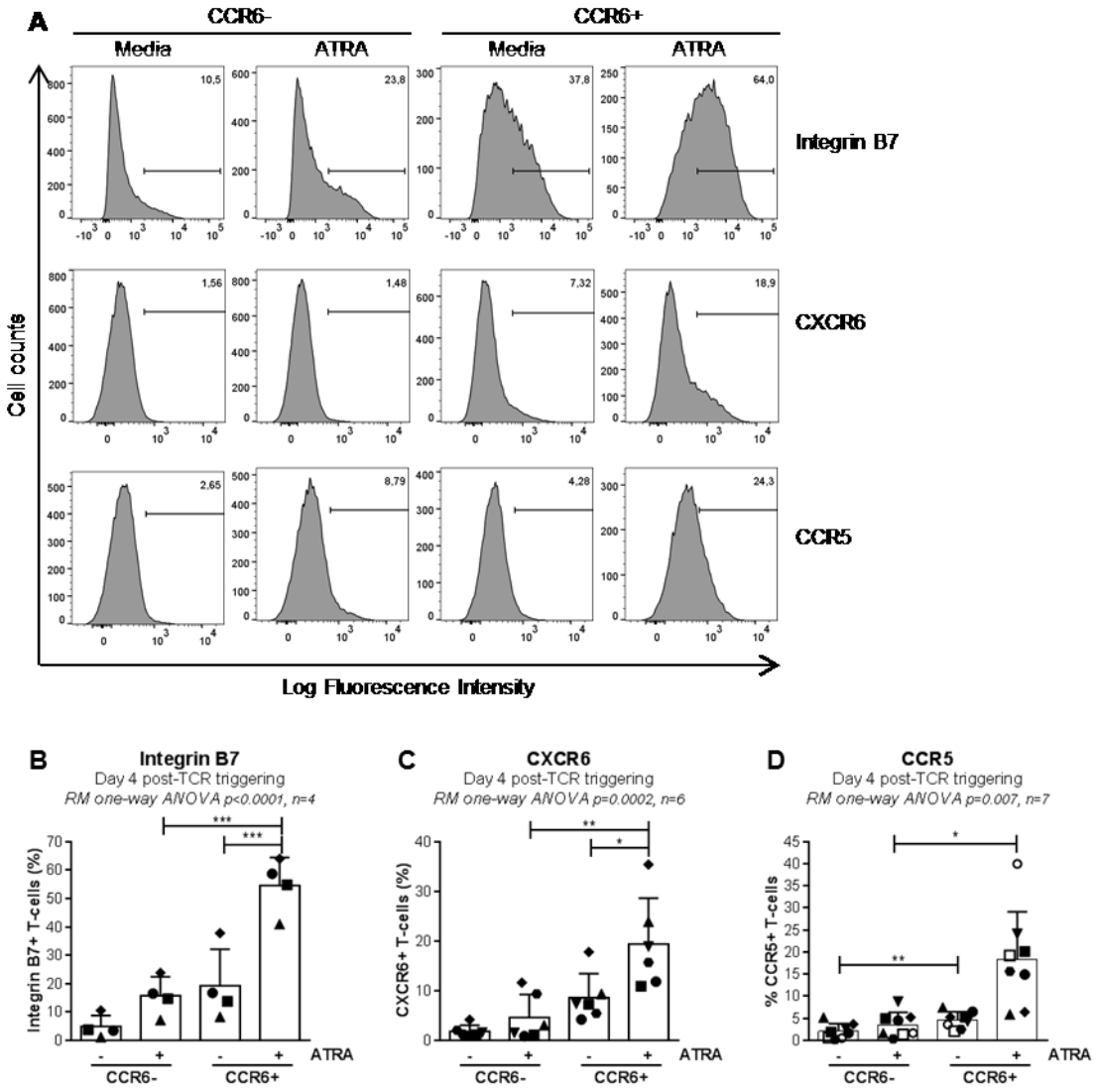
Suppl. Figure 1: Proliferation and IL-2 production of CCR6^{+} *versus* CCR6^{-} T-cells upon exposure to ATRA. (A-B) FACS-sorted memory CCR6^{+} and CCR6^{-} T-cells were loaded with CFSE (0.5 µM) and stimulated *via* CD3/CD28 for five days in the presence/absence of ATRA (10 nM). Shown are **(A)** flow cytometry histograms and **(B)** bar graph representation of CFSE dilution (CFSE^{low}) in n=2 independent donors; each point represent one experimental triplicate (mean±SD of triplicate wells). **(C)** Levels of IL-2 were quantified by ELISA in cell culture supernatants of FACS-sorted memory CCR6+ and CCR6- T-cells at day 4 post-TCR triggering in presence or absence of ATRA. Shown are results from n=2 different donors (mean±SD). **(D-G)** Total memory CD4+ T-cells were stimulated *via* the TCR in the presence or absence of ATRA (10 nM) for 4 days. The expression of CCR6 and CD25 was analyzed by flow cytometry. Shown are histograms from one representative donor for CCR6 expression on total memory T-cells **(D)** and CD25 expression on CCR6+/CCR6- T-cells **(F)**, as well as statistical analysis on n=3 different HIV-uninfected individuals (mean±SEM; each symbol represents one different donor) **(E, G)**. RM one-way ANOVA p-values **(G)** or ordinary one-way ANOVA **(B, C, D),** with the significance of the Tukey's multiple comparisons test, or Paired t-Test p-values **(F)** are indicated on the figures. Each symbol represents one triplicate value **(B)** or one different donor **(C, E, G)**.



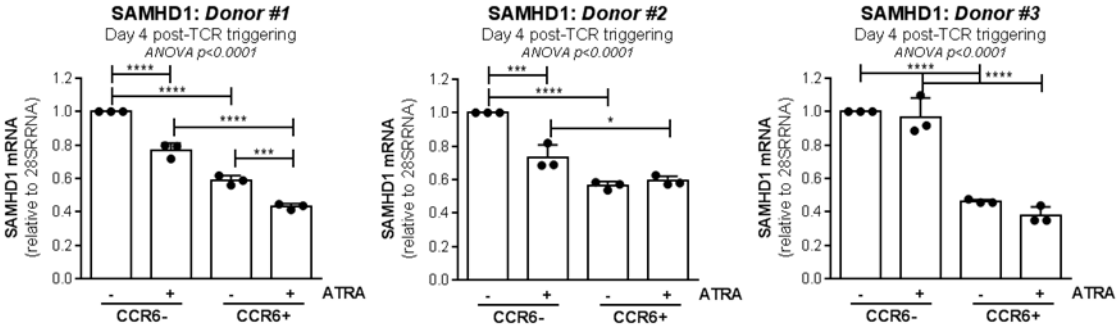
Suppl. Figure 2: TNF-α production and NF-κB DNA-binding activity in CCR6$^+$ *versus* CCR6$^-$ T-cells in the presence or absence of ATRA. Memory CCR6$^+$ and CCR6$^-$ were sorted from HIV-uninfected donors, as described in Figure 1, and stimulated for 4 days *via* CD3/CD28 in presence/absence of ATRA (10 nM). **(A)** TNF-α levels were measured by ELISA (mean±SEM) in cell culture supernatants (n=10). **(B)** Nuclear and cytoplasmic fractions were separated using the BD transfactor extraction kit. The active form of NF-κB-p65 was quantified in the nuclear fractions using an activity-based ELISA assay; the specificity of NF-κB-p65 DNA-binding activity was determined using wild type and mutated NF-κB-p65 duplex competitors (data not shown). Shown is the NF-κB-p65 DNA-binding activity in nuclear extracts (n=3; mean±SD of triplicate wells). RM one-way ANOVA p-values **(A)** or ordinary one-way ANOVA **(B)** with the significance of the Tukey's multiple comparisons test are indicated on the figures. Each symbol represents one different donor **(A) or** one triplicate value **(B)**.


CCR6n
CCR6p
RORC expression (log2)
ILMN_1734966
ARNTL expression (log2)
ILMN_2405305
NFIL3 expression (log2)
ILMN_1707312
ITGA4 expression (log2)
ILMN_1747062
ITGB7 expression (log2)
ILMN_1777519
CCR9 expression (log2)
ILMN_2337386
CCR6 expression (log2)
ILMN_1660907
CXCR6 expression (log2)
ILMN_1674640
KLF2 expression (log2)
ILMN_1735930
CCL3 expression (log2)
ILMN_1671509
CCL3L1 expression (log2)
ILMN_2218856
CD38 expression (log2)
ILMN_2233783
MAP3K4 expression (log2)
ILMN_1678087
PTPN13 expression (log2)
ILMN_2374687
PPARG expression (log2)
ILMN_1800225
neg
ATRA
Treatment
Donor
1
2
3
4
5
6

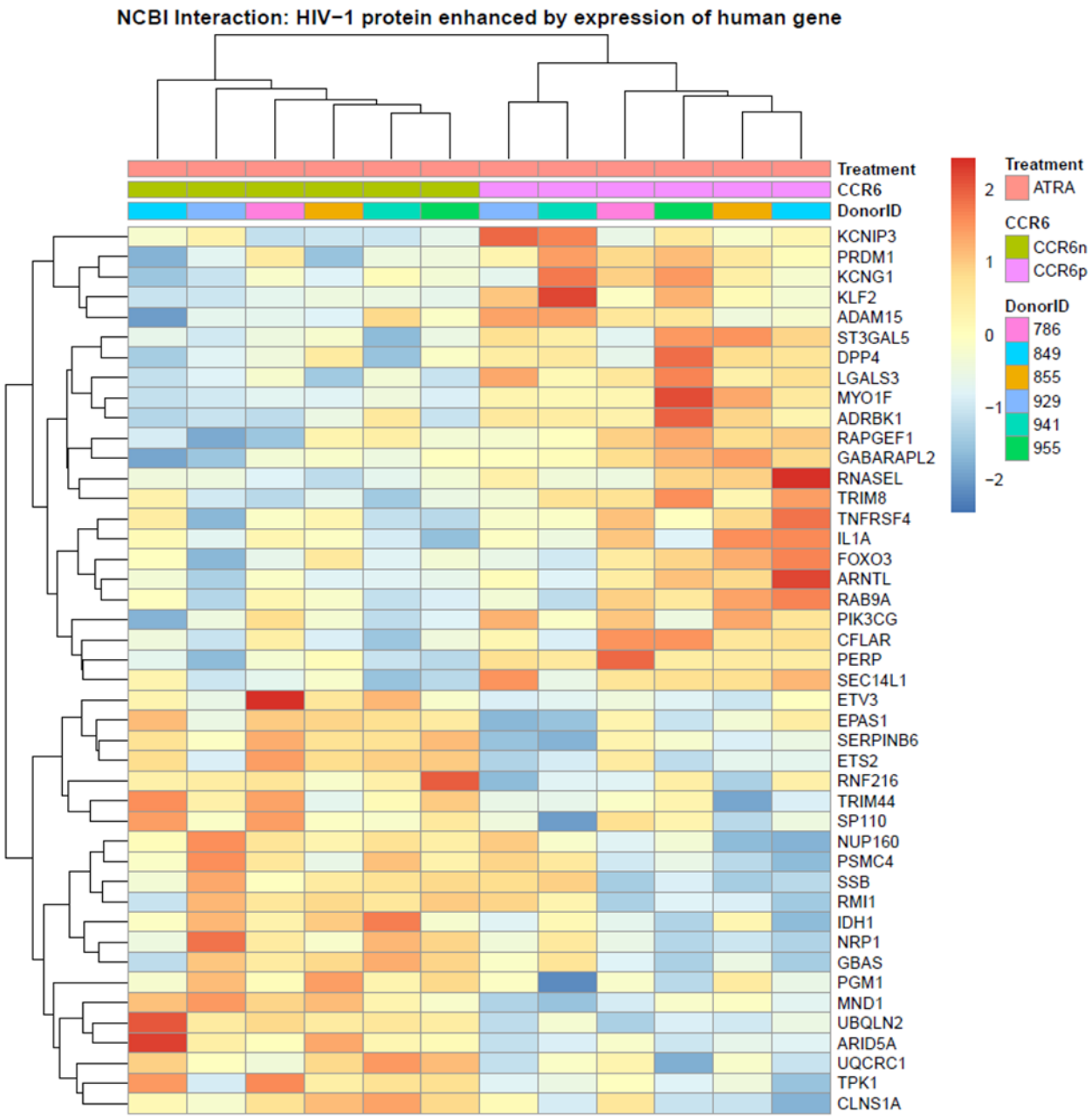
Suppl. Figure 3: ATRA promotes differential gene expression in CCR6$^-$ and CCR6$^+$ T-cells. Genome-wide transcriptional profiles were generated as described in Figure 3. Probe sets differentially expressed between CCR6$^+$ *versus* CCR6$^-$ T-cells were identified based on p-values (<0.05) and FC ratios (1.3 cut-off). Shown are line plots of Log_2 FC values in gene expression of n=15 selected genes. Results from each donor are indicated with a different color code (n=6).



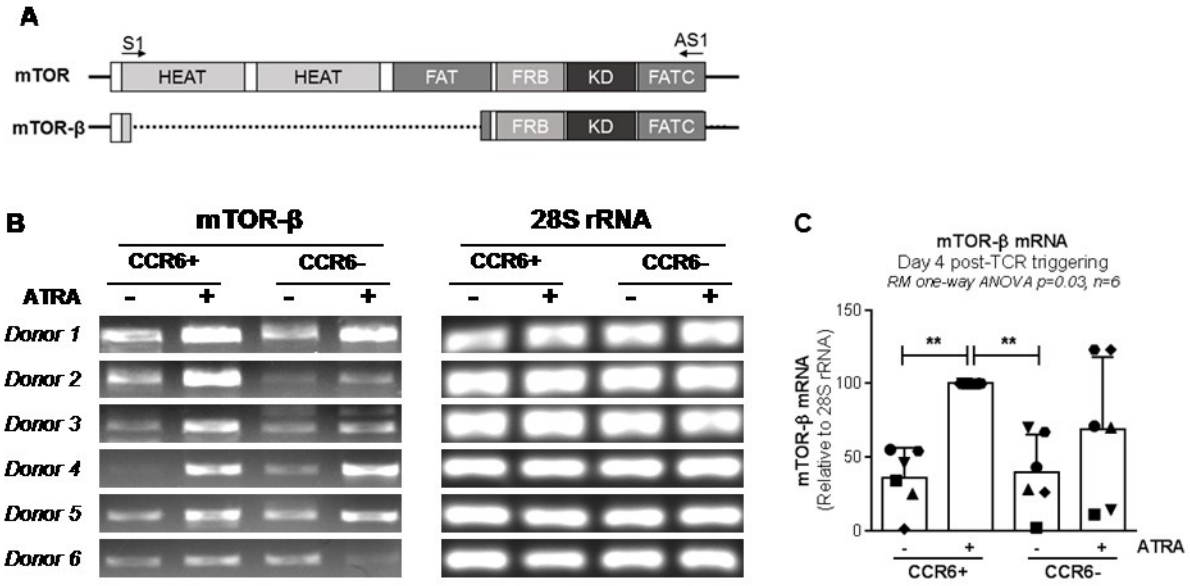
Suppl. Figure 4: ATRA preferentially up-regulates integrin β7, CXCR6, and CCR5 expression on CCR6$^+$ T-cells. Memory CCR6$^+$ and CCR6$^-$ T cells were sorted and stimulated *via* CD3/CD28 in the presence/absence of ATRA (10 nM) for 4 days as described in Figure 1. The expression of integrin β7, CXCR6 and CCR5 was analyzed by flow cytometry. Shown are histograms from one representative donor **(A)** and statistical analysis of integrin β7 **(B**, n=4**)**, CXCR6 **(C**, n=6**)** and CCR5 **(D**, n=7**)** expression in different HIV-uninfected individuals (each symbol represents one different donor; mean ± SEM). **(B, C, D)** RM one-way ANOVA p-values with the significance of the Tukey's multiple comparisons test are indicated on the figures. Each symbol represents one different donor.



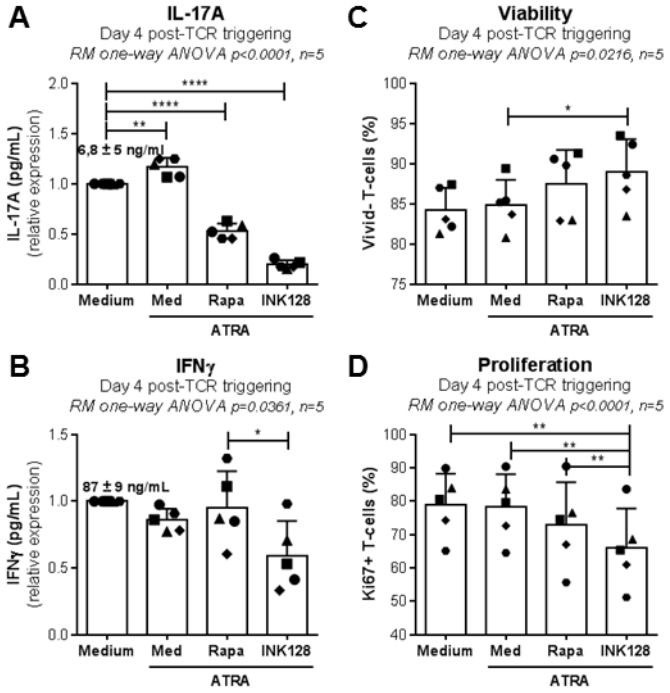
Suppl. Figure 5: CCR6$^+$ *versus* CCR6$^-$ T-cells express lower levels of SAMHD1 mRNA. Memory CCR6$^+$ and CCR6$^-$ T cells were sorted and stimulated as described in Figure 1. Four days post-TCR-triggering, RNA from cells was extracted and SAMHD1 mRNA quantification was performed by SYBR Green real-time RT-PCR. Results were generated with cells from n=3 different HIV-uninfected individuals (mean±SD of triplicate wells). One-way ANOVA p-values and the significance of the Tukey's multiple comparisons test are indicated on the figures. Each symbol represents one triplicate value.



<u>Suppl. Figure 6</u>: Meta-analysis using the NCBI HIV interaction database identifying HIV-1 proteins enhanced by expression of human genes in ATRA-treated T-cells. Genome-wide transcriptional profiles were generated as described in <u>Figure 3</u>. Differentially expressed genes between ATRA-treated CCR6^{+} and ATRA-treated CCR6^{-} ($p < 0.05$, FC cut-off 1.3) were matched to the lists of human genes previously identified to interact with HIV-1 proteins. Shown are significant probes with the smallest p-values for each overlapping gene. Heatmap cells are scaled by the expression level z-scores for each probe individually. Results from each donor are indicated with a different color code (n=6).



Suppl. Figure 7: Increased expression of mTOR-β mRNA in memory CCR6+ T-cells upon exposure to ATRA. (A) Schematic representation of domain organization of the full-length mTOR (2,549 amino-acids) and the mTOR-β splicing isoform (706 amino-acids) and the localization of primers used for semi-quantitative RT-PCR. **(B)** Memory $CCR6^+$ and $CCR6^-$ T cells were sorted and stimulated as described in Figure 1. Four days post-TCR-triggering, cells were harvested and total RNA was extracted. A set of mTOR primers previously described (S1 and AS1; Panasyuk *et al.,* JBC, 2009) to amplify the coding sequence of mTOR-β was used for semi-quantitative RT-PCR reactions. A set of primers specific for 28S rRNA were used in parallel for the normalization of mTOR-β mRNA. **(B)** Shown are levels of mTOR mRNA (left panels; 2,118 Kb) and 28S rRNA (right panels; 100 Kb), as visualized by agarose gel electrophoresis, and **(C)** statistical analysis of mTOR-β expression relative to 28S rRNA quantified by densitometry. Results were generated with cells from n=6 different HIV-uninfected individuals (mean±SEM). **(C)** RM one-way ANOVA p-values and the significance of the Tukey's multiple comparisons test are indicated on the figures. Each symbol represents one different donor.



Suppl. Figure 8: mTOR inhibitors strongly inhibit IL-17A production without interfering with cell viability, cell cycle progression, and IFN-γ production. Memory CD4$^+$ T-cells were sorted and stimulated in the presence/absence of ATRA, rapamycin and/or INK128 for 4 days, as in Figure 4. **(A-B)** Levels of IL-17A and IFN-γ were quantified in cell culture supernatants by ELISA. Shown are IL-17A **(A)** or IFN-γ **(B)** production (mean±SEM; n=5) relative to memory CD4$^+$ T-cells untreated (Medium; considered as 1), with the range of IL-17A and IFN-γ absolute values and paired t-test p-values being indicated. In parallel, cells were stained on the surface with a viability dye and intracellular using Ki67 Abs at day 4 post-stimulation. Shown are statistical analysis of **(C)** cell viability (vivid-) and **(D)** cell cycle progression (Ki67$^+$) (mean±SEM; n=5) . RM one-way ANOVA p-values and the significance of the Tukey's multiple comparisons test are indicated on the figures. Each symbol represents one different donor.

TABLES

Supplemental Table 1: Transcripts differentially expressed by CCR6+ versus CCR6- T-cells upon TCR triggering in the absence of ATRA
Supplemental Table 2: Transcripts modulated by ATRA in CCR6+ T-cells
Supplemental Table 3: Transcripts modulated by ATRA in CCR6- T-cells
Supplemental Table 4: Transcripts differentially expressed by CCR6+ versus CCR6- T-cells upon TCR triggering in the presence of ATRA

View Supplemental data

Supplemental Table 5: Clinical parameters of HIV-infected study participants receiving viral-suppressive antiretroviral therapy (ART).

Participant ID	CD4 counts[#]	CD8 counts[#]	Plasma viral load[&]	Time since infection*	ART	Time of aviremia[$]
ART #1	391	620	<40	165	Kivexa	54
ART #2	581	1060	<40	96	Sustiva, Truvada	5
ART #3	796	399	<40	8	Stribild	1
ART #4	459	545	<40	189	Truvada, Raltegravir	>12
ART #5	616	330	<40	180	Viracept, Truvada	30
ART #6	963	644	<40	123	Prevista, Kivexa, Norvir	111
COL #1	601	819	<40	267	Darunavir, Ritonavir, Raltegravir	58
COL #2	528	501	<40	248	Tivicay	84
COL #3	428	1035	<40	351	Etravirine, Raltegravir, Norvir, Darunavir	109
COL #4	935	581	<40	364	Truvada, Darunavir, Norvir	111
COL #5	439	685	<40	297	Norvir, Raltegravir, Darunavir, Maraviroc	100
COL #6	628	1063	<40	71	Tivicay	51
COL #7	254	689	<40	287	Dolutegravir, Etravirine, Darunavir, Norvir	86
COL #8	137	481	<40	321	Tenofovir, Ritonavir	103
COL #9	512	615	<40	194	Raltegravir, Truvada	103
COL #10	612	1104	<40	271	Reyataz, Complera, Raltegravir	121

[#], cells/µl; [&], HIV RNA copies per ml plasma; *, months; ART, antiretroviral therapy; [$], months; NA, information not available; COL, colonoscopy

Supplemental Table 6: Antibodies used for flow cytometry analysis

	Fluorochrome	Clone	Vendor
CCR6	PE	11A9	BD Pharmingen
CCR5	FITC	2D7/CCR5	(San Diego, CA, USA)
Ki67	FITC	B56	
CD25	BV421	M-A251	
Integrin β7	FITC	FIB504	eBioscience
phospho-mTOR	FITC	MRRBY	(San Diego, CA, USA)
CD56	FITC	MEM188	
CD8	PerCP-Cy5.5	RPA-T8	BioLegend
CD19	PerCP-Cy5.5	HIB19	(San Diego, CA, USA)
CD66b	PerCP-Cy5.5	CG10F5	
CD8	FITC	BW135/80	Miltenyi Biotech
CD19	FITC	LT19	(Auburn, CA, USA)
CXCR6	PE	56811	R&D Systems, (Minneapolis, MN, USA)
HIV-p24	FITC	KC57	Beckman Coulter (Brea, CA, USA)

CHAPITRE 4: METFORMIN TREATMENT IN NON-DIABETIC HIV-INFECTED INDIVIDUALS ON ART (MANUSCRIT #2)

En préparation pour soumission au Journal of Clinical Investigation, section Clinical Medicine

Delphine Planas, Amélie Pagliuzza, Rosalie Ponte, Augustine Fert, Laurence Raymond Marchand, Marta Massanella, Annie Gosselin, Vikram Mehraj, Franck Dupuy, Sylvie Lesage, Eric A. Cohen, Maged Peter Ghali, Jonathan B. Angel, Nicolas Chomont, Jean-Pierre Routy and Petronela Ancuta

Contribution des auteurs:

DP a conçu et effectué les travaux de recherche, analysé les résultats, préparé les figures et a rédigé le manuscrit. AP a effectué les expériences et analysé des données relatives aux mesures des réservoirs du VIH. RP, AF, LRM, MM, AG, VM et FD ont contribué à la conception des études cliniques, à la collecte/préparation d'échantillons et/ou aux mesures de réservoir du VIH. SL et EAC ont fourni des protocoles et ont contribué à la conception de la recherche. MPG a permis l'accès à des échantillons de sang et des biopsies du côlon. JBA a contribué à la conception de l'étude clinique et a recruté des participants. NC a fourni les protocoles et les réactifs, a contribué au design de la recherche sur les réservoirs du VIH et à la rédaction du manuscrit. JPR a conçu l'étude clinique LILAC, a organisé l'accès à des échantillons/informations cliniques, a mis en place des protocoles de recherche clinique et a réalisé le recrutement des participants. PA a conçu le design de l'étude de recherche, dirigé la recherche, analysé les données et a rédigé le manuscrit.

Expériences nécessaires pour compléter le manuscrit:

1. **RNA-Seq dans les cellules totales extraites des biopsies en collaboration avec Genome Quebec.** Les cellules extraites des biopsies de colon collectées avant/après 12 semaines de traitement à la metformine ont été utilisées pour extraire l'ARN total. Les échantillons seront envoyés à Genome Quebec la semaine du 14 mai 2019 pour le séquençage ARN, en utilisant la Technologie Illumina (*NovaSeq6000 S4 PE100 Sequencing lane*, après déplétion de l'ARN ribosomal). Nous prévoyons la réception des résultats avant le 30 juin 2019.

2. **HIV-RNA/DNA Scope en collaboration avec Dr. Jacob Estes (Vaccine and Gene Therapy Institute, Beaverton, OR, USA).** Les biopsies de colon collectées avant/après 12 semaines de traitement à la metformine ont été envoyées à Dr. Estes. L'objectif est ***i)*** de visualiser *in situ* la co-expression de l'ARN/ADN du VIH et du mTOR phosphorylé

et ii) de déterminer des changements induits par le traitement à la metformine dans l'expression de l'ARN/ADN viral et de mTOR phosphorylé *in situ*. Dans des expériences préliminaires, Dr. Estes a validé ce marquage. Ces manips seront réalisées sur des échantillons appariés de n=11 participants. Nous prévoyons la réception des résultats avant août 2019.

Metformin Treatment in Non-Diabetic HIV-Infected Individuals on Antiretroviral Therapy

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Running title: Metformin for HIV remission
Abstract word count: 250 words
Text word count: 12,143 words
Number of figures: 8
Number of supplemental figures: 5
Number of Supplemental Tables: 3
References: 134

ABSTRACT

Background: Although antiretroviral therapy (ART) improves the health of people living with HIV (PLWH), chronic inflammation and residual HIV transcription persist and cause non-AIDS co-morbidities. In this study, we evaluated metformin, a drug used to treat type 2 diabetes, for its ability to reduce immune activation and HIV reservoirs.

Methods: Metformin (500 to 850 mg bid) was administered orally for 12 weeks in n=22 ART-treated PLWH, non-diabetic, on ART >3 years, virally-suppressed >3 months (<40 HIV-RNA copies/ml plasma), and CD4/CD8 ratios ≤0.7. Blood was collected at Baseline, after 12 weeks of metformin (Week 12), and 12 weeks after metformin discontinuation (Week 24) (n=22). Matched sigmoid colon biopsies were collected at Baseline/Week 12 (n=13). The primary outcome was the restoration of CD4 counts and CD4/CD8 ratios. The secondary outcome was the HIV reservoir size reduction. Safety was assessed throughout the study.

Results: Metformin supplementation of ART was well-tolerated. Metformin prompted a subtle reduction in plasma markers of inflammation and gut-barrier impairment. In the blood, the frequency/phenotype of naive/memory $CD4^+$ T-cell subsets and their HIV-DNA/RNA contents were stable under metformin; however, a quantitative viral outgrowth assay revealed reduced replication-competent reservoirs in 3/4 participants. In colon biopsies, metformin significantly reduced $CD4^+$ T-cell infiltration, phosphorylated-mTOR expression in $CCR6^+CD4^+$ T-cells, and HIV-RNA/DNA ratios.

Conclusion: This study revealed metformin-mediated benefits in diminishing immune activation/residual HIV transcription, mainly in the colon, likely by interfering with mTOR activation. This opens the path for future studies aimed at exploring the effects of long-term metformin supplementation for HIV cure/remission strategies.

Trial name and registration: The Lilac study - NCT02659306

Keywords: Metformin, ART, HIV reservoirs, Th17, mTOR

INTRODUCTION

Current antiretroviral therapies (ART) potently suppress HIV-1 replication and transformed HIV infection from a deadly condition into a chronic disease successfully managed on a long-term basis (1). Nevertheless, long-term ART administration in people living with HIV (PLWH) does not eliminate HIV-infected cells (2-6), and rapid viral rebound occurs upon ART interruption (7, 8). In addition, the persistence of HIV reservoirs on ART is associated with chronic inflammation/immune activation, alteration of intestinal mucosal homoeostasis, and metabolic disorders (9). Only ART initiation during early acute stages of infection (Fiebig I stage) prevents gut damages and HIV-related immune activation (10, 11). Subjects treated early during the early acute phase also have a smaller HIV reservoir size compare to subjects treated during chronic infection (12, 13). Weather a small HIV reservoir in early ART-treated individuals is a cause or a consequence of reduced immune activation and inflammation remains unknown. In the HIV/AIDS research field there is a consensus for the need to discover/implement novel adjunctive therapeutic strategies to reduce inflammation/immune activation and to improve the host responses thus enabling HIV reservoir purging toward remission and/or eradication (14-20).

Metabolic disorders observed in PLWH on ART may be linked to antiviral drugs themselves but also to the persistent inflammation/immune activation (21). Specific metabolic changes are required for efficient T-cell activation/differentiation, with oxidative metabolism in naive T-cells switching for aerobic glycolysis during differentiation to overcome the energy demands of immune function and proliferation (22-24). Innate and adaptive immune responses depend on the cellular metabolic state (25). Therefore, metabolic alterations likely affect immune functions and contribute to disease progression and occurrence of non-AIDS comorbidities. In line with the advantage of an active cellular metabolic state for HIV replication, therapies targeting this process need to be investigated.

Changes in T-cell metabolic activity during HIV infection have been associated with viral replication and reservoir persistence, as well as $CD4^+$ T-cell depletion (26). Higher glucose uptake was observed in HIV-infected $CD4^+$ T-cells compared to uninfected cells (27). Increased glucose uptake in $CD4^+$ T-cells is mediated in part by the overexpression of several glucose transporters and glycolytic enzymes in response to HIV-1 exposure (28, 29), indicative that HIV infection itself influences the cellular metabolism to the benefit of viral replication. Among memory $CD4^+$ T-cell populations, HIV preferentially infects cells expressing high level of glucose transporter 1 (GLUT1) which exhibit a high glycolytic flux (30). Recently, it was demonstrated that HIV targets for infection memory $CD4^+$ T-cell subsets with high metabolic activity (31). Indeed, $CD4^+$ T helper cell subsets differ in they metabolic status and their permissiveness to HIV infection (32). Particularly Th17 cells distinguish from Th1 and Th2 cells as they express high levels of GLUT1, exhibit an active glucose metabolism (33), selectively express the hypoxia-inducible factor-1 (HIF-1α) a key mediator of glucose metabolism (34), and are highly permissive to HIV infection (reviewed in (20, 35)). Finally, inhibition of glycolysis reduces viral replication *in vitro* (26, 31).

The mechanistic target of rapamycin (mTOR) is a key regulator of energy balance and metabolism at the cellular level (36, 37). mTOR is a conserved serine/threonine kinase that forms two complexes with different protein components [mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2)] and acts as a nutrient sensor. The mTORC1 stimulates nucleotide synthesis, glucose uptake and glycolysis, while it inhibits the autophagy process. The mTORC2 also regulates glucose metabolism, as well as cell survival and cytoskeletal organization (38). Of note, mTOR was reported to be a positive regulator of HIV replication by acting at the level of viral entry (39) and transcription (40, 41), as well as indirectly *via* the modulation of autophagy (42, 43). Of particular importance, our group previously reported that phosphorylated mTOR is mainly expressed by $CCR6^+$ Th17 cells infiltrating the colon or exposed *in vitro* to retinoic acid, a gut-homing promoter, thus, providing a molecular explanation for the preferential HIV infection of gut-homing Th17 cells (44). The gut-associated lymphoid tissues (GALT) are enriched in Th17 cells that represent an important site for HIV persistence

during ART (45). Noteworthy, a faction of Th17 cells are long-lived (46) and contribute to HIV persistence on ART-treated PLWH (45, 47, 48).

In addition to $CD4^+$ T-cells, metabolic alterations were reported in HIV-specific $CD8^+$ T-cells during primary HIV infection and linked to persistent immune activation leading to immune exhaustion (49-51). Of note, an asymmetric inheritance of mTORC1 during $CD8^+$ T-cells differentiation was also reported, consistent with the differential expression of mTOR in functionally distinct T-cell subsets (52). Conversely, elite controllers present poly-functional HIV-specific cytotoxic T-lymphocytes and a smaller HIV reservoir compared to ART-treated PLWH (53-56). Studies in mice demonstrated that glucose metabolism drives $CD8^+$ T-cells fate and cytotoxic capacities (57). Finally, the inhibition of mTORC1 signaling pathway with rapamycin is associated with improved virus-specific cytotoxic T-cells capacity in mice (58).

Metformin (dimethylguanide) has well-established immuno-metabolic regulator properties and is one of the widely used drugs in clinics for type II diabetes (59). This drug isolated from the French lilac presents limited side effects. Metformin mainly targets the liver, kidney and intestines to limit hepatic glucose production and intestinal glucose absorption, while improving glucose uptake in peripheral tissues. Metformin inhibits the mitochondrial complex I enzyme which decreases ATP production. The increased AMP:ATP ratio activates the energetic sensor AMP-activated protein kinase (AMPK), leading to an inhibition of mTORC1 signaling pathway (60, 61). Metformin has been shown to improve the life-span extension in worms, rodents and diabetic individuals (62) and to reduce the age-related diseases including cancer, inflammation, cardiovascular, and Alzheimer disease (63). Aging-relevant actions of metformin also include the modulation of microbiota composition and microbial metabolism (64-69). Of particular notice, metformin was reported to inhibit Th17 polarization and, this way, it exerts a positive effect on decreasing tumor growth (70) and autoimmunity symptoms (71).

Considering the role of mTOR in the positive regulation of HIV transcription/replication (39-41, 44) and the well-established ability of metformin to inhibit mTOR-mediated Th17 effector functions (70, 71), in this manuscript, we explored the benefits of metformin supplementation of ART in PLWH, in view of its recommendation in HIV remission/cure strategies. This non-randomized pilot clinical study (The Lilac Study - NCT02659306) was performed on non-diabetic virally-suppressed ART-treated PLWH. This study revealed metformin-mediated benefits in controlling immune activation and residual HIV transcription under ART especially in the colon, likely via the metformin-mediated decrease in mTOR activation. These results support the need for future clinical studies exploring the long-term metformin supplementation of ART in HIV remission/cure strategies.

RESULTS

Clinical outcomes. Detailed inclusion criteria for study participants, study objectives and safety outcomes are included in a recent publication by our group (72). The study design is schematically represented in Figure 1. For this study, we had access to blood [before metformin treatment (Baseline), after 12 weeks of metformin treatment (Week 12), and 12 weeks after metformin discontinuation (Week 24); n=22] and/or matched sigmoid colon biopsies (Baseline and Week 12; n=13) from non-diabetic virally-suppressed ART-treated PLWH (Supplemental Table 1). The first study outcome was to determine whether 12 weeks of metformin treatment promotes the normalization of CD4 counts and CD4/CD8 ratios (72). In contrast to our expected outcome, absolute CD4 and CD8 counts, as well as CD4/CD8 ratios, remained stable between Baseline, Week 12, and Week 24 (Supplemental Figure 1).

Metformin promotes subtle changes in plasma markers of systemic inflammation and gut-barrier dysfunction. Multiple plasma markers indicative of systemic inflammation [soluble CD14 (sCD14), lipopolysaccharide-binding protein (LBP)] and mucosal barrier dysfunction [intestinal fatty acid-binding protein (I-FABP)] were previously linked to HIV disease progression (73-76). To get first insights into the benefit of metformin treatment, plasma

samples were used to quantify soluble factors by ELISA and R&D Systems Multiplex Assay. Quantifications by ELISA revealed no changes in LBP and I-FABP levels, while a statistically significant decrease in sCD14 levels was observed at Week 24 *vs.* Baseline (Figure 2A). Quantifications by multiplex assay of plasma markers associated to inflammation and immune activation revealed multiple changes, with top up-regulated and down-regulated chemokines at Week 12 *vs.* Baseline identified as CCL11/eotaxin (binding to CCR3, a reported minor HIV receptor (77, 78)) and CXCL10/IP-10 (binding to CXCR3, associated with poor HIV/SIV prognosis (79)), respectively (Figure 2D-E-F). Finally, CCL20/MIP-3α (binding to CCR6, overexpressed during HIV infection (80)), was found down-regulated by metformin (Week 12 *vs.* Baseline) in the majority of study participants (Figure 2D, G). In conclusion, 12 weeks of metformin treatment promoted a subtle decrease in the expression of markers associated with systemic inflammation and an increase of CCL11 in ART-treated PLWH.

Effects of metformin treatment on HIV reservoirs size in blood CD4$^+$ T-cells. The secondary study outcome was to determine whether metformin treatment decreases the HIV reservoir size. To this aim, we performed virologic measurements in total CD4$^+$ T-cells isolated from the peripheral blood at Baseline, Week 12 and Week 24 (Figure 1). The quantification of integrated HIV-DNA demonstrated no significant changes between Baseline and Week 12/Week 24 (Figure 3A). To assess transcription-competent HIV reservoirs, we quantified unspliced (US) HIV-RNA (Figure 3B); the frequency of cells harboring inducible multiply spliced (MS) HIV-RNA by Tat/Rev induced limiting dilution assay (TILDA) upon activation *in vitro* (Figure 3C). Results in Figures 3B-C demonstrated that HIV-RNA *ex vivo* and upon stimulation *in vitro* were detectable in the majority of donors, with important donor-to-donor variations, but no significant changes induced by metformin treatment. To measure replication-competent HIV reservoirs, a quantitative viral outgrowth assay (QVOA) was performed on n=7 participants for which sufficient numbers of total CD4$^+$ T-cells were available at the 3 time-points (*i.e.,* 6-8.10^{e6} CD4$^+$ T-cells) (Figure 3D). In 3/7 participants (LILAC #20, #16 and #17; Supplemental Table 1), HIV outgrowth was not detected at any time-point (data not shown). In 4/7 participants, the number of infectious units per millions (IUPM) was determined with precision (LILAC #11, #15,

#21, #22; Supplemental Table 1; Figure 3D). Noteworthy, in 3/4 participants the IUPM number decreased after 12 weeks of metformin and increased back to basal levels at Week 24 (LILAC #11, #15, #22, Supplemental Table 1); while in 1/4 participants, the IUPM number decreased at Week 24 vs. Baseline (LILAC # #21; Supplemental Table 1; Figure 3D). In conclusion, while the PCR/RT-PCR-based assays revealed no changes upon 12 weeks of metformin treatment, the QVOA reveal that, in a fraction of participants, metformin decreases the size of replication-competent HIV reservoirs.

Spearman correlation (SC) and Linear regression (LR) models were used to study the correlation between Baseline and Week 12/Week 24 for integrated HIV-DNA, US HIV-RNA *ex vivo*, and inducible MS HIV-RNA (depicted in Figure 3A-C). Results in Supplemental Figure 2 reveal a positive correlation for these virological parameters Baseline *vs*. Week 12 and Baseline *vs*. Week 24, indicative of limited changes promoted by metformin in the blood compartment.

Effects of metformin on the frequency/phenotype of blood naive/memory T-cell subsets. Frozen PBMCs of n=22 participants (Supplemental Table 1), collected at Baseline, Week 12 and Week 24, were first analyzed for the frequency of naive (N, $CD45RA^{+}CCR7^{+}CD27^{+}$), central memory (CM, $CD45RA^{-}CCR7^{+}CD27^{+}$), transitional memory (TM, $CD45RA^{-}CCR7^{-}CD27^{+}$), effector memory (EM, $CD45RA^{-}CCR7^{-}CD27^{-}$), and terminally differentiated (TD, $CD45RA^{+}CCR7^{-}CD27^{-}$) within $CD4^{+}$/$CD8^{+}$ T-cell subsets (Supplemental Figure 3A). The frequency of naive and memory subsets within $CD4^{+}$ T-cells (Supplemental Figure 3B) and $CD8^{+}$ T-cells (Supplemental Figure 3C) was similar at Baseline, Week 12 and Week 24. Further, the expression of the HIV co-receptor CCR5 and the immune checkpoint molecules PD-1, LAG3, and TIGIT on naive and memory $CD4^{+}$/$CD8^{+}$ T-cell subsets was stable between Baseline and Week 12 or Week 24 (Supplemental Figure 4). Finally, the expression of tissue-specific homing integrins α4β7, α4β1 and αEβ7 on naive/memory $CD4^{+}$ T-cell subsets was stable between Baseline and Week 12 or Week 24 (Supplemental Figure 5). In conclusion, these results demonstrate that metformin did not promote significant changes in the frequency of naive/memory $CD4^{+}$/$CD8^{+}$ T-cell subsets

circulating in the blood, nor in their expression of functional markers linked to HIV permissiveness, T-cell exhaustion, and tissue-specific homing.

Metformin reduces the frequency of CD4$^+$ T-cells in the colon, with a minor impact on classical activation markers. It is well established that T-cells present in the GALT are more activated compared to blood cells and that HIV infection increases mucosal T-cell activation (81). Moreover, GALT CD4$^+$ T-cells represent key targets for HIV replication/persistence and their persistent depletion in ART-treated PLWH is considered a major cause for mucosal barrier impairment, chronic immune activation, and occurrence of non-AIDS co-morbidities (82-89). Furthermore, the intestine is one principal organ targeted by metformin (90). For all these reasons, matched blood and colon biopsies were collected at Baseline and. Week 12 (LILAC #1-13; Supplemental Table 1). Freshly isolated CD4$^+$/CD8$^+$ T-cells were analyzed for the expression of multiple parameters (Figure 4A**)**. Of note, metformin significantly reduced the frequency of CD4$^+$ T-cells in the colon but not in the blood (Figure 4B-C). Both memory CD4$^+$ and CD8$^+$ T-cells expressed higher level of classical activation markers CD38 and HLA-DR in the colon *vs.* blood (Figure 4D-E). Metformin did not significantly interfere with the frequency of memory CD4$^+$ (Figure 4D) and CD8$^+$ (Figure 4E) T-cells co-expressing CD38 and HLA-DR neither in blood nor in the colon, as reflected by the Week 12 *vs*. Baseline comparison. Nevertheless, an important inter-individual heterogeneity was observed, with the frequency of colon-infiltrating CD38$^+$HLA-DR$^+$CD4$^+$ T-cells and CD38$^+$HLA-DR$^+$CD8$^+$ T-cells decreasing robustly in 9/13 participants and 8/13 participants, respectively, Week 12 *vs.* Baseline (Figure 4D-E). In conclusion, metformin treatment was associated with a significant decrease in the frequency of colon-infiltrating CD4$^+$ T-cells and, in a fraction of participants, colon-infiltrating CD4$^+$/CD8$^+$ T-cells were less activated.

Metformin decreases expression of phosphorylated mTOR in colon-infiltrating but not blood memory CD4$^+$ T-cells. Metformin indirectly inhibits mTOR signaling pathway *via* the activation of AMPK (91). Recent reports by our group and others demonstrated the key role played by mTOR in the positive regulation of HIV transcription/replication (39, 40, 44, 92). Thus, the

expression of the active phosphorylated form of mTOR (p-mTOR) was analyzed in blood and colon-infiltrating $CD4^+$/$CD8^+$ T-cells, using the gating strategy depicted in Figure 4A. A significant decrease in the expression of p-mTOR was observed in both colon-infiltrating $CD4^+$ T-cells (median: 20.4% *vs*. 15.5%; p=0.064) and $CD8^+$ T-cells (median: 17.6% *vs*. 8.27%, Baseline *vs*. Week 12; p=0.039) (Figure 5A). Compared to the colon, p-mTOR expression was remarkably lower in the blood $CD4^+$/$CD8^+$ T-cells, with no significant differences observed upon metformin treatment (Figure 5A).

Considering our previous findings that p-mTOR expression is significantly higher in colon-infiltrating memory $CCR6^+$ *vs*. $CCR6^-CD4^+$ T-cells (44), we further investigated the effect of metformin separately in these subsets. Results in Figure 5B-C demonstrate that at Baseline p-mTOR levels are indeed the highest in colon $CCR6^+$ compared to colon $CCR6^-$ and blood $CCR6^+$/$CCR6^-CD4^+$ T-cells. Of particular importance, metformin treatment resulted in a significant decrease in p-mTOR expression on memory $CCR6^+CD4^+$ T-cells (median: 13% *vs*. 7.87%, Baseline *vs.* Week 12; p=0.0129) (Figure 5C). Exceptionally, in 2/14 participants, the frequency of p-$mTOR^+CCR6^+CD4^+$ T-cells increased after 12 weeks of metformin (LILAC#6; 6.08% *vs*. 14.7%; LILAC#8 4.15% *vs*. 5.38%). Despite the low expression of p-mTOR in $CCR6^-$ $CD4^+$ T-cells, metformin also significantly reduced the frequency of colon infiltrating p-$mTOR^+CCR6^-CD4^+$ T-cells (median: 4.07% *vs*. 1.44%, Baseline *vs*. Week 12; p=0.0139) (Figure 5C).

To get further insights into functional changes promoted by metformin, blood/colon cells were stained in parallel for the expression of the cell death regulator Bcl2 (93), the HIV restriction factor SAMHD1 (94), the HIV co-receptor CCR5 (95), and the gut-homing and HIV binding molecule integrin β7 (96). Metformin treatment significantly increased the expression of Bcl-2 in colon-infiltrating $CCR6^+$ T-cells (median: 24.7% *vs.* 38.3%, Baseline *vs.* Week 12; p=0.039) (Figure 6A). At Baseline, SAMHD1 was expressed at lower levels in blood $CCR6^+$ (median: 5.34 %) compared to blood $CCR6^-CD4^+$ T-cells (median: 8.06%) and at higher levels in colon-infiltrating $CCR6^+$ (median: 15.55 %, n=8) compared to blood $CCR6^+$ T-cells (median: 5.34 %,

n=8) (Figure 6B). Of note, blood/colon-infiltrating CCR6$^-$ T-cells expressed similar levels of SAMHD1 (Figure 6B). Of particular interest, metformin significantly increased levels of SAMHD1$^+$CCR6$^+$ T-cells in blood (median: 5.35% *vs*. 11%; p=0.007) (Figure 6B), with a similar tendency that did not reach statistical significance being observed in the colon, with 6/8 participants showing a robust increase in the frequency of colon SAMHD1$^+$CCR6$^+$CD4$^+$ T-cells upon metformin treatment (median: 24.7% *vs.* 38.3%) (Figure 6B). Finally, no significant changes between Baseline and Week 12 were observed in terms of frequency of CCR6$^-$/CCR6$^+$ T-cells expressing CCR5 (Figure 6C) and integrin β7 (Figure 6D), neither in blood nor in the colon. Of note, the highest frequency of CCR6$^+$CCR5$^+$ and CCR6$^+$integrinβ7$^+$CD4$^+$ T-cells was observed in the colon (Figure 6C-D), in line with the knowledge that cells in the colon are preferential sites of HIV replication (reviewed in (20, 35)).

Metformin is associated with decreased HIV transcription in colon-infiltrating memory CD4$^+$ T-cells. Considering that significant changes upon metformin supplementation of ART were only observed in colon CD4$^+$/CD8$^+$ T-cells, changes in the size of HIV reservoirs in memory CD4$^+$ T-cells from colon biopsies were investigated at Baseline *vs.* Week 12. Consistent with results obtained in blood (Figure 3), levels of total HIV-DNA were stable in memory CD4$^+$ T-cells from the colon between Baseline and Week 12 (Figure 7A). Of note, donor-to-donor variations were observed in HIV-DNA reservoirs in colon memory CD4$^+$ T-cells, with a decrease in 8/13 participants and an increase in 5/13 participants between Baseline and Week 12 (Figure 7A).

We further evaluated the effect of metformin on HIV transcription. Considering the stochastic biopsy sampling, HIV transcription in colon-infiltrating memory CD4$^+$ T-cells was evaluated as the HIV-RNA/DNA ratio upon RT-PCR/PCR quantification performed on RNA/DNA dually extracted from the same cell pellets. The HIV-RNA/DNA ratios are depicted in Figure 7B, with Baseline values considered 1 for each participant. Of note, for 8/13 participants, a 15-92% decrease in HIV transcription was observed (LILAC #1, #2, #3, #4, #5, #7, #8, #12), while for 4/13 participants an important increase in HIV transcription occurred (LILAC #6, #9, #11, #13) (Figure 7B). For 1/13 participants, HIV transcription remained stable between Baseline and

Week 12 (LILAC #10, Supplemental Table 1) Thus, in the majority of study participants, metformin exerted a positive effect in controlling residual HIV transcription during ART in the colon.

HIV transcription in colon-infiltrating memory CD4$^+$ T-cells positively correlated with the co-expression of HLA-DR, CD38, CCR6, CCR5 and p-mTOR. Considering the established link between HIV replication and cell activation, as well as CCR6, CCR5 and p-mTOR expression (reviewed in (20, 35)), the correlation between HIV-RNA/DNA ratios (measured in Figure 7B) and the frequency of colon-infiltrating memory CD4$^+$ T-cells co-expressing these markers (measured in Figures 4D, 5C, 6C) was studied using SC and LR models. At baseline, HIV-RNA/DNA ratios in colon-infiltrating memory CD4$^+$ T-cells correlated with the frequency of cells co-expressing HLADR/CD38 (r=0.69; p=0.0084), CCR6/p-mTOR (r=0.49; p=0.08), and CCR6/CCR5 (r=0.57; p=0.04). (Figure 8A). Of note, no significant association was observed between the levels of HIV transcription (reflected by the HIV-RNA/DNA ratios) and those of different markers of microbial translocation (sCD14, LBP) (data not shown). In contrast to Baseline, no significant correlations were observed between these parameters at Week 12 (Figure 8B). In conclusion, HLA-DR, CD38, CCR6, CCR5, and p-mTOR expression predicts residual HIV transcription under ART in colon-infiltrating CD4$^+$ T-cells.

Finally, while total HIV-DNA levels in colon-infiltrating CD4$^+$ T-cells (measured in Figure 7A) positively correlated between Week 12 and Baseline, HIV transcription reflected by the HIV-RNA/DNA ratios (measured in Figure 7B) failed to correlate between Week 12 and Baseline (Figure 8C). The later results are in line with the metformin-mediated reduction in residual HIV transcription but not HIV-DNA reservoir in the colon of ART-treated PLWH.

DISCUSSION

In this manuscript, we report results from a non-randomized pilot clinical trial, LILAC NCT02659306, in which metformin was administered for 12 weeks in ART-treated PLWH (72). Metformin supplementation of ART was safe/well-tolerated and resulted in subtle immunological/virological changes in the expression of plasma markers of immune activation/gut-barrier integrity and in the frequency/phenotype of peripheral blood $CD4^+$/$CD8^+$ T-cell subsets. Noteworthy, investigations on colon biopsies revealed that metformin treatment had a positive impact by decreasing the frequency of total and activated $CD4^+$/$CD8^+$ T-cells, reducing the p-mTOR expression in $CCR6^+$ T-cells, and downsizing transcription-competitive HIV reservoirs in colon $CD4^+$ T-cells. Considering the results of this pilot study, the metformin supplementation of ART may represent a new safe/sell tolerated and simple strategy to be exploited in view of achieving the goal of immunological restoration and HIV reservoir purging in PLWH.

The implementation of adjunctive therapeutic strategies aimed at HIV remission/cure requires the identification of immunomodulators able to limit HIV residual transcription/replication in $CD4^+$ T-cells and boost $CD8^+$ T-cells cytotoxic functions require active investigations (16, 20, 97, 98). Metformin, an AMPK activator and subsequent mTOR inhibitor (60, 61), is a drug successfully used for the treatment of type II diabetes (59) and proved efficacy in cancer therapy (63). Considering metformin-mediated mTOR activity inhibition in Th17 cells (70) and the role of mTOR in promoting HIV replication/persistence in these cells (20, 44), in this study we evaluated the beneficial effects of ART supplementation by metformin. The primary outcome of the study was the restoration of CD4 counts and CD4/CD8 ratios, while the secondary outcome was the HIV reservoir size reduction. To this aim, multiple clinical, immunological and virological measurements were performed on plasma and T-cells from peripheral blood and matched sigmoid colon biopsies in a cohort of ART-treated PLWH.

We first investigated the ability to metformin supplementation to reduce plasma levels of inflammation/activation markers and gut-barrier dysfunction. In addition to decreased levels of sCD14 at Week 24, indicative of reduced systemic inflammation, metformin treatment was associated with subtle changes in multiple soluble factors, including an increase in CCL11/eotaxin and a decrease in CXCL10/IP-10 and CCL20/MIP-3α at Week 12. These chemokines play key roles in regulating immune cell trafficking and innate/adaptive immune responses (99). CCL11 binds to CCR3, which is expressed on HIV permissive T-cells (100), represents a minor receptor for HIV entry (77, 78), and acts as a CCR5 antagonist (101). Thus, CCL11 up-regulation by metformin may limit CCR3/CCR5-mediated viral entry *in vivo* (101, 102). Of note, CCL11 overexpression is associated with aging (103) and cannabis use (104)). There is documented interaction between diabetes drugs and cannabis use (105), but whether study participants with the highest CCL11 levels are cannabis users remains to be determined. In contrast to CCL11, CXCL10 levels were decreased in the majority of participants, with some outliers for which plasma CXCL10 levels increased. Considering the pro-inflammatory potential of CXCL10 and its identification as a marker of disease progression (79), such a decrease may limit HIV transcription and inflammation (106). The small decrease observed in CCL20 (80) and IL-6 (107) point to the same scenario or reduced inflammation under metformin. Despite these encouraging tendencies toward decrease in plasma levels of well-established disease progression markers, the primary study objective was not reached, since CD4 counts and CD4/CD8 ratios remained stable upon metformin treatment.

In order to evaluate metformin-mediated changes in the size of HIV reservoirs in blood $CD4^+$ T-cells, we used several assays including PCR/RT-PCR quantification of integrated HIV-DNA and HIV-RNA *ex vivo*, TILDA, and QVOA. Levels of integrated HIV-DNA and HIV-RNA, as well as the frequency of cells carrying inducible MS HIV-RNA remained stable between Baseline, Week 12 and Week 24. These results are in line with the well-described stability of HIV reservoirs under ART and the difficulty to perturbed HIV reservoirs upon short time interventions (4, 19, 108, 109). However, results from the QVOA revealed, for a few donors (3/4 showing positive IUPMs at Baseline; n=7 tested in all), a decreased frequency of cells carrying replication-competent

proviruses after metformin treatment. The discrepancy between PCR/RT-PCR-based assays and functional assays is consistent with the literature (14, 110). In contrast to "gold-standard" QVOA set up by Siliciano's group (111, 112), our QVOA does not include feeder cells, and therefore, IUPM differences we observed may be linked as well to metformin-mediated changes in the metabolic status of the cells that may be less permissive to cell-to-cell HIV transmission in culture. Although we acknowledge the limit of our QVOA results generated only with cells from 4 participants, one cannot oversee the message emerging that functional assays are more informative that PCR/RT-PCR-based assays. Therefore, future studies should prioritize QVOA measurements when evaluating the performance of immunomodulatory adjuvant therapies.

The gut mucosa is the most important site of T-cells activation and also the portal site of HIV entry (113, 114). It is well documented that gut mucosa contain the majority of HIV reservoirs T-cells (115). Previously, we demonstrated that colon-infiltrating $CCR6^+$Th17, express the highest levels of phosphorylated mTOR (44). Therefore, in this study we also investigated the effect of metformin treatment at the intestinal level, particularly in the sigmoid colon. Our main findings included ***i)*** decreased infiltration of memory $CD4^+$ T-cells in the colon, despite no significant changes in HLA-DR/CD38 expression on colon-infiltrating $CD4^+$/$CD8^+$ T-cells; ***ii)*** decreased mTOR phosphorylation and increased Bcl-2 expression in colon-infiltrating $CCR6^+CD4^+$ T-cells; and ***iii)*** decreased HIV-RNA/DNA ratios in colon-infiltrating memory $CD4^+$ T-cells. These findings point to the beneficial effect of metformin in decreasing colon inflammation, improving cell survival, and decreasing residual HIV transcription under ART, likely *via* mechanisms involving a decrease in mTOR activation. Indeed, cell survival is regulated by cell metabolism, with mTOR being reported to inhibit Bcl-2 expression (116).

The quantification of total HIV-DNA in matched blood/colon memory $CD4^+$ T-cells shown again the stability of HIV reservoirs. Nevertheless, HIV transcription reflected by the HIV-RNA/DNA ratios in colon memory $CD4^+$ T-cells was remarkably decreased in 8/13 study participants. This significant effect could be the direct consequence of a reduced mTOR phosphorylation,

consistent with the role of mTOR in the positive regulation of HIV transcription/latency in $CD4^+$ T-cells (40, 41). In contrast, an increase in HIV transcription was observed in colon-infiltrating memory $CD4^+$ T-cells of 3/13 participants. Of note, these 3 participants the ART regimen included the integrase inhibitor dolutegravir, previously described to significantly increased metformin plasma exposure but well-tolerated with dose adjustments of metformin (117). This aspect was taken in consideration in this clinical trial and metformin doses were adjusted at 500 mg bid. Noteworthy, studies mainly investigated the pharmacokinetic of metformin and dolutegravir interaction and the potential deleterious effect on glycemia and renal function, but did not investigated the interaction between dolutegravir and metformin on the GALT of PLWH. Our results point to the necessity to avoid the co-administration of metformin with dolutegravir, as it was proposed recently (118).

Other observations in colon-infiltrating $CCR6^+CD4^+$ T-cells that did not reach statistical significance but were remarkable in a subset of participants included an increased SAMHD1 expression induced by metformin in colon-infiltrating $CCR6^+CD4^+$ T-cells. Previous studies reported that, in the blood, SAMHD1 is expressed at higher levels in $CCR6^-$ *vs.* $CCR6^+CD4^+$ T-cells, thus providing an explanation for the relative resistance of $CCR6^-$ T-cells to HIV infection (119). In our study, whereas in the blood a similar tendency was also observed, in the colon, SAMHD1 expression was surprisingly higher in colon-infiltrating $CCR6^+$ *vs*. $CCR6^-CD4^+$ T-cells. We acknowledge the fact that the SAMHD1 Abs used recognize total but not the phosphorylated inactive form of SAMHD1 (120). A direct link between mTOR and SAMHD1 is not described in the literature, but SAMHD1 is well-established to play a role in cell metabolism by decreasing the pool of intracellular dNTPs (120)(94)(121). Metabolically active cells express low level of SAMHD1, therefore creating an environment in favor for RNA synthesis which also favors HIV reverse transcription (31). Additionally, it was recently demonstrated that SAMHD1 suppress HIV-LTR transcription (122). Thus, increased SAMHD1 expression in colon $CCR6^+$ T-cells may result in decreased HIV transcription. In this sense, increased SAMHD1 expression after metformin administration could reflect a reduction in the metabolic activity at the cellular

level, in line with the decreased expression of phosphorylated mTOR in colon-infiltrating $CCR6^{+}CD4^{+}$ T-cells.

Overall, our results highlight the importance of investigating the GALT, and in particular intestinal Th17 cells, during clinical intervention aimed at HIV remission/cure. Consistent with previous studies by our group demonstrating that gut-homing Th17 cells exhibit a transcriptional program prone to HIV replication/persistence (reviewed in (20, 35)), in this study we confirmed superior expression of the activation markers HLADR/CD38, the HIV co-receptor CCR5, the gut-homing integrin β7, and the metabolic regulator mTOR by colon-infiltrating $CCR6^{+}$ *vs.* $CCR6^{-}$ T-cells. Our finding that mTOR phosphorylation in $CCR6^{+}$ T-cells correlated positively with HIV transcription in the colon, and that metformin significantly reduce these two parameters, support previous finding showing that mTOR boosts HIV transcription (40, 41). Another reason for prioritizing investigations in the GALT during clinical trials is provided by a recent study highlighting the existence of distinct mechanisms controlling HIV latency in cells form the blood *vs.* gut tissues (123).

In conclusion, this pilot study reveals metformin-mediated benefits in controlling inflammation, in part via mTOR regulation, that could represent an interesting new Th17-specific therapeutic strategies. Our results highlight the important heterogeneity between blood and colon in terms of cell populations, expression of HIV permissiveness/resistance markers, size of HIV reservoirs, and response to metformin . Further investigation are ongoing with sample accumulated in this trial. First, in collaboration with Dr. Jacob Estes, we are now performing HIV-RNA/DNA scope in combination with p-mTOR staining in colon biopsies collected at Baseline *vs.* Week 12. This will allow us to visualize in situ HIV transcription to p-mTOR expression at the single-cell level. In addition to HIV-RNA/DNA and p-mTOR, the effect of metformin on colon neutrophil infiltration, indicative of tissue damage, will also be visualized and quantified in situ. Secondly, transcriptional profiling using the Illumina RNA-Seq technology in total cell isolated from biopsies at Baseline vs. Week 12 will be performed to identify a transcriptional signature associated with metformin treatment in the colon.

Together these results prompt us to further investigate the immunological/virological benefits of long-term metformin supplementation in ART-treated PLWH.

MATERIALS AND METHODS

Study design and participants

The LILAC study was conducted between October 2016 and August 2018. Study participant enrollment and biological sample collection was performed at the McGill University Health Centre (MUHC), Glen site, Montréal, QC, Canada, for matched blood and sigmoid colon biopsies, and at The Ottawa Hospital, Ottawa, ON, Canada, for blood collection. Research investigations were performed at the CHUM Research Centre, Montréal, QC, Canada. This study was approved by the Research Ethics Boards of the Research Institute of the McGill University Health Centre (MUHC) number MP-37-2016-2456, and by the Health Canada Therapeutic Products Directorate. The study was also approved by the Internal Review Board (IRB) of the Ottawa Hospital Research Institute, ON, Canada (IRB No. 20160433-01H) and CHUM Research Centre, Montréal, QC, Canada (IRB No. 17.074). This study was conducted in accordance with the Declaration of Helsinki of 1975. Each participant provided written informed consent before any study procedure. Canadian CIHR/CTN protocol CTNPT027 - Trial registration: NCT02659306.

For this study, n=22 HIV-infected individuals (20 males, 2 females; Supplemental Table 1) were recruited, based on the inclusion criteria recently published by our group describes in (72). Briefly, participants were virologically suppressed (<40 copies per ml, at least two measurements per year) under ART for a minimum of 3 years and exhibited CD4/CD8 ratio ≤ 0.7 (indicative of high risk of inflammation and non-AIDS events). They are not diabetic as defined by HbA1c < 5.9% (72). They received orally twice daily as 500 mg metformin (Glucophage®; Sanofi-Aventis Canada Inc.) to assess tolerability during the first week, following which the dose was increased to 850 mg twice daily (weeks 2 to 12). These doses were chosen based on the mean dose used in diabetic individuals, with metabolic improvement being observed (59). The treatment duration was chosen based on a previous clinic study in non-diabetic patients treated for polycystic ovary syndrome and in which 12 weeks of metformin was sufficient to decrease inflammation (124). Drug-to-drug interaction between dolutegravir

and metformin was reported and study participants using dolutegravir (Table 1; LILAC #4, #6, #9, #11, #13, #14 #18 #22) maintained the 500 mg dose metformin twice daily throughout the study (125, 126). Blood was collected at baseline, after 12 weeks of metformin administration (week 12) and 12 weeks post-metformin discontinuation (week 24). 13 individuals (12 males and 1 female) consented to the optional endoscopic collection of sigmoid colon biopsies at baseline and 12 weeks post-metformin administration (Figure 1). The later study participants benefitted from colon cancer screening in the same time (72, 127).

Blood and sigmoid colon biopsies

PBMCs were collected by leukapheresis (128) and cryopreserved until use. Sigmoid biopsies (≈32 biopsies/donor) were collected from HIV-infected individuals receiving ART (Supplemental Table 1) during colonoscopy and processed using Liberase DL (Roche Diagnostics), as previously described (45, 129, 130). Matched peripheral blood (20 ml/donor) was collected the same day from biopsy donors and immediately processed by Ficoll for PBMC isolation, performed in parallel of the cell extraction from biopsies pieces.

Isolation of total $CD4^+$ T-cells from cryopreserved PBMC

Total $CD4^+$ T cells were isolated from cryopreserved PBMCs using magnetic depletion as per the manufacturer's protocol (Stem Cell Technologies, Vancouver, Canada).

Cell sorting of memory $CD4^+$ T-cells from cells extracted from fresh blood and sigmoid colon biopsies

Cells extracted from sigmoid biopsies and matched PBMCs were stained on the surface as previously described (44) using specific Abs (Supplemental Table 2) Blood memory $CD4^+$ T-cells ($CD45RA^-CD3^+CD4^+$) and colon memory $CD3^+$ ($CD45RA^-CD3^+CD4^{+/low}$) lacking CD8 T-cell (CD8), B cell (CD19), NK cell (CD56), and epithelial cell (CD326) lineage markers were sorted using the BD-FACSAriaIII (BD Biosciences) in the Biosafety level 3 laboratory. The viability dye LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Invitrogen) was used to exclude dead cells. Flow cytometry

gates were defined using the fluorescence minus one strategy, as previously described (131, 132).

Plasma biomarker measurements

Plasma was collected from peripheral blood processed and subsequently cryopreserved. Thawed plasma was inactivated with disruption buffer (PBS, 0.05% Tween-20, 2.5% Triton X-100, 1% trypan blue, 0.02% thimerosal) at 37°C for 1 hour. The concentration of 45 biomarkers was quantified using Human XL Cytokine Discovery Luminex (R) High Performance Assay (R&D Systems) according to the manufacturer's instructions.

Flow cytometry staining and analysis

Surface and intracellular staining (using the Fixation/Permeabilization Solution Kit (BD) were performed with specific fluorochrome-conjugated Abs (Supplemental Table 2), as previously described (44, 132). Cells were analyzed using a LSRII cytometer (Diva, version 6, BD Biosciences), and FlowJo (version 10.0.6, Tree Star Inc.). All Abs were titrated for an optimal noise/signal ratio and Ab cocktails were validated by comparing single with multiple staining. Positive gates were placed based on fluorescence minus one control (131, 132). Cells were analyzed using a LSRII cytometer (Diva, version 6, BD Biosciences), and FlowJo (version 10.0.6, Tree Star Inc.).

Quantification of cell-associated unspliced HIV-RNA in total blood $CD4^+$ T-cells

Total RNA was extracted from total $CD4^+$ T-cells using RNeasy Mini Kit with on-column DNase treatment (Qiagen), according to the manufacturer's instructions.

The quality (260nm/280nm ratio) and quantity of RNA collected were evaluated by spectrophotometry on a Nanodrop instrument. Quantification of LTR-Gag HIV-RNA was performed by nested real-time RT-PCR. The external primers for the first amplification were ULF1 and UR1 (Supplemental Table 3). The internal primers for the second amplification PCR were Lambda (λ) T (Forward) and UR2 (Reverse) (Supplemental Table 3). The probe used was: UHIV FamZen (Supplemental Table 3). The first amplification was performed on Proflex PCR

System (Applied Biosystem), while the second amplification was performed on the RotorGene instrument (Qiagen). LTR-gag (unspliced) HIV-RNA standards were generated from plasmids by *in vitro* transcription (MEGAscript™ T7 Transcription Kit, ThermoFisher). The numbers of copies of each transcript were normalized to the levels of the reference gene beta glucuronidase (GUSB). The oligonucleotides used for GUSB amplification were GusbF1 and GusbR1 for the first amplification and GusbF2 and GusbR2 for the second amplification (Supplemental Table 3). The probe sequence was: GUSB-HEX (Supplemental Table 3). All measures were performed in triplicate

Quantification of integrated and Gag HIV-DNA

Levels of integrated and Gag HIV-DNA were quantified in cell lysates by ultrasensitive nested real-time PCR (10^5 cells/test in triplicates; detection limit, 3 HIV-DNA copies), as previously described (44, 132, 133). The ACH2 cells carrying one copy of integrated HIV-DNA per cell (NIH AIDS Reagent Program) were used as a standard curve, as previously described (44, 132, 133). For integrated HIV-DNA amplification, the external primers were ULF1 and Alu1/Alu2; the internal primers were: Lambda (λ) T (Forward) and UR2 (Reverse); the probe used was: UHIV FamZen (Supplemental Table 3). For Gag HIV-DNA amplification, the external/internal primers and the probe used was the same as those used for LTR-Gag HIV-RNA quantification (Supplemental Table 3). For CD3 amplification, the external primers were HCD3OUT 5' and HCD/OUT 3'; the internal primers for the real-time PCR were: HCD3IN 5' and HCD3IN 3'; the probe used was: CD3 FamZen (Supplemental Table 3). All measures were performed in triplicate.

Quantification of cell-associated unspliced HIV-RNA in colon memory $CD4^+$ T-cells

Total DNA and RNA were dually extracted from colon memory $CD4^+$ T-cells using the AllPrep DNA/RNA Mini Kit (Qiagen), according to the manufacturer's instructions. Cell-associated HIV-RNA and HIV-DNA extracted from each sample ere quantified as described above. The quantification of CD3-DNA copy numbers allowed us to determine the number of cells per sample, thus allowing the normalisation of HIV-RNA copy numbers per 10e6 cells (2 CD3-DNA

copies/cell). Finally, the ratio between HIV-RNA/10e6 cells and HIV-DNA/10e6 cells was calculated, indicative of HIV transcription.

Quantification of Tat/rev inducible multiply spliced HIV-RNA (TILDA)

The frequency of $CD4^+$ T cells with inducible multiply spliced HIV-RNA was determined using modify version of Tat/rev inducible limiting dilution assay (TILDA), as previously described (134). Briefly, enriched $CD4^+$ T-cells were rested for 3–5 h at 37 °C, 5% CO_2, in the presence of antiretroviral drugs (200 nM raltegravir, 100 nM efavirenz and 180 nM AZT). $CD4^+$ T-cells were stimulated for 18 h with PMA (100 ng/ml) and Ionomycin (1 µg/ml) (Sigma). After stimulation, cells were washed in RPMI, counted and serially diluted to 18×10^6 cells/ml, 9×10^6 cells/ml, 3×10^6 cells/ml and 1×10^6 cells/ml in culture medium. Primers for tat/rev pre-amplification were: Tat1.4 and Rev (Supplemental Table 3). The tat/rev primers for the real-time PCR were Tat2 and Rev (Supplemental Table 3). The probe used in these reactions was msHIV FamZen (Supplemental Table 3). Positive wells at each dilution were counted and the maximum likelihood method was used to calculate the frequency of cells with inducible HIV msRNA (http://bioinf.wehi.edu.au/software/elda) (134).

Quantitative viral outgrowth assay

A new simplified quantitative viral outgrowth assay (QVOA) was set up, using a cell culture procedure previously established in the lab (44, 48). Briefly, total $CD4^+$ T-cells were cultured in complete medium (RPMI1640, 10% FBS, 1% antibiotics) in the presence of immobilized CD3 and soluble CD28 Abs (1 µg/ml) and ATRA (100 nM) up to 12 days at 37 ºC. To perform three serial dilutions, cells were cultured in 48-well plates at $1x10^6$ cells/ml/well (2-3 replicates) and in 96-well plates at $2x10^5$ or $1x10^5$ cells/mL/well (6-8 replicates). At day 3 post-TCR triggering cells were harvested, washed, split into two new wells, and cultured in fresh complete media containing IL-2 (5 ng/ml) and ATRA (100 nM). At days 6 and 9, cells from each well were split in two new wells and media containing IL-2 (5 ng/ml) and ATRA (100 nM) was refreshed. Supernatants were collected at days 3, 6, 9 and 12 for HIV-p24 quantification by ELISA. HIV-p24 positive wells at each cell dilution were considered positive if the HIV-p24 levels were

detected at 2 consecutive time points. The number of infectious units per millions cells (IUPM) was calculated using the IUPMStats v1.0Infection Frequency Calculator (http://silicianolab.johnshopkins.edu/)

Statistics

All statistical analyses were performed using the Prism 8 (GraphPad software). Specifications are included in Figure legends. P-values are indicated on the graphs as follow: *P< 0.05; **P< 0.01; ***P< 0.001; ****P< 0.0001.

Competing interest statement

EAC is a member of the Scientific Advisory Board of Theratechnologies. JBA performed contract research and/or served on Advisory Boards for Gilead Sciences Canada Inc., Merck Canada Inc., Abbvie Corp., ViiV Healthcare, Bristol Myers Squibb, Janssen Inc., and Argos Pharmaceuticals. NC received research funding from Merck & Co. and from InnaVirVax and served on the Advisory Board of Theravectys. JPR performed contract research and/or served on Advisory Boards for Gilead Sciences Canada Inc., Merck Canada Inc., Abbvie Corp., ViiV Healthcare, Bristol Myers Squibb, Janssen Inc., Argos Pharmaceuticals from InnaVirVax, and Theravectys. PA's laboratory receives research funding from Glaxo Smith Klein/NeoMed for projects different from the present study. PA serves as a Consultant at Merck Canada Inc. relative to projects different from the present study. DP, AP, RP, AF, LRM, MM, AG, VM, FD, SL, and MPG have no financial or non-financial competing interests to disclose.

Authors' contributions

DP designed and performed research, analyzed data, and wrote the manuscript. AP performed research and analyzed data related to HIV reservoir measurements. RP, AF, LRM, MM, AG, VM and FD performed research related to clinical study design, sample collection/preparation and/or HIV reservoir measurements. SL and EAC provided protocols and contributed to research design. MPG provided access to matched blood and colon biopsies. JBA contributed to clinical study design and recruited participants. NC provided protocols and reagents, contributed to research design and manuscript writing. JPR conceived the clinical study

hypothesis and design, provided access to clinical samples/information, set up clinical research protocols and recruited participants. PA conceived the research study hypothesis, designed research, analyzed data, and wrote the manuscript.

Acknowledgements

The authors thank Dr. Dominique Gauchat and Philippe St Onge (Flow Cytometry Core Facility, CHUM-Research Center, Montréal, QC, Canada) for expert technical support with polychromatic flow cytometry sorting. The authors thank Olfa Debbeche (NLC3 Core Facility CHUM-Research Center, Montréal, QC, Canada); Mario Legault for help with ethical approvals and informed consents, and Josée Girouard, Angie Massicotte, Cynthia Dion, and Dr. Stéphane Isnard for their key contribution to LILAC study participant recruitment and access to matched blood and sigmoid biopsies and clinical information from HIV-infected and uninfected donors. The authors address a special thanks to all PLWH study participants from Montréal and Ottawa for their gift of blood and colon biopsies and key contribution to this work.

Funding

This study was supported by grants from the Canadian Institutes of Health Research (CIHR) (#IBC-154053 to PA), the CIHR Canadian HIV Trials Network (CTN #247 to JPR), the *Fonds de Recherche du Québec-Santé* (FRQ-S)/AIDS and Infectious Diseases Network, Québec, Canada (to PA and JPR). The study was also supported by The Canadian HIV Cure Enterprise Team Grant (CanCURE 1.0) funded by the CIHR in partnership with CANFAR and IAS (CanCURE 1.0; # HIG-133050 to PA, EAC, JBA, JPR), and The Canadian HIV Cure Enterprise Team Grant (CanCURE 2.0) funded by the CIHR (#HB2–164064 to PA, EAC, JBA, JPR). JPR holds a Louis Lowenstein Chair in Hematology and Oncology, McGill University. The funding institutions played no role in the design, collection, analysis, and interpretation of data. DP received a Doctoral award from the Université de Montréal and the FRQ-S. JPR holds a Louis Lowenstein Chair in Hematology and Oncology, McGill University. Core facilities and HIV-infected patients' cohorts were supported by the *Fondation du CHUM* and the FRQ-S Network.

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FIGURES

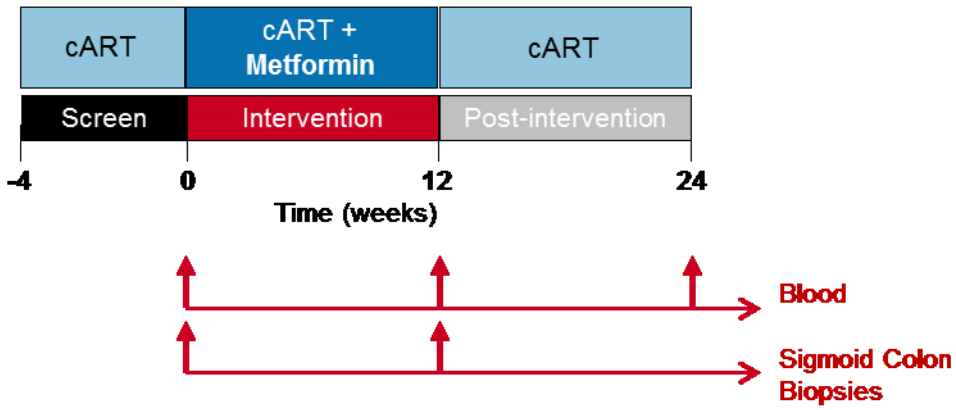


Figure 1: Flow chart of the clinical trial. Depicted is the timing of metformin intervention and sample collection. Briefly, blood and plasma from n=22 HIV-infected individuals with undetectable plasma viral load (<40 HIV-RNA copies/ml) under ART were collected at baseline (Baseline), after 12 weeks of metformin treatment (500 to 850 mg bid) (Week 12) and after 12 week of metformin discontinuation (Week 24). Matched colon sigmoid biopsies from n=13 study participants were collected at Baseline and Week 12. Blood and colon biopsies were processed immediately for immunological/virological measurements. A fraction of PBMC was frozen in DMSO for subsequent immunological/virological measurements.

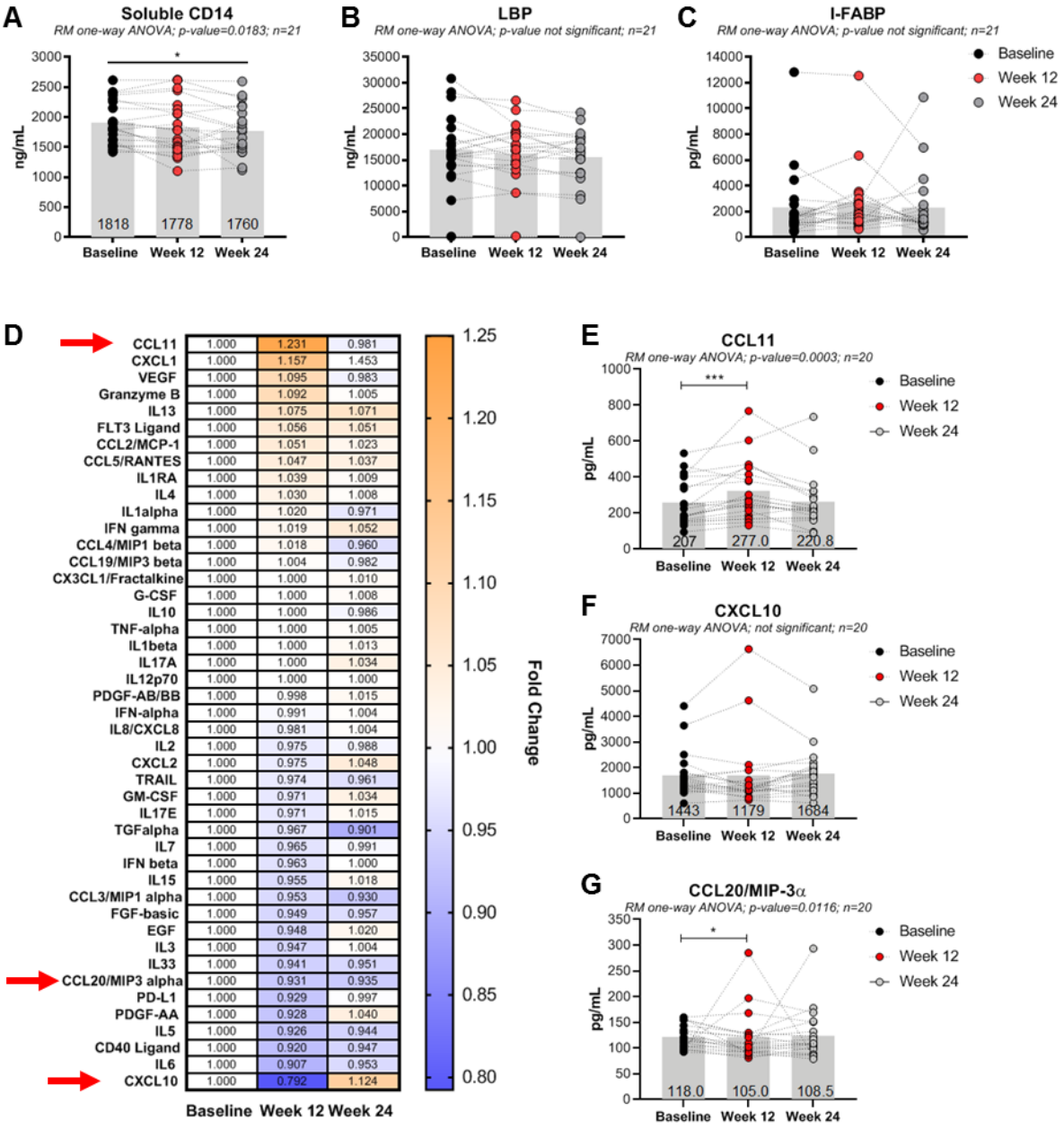


Figure 2: Effects of metformin treatment on plasma markers of systemic inflammation and gut barrier dysfunction. Plasma levels of **(A)** soluble CD14 (sCD14), **(B)** lipopolysaccharide binding protein (LBP), and **(C)** intestinal fatty-acid binding protein (I-FABP) were quantified by ELISA at baseline (**black circles**), week 12 (**red circles**) and week 24 (**grey circles**). **(D-G)** Forty-five soluble biomarkers were measured in plasma using the Luminex assays. **(D)** The heatmap depicts relative changes in biomarker abundance between Baseline (considered 1) and Week 12 or Week 24. Colors indicate normalized row min and max shaded blue and orange, respectively. Fold change (FC) values between Baseline and Week 12 or Week 24 are indicated in the heatmap for each soluble factor. **(E-F-G)** Shown are statistical analysis of plasma concentrations of CCL11 **(E),** CXCL10 **(F)** and CCL20 **(H)** measured by Luminex between Baseline and Week 12 or Week 24. Grey bars represent the mean values, and median values are indicated on

the bars. One-way ANOVA (Friedman) with Tukey's multiple comparisons test p-values are indicated on the graphs.

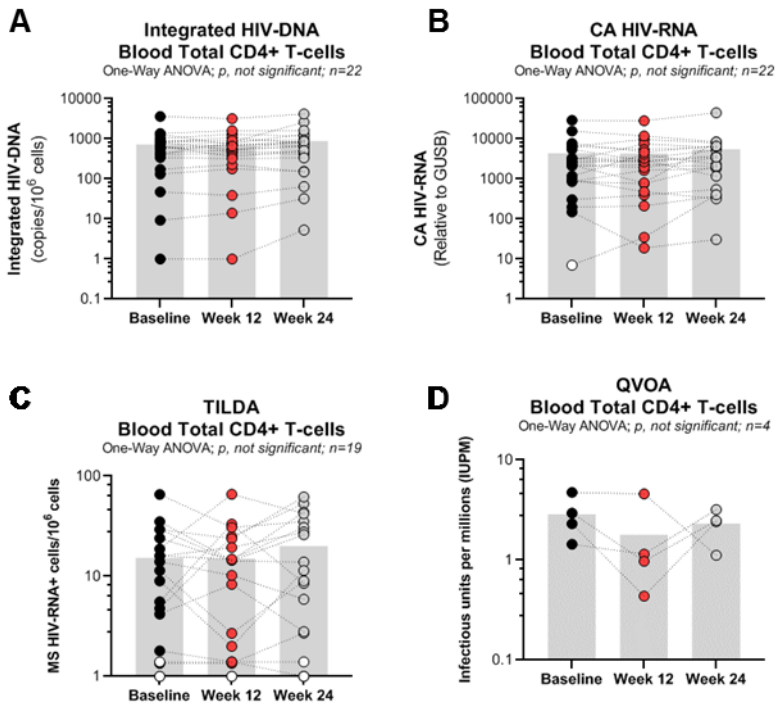


Figure 3: Effects of metformin treatment on viral reservoirs in blood CD4⁺ T-cells. Total CD4⁺ T-cells were enriched from fresh PBMCs isolated at baseline (**back circles**), week 12 (**red circles**) and week 24 (**grey circles**) from the blood of study participants (n=22, Supplemental Table 1) by negative selection. **(A)** Levels of integrated HIV-DNA (Alu/HIV-LTR primers) were quantified by real-time nested PCR in cell lysates. Shown are HIV-DNA copy numbers per 10e6 cells (two CD3-DNA copies/cell) **(B)** In parallel, RNA was extracted from total CD4⁺ T-cells and cell-associated (CA) HIV-RNA (LTR/Gag primers) levels were quantified by real-time RT-PCR. Shown are (CA HIV-RNA copy numbers relative to GUSB mRNA (housekeeping gene). **(C)** The frequency of inducible multiply spliced (MS) HIV-RNA in CD4⁺ T cells was quantified using the Tat/rev induced limiting dilution assay (TILDA). Shown are values normalized *per* 10e6 cells. **(D)** A quantitative viral outgrowth assay (QVOA) was performed in total CD4⁺ T-cells from 8/22 study participants. Viral reactivation was assayed by HIV-p24 ELISA quantification at day 3, 6, 9 and 12. HIV outgrowth was considered positive when HIV-p24 was detectable sequentially at day 9 and 12. Shown are infectious units per million (IUPM) values calculated using the online IUPMStats v1.0Infection Frequency Calculator (http://silicianolab.johnshopkins.edu/). Of note, reactivation was detected in 4/8 participants (filled circles), while in 4/8 participants HIV outgrowth was not detected at any time point (not shown). **(A-D)** Grey bars represents mean values of different virological measurements. One-way ANOVA (Friedman) with Tukey's multiple comparisons test P-values are indicated on the graphs.

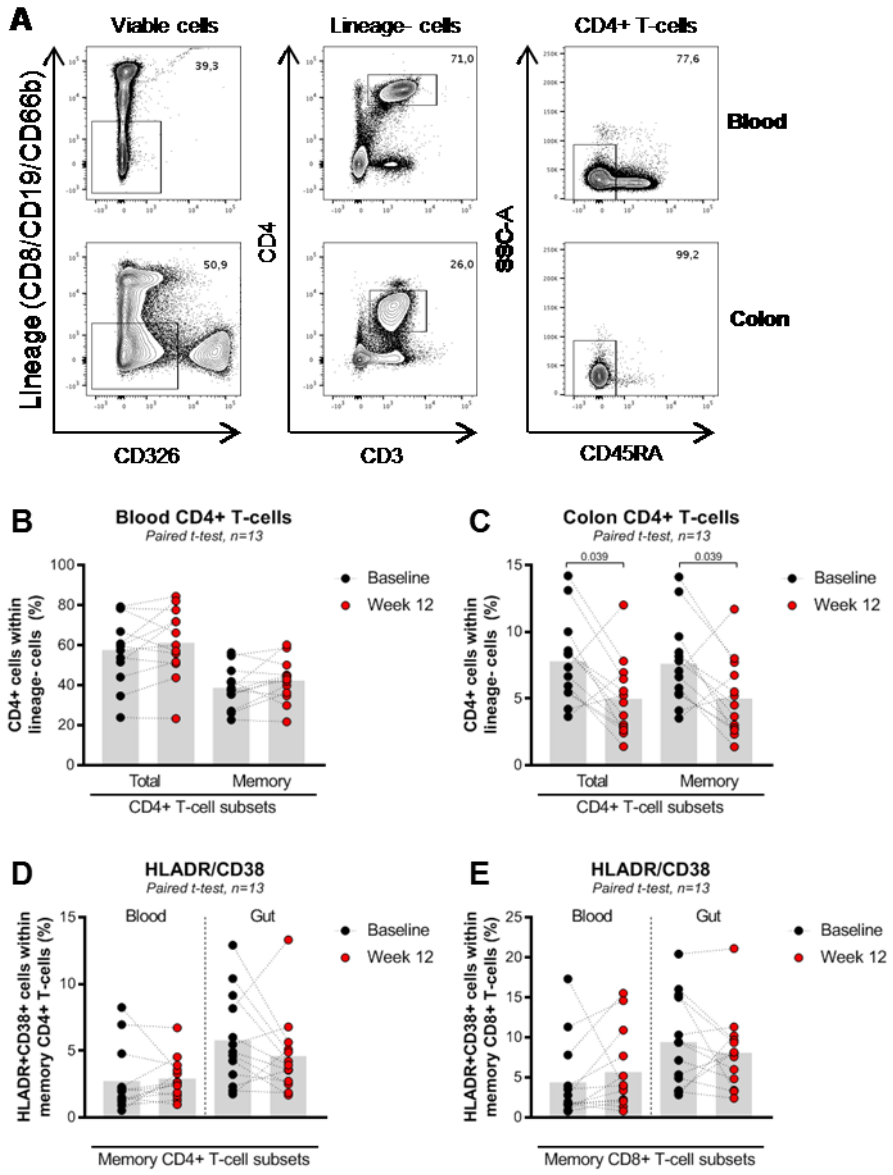


Figure 4: Effects of metformin treatment on blood and colon CD4⁺ T-cell frequencies and expression of classical activation markers. Matched blood and sigmoid colon biopsies were collected from n=13 participants (Supplemental Table 1) at baseline (**black circles**) and after 12 weeks of metformin treatment (**red circles**). Cells were extracted and stained with a cocktail of specific Abs for flow cytometry analysis, as described in Material and Methods. A viability dyes was used to exclude dead cells from the analysis. **(A)** Shown is the gating strategy for flow cytometry identification of total ($Lineage^-CD326^-CD3^+CD4^+$) and memory ($CD45^-$) $CD4^+$ T-cells in blood (upper panels) and colon (lower panels). **(B-C)** Shown are statistical analysis of the frequency of total and memory $CD4^+$ T-cells within live $Lineage^-CD326^-$ cells. Memory $CD8^+$ T-cells were identified as $CD326^-$ $Lin^+CD3^+CD45^-$ cells (gating strategy not shown). **(D-E)** Shown are statistical analysis of the frequency of $HLA\text{-}DR^+CD38^+$ within memory $CD4^+$ **(D)** and $CD8^+$ **(E)** T-cells from blood and colon at baseline and week 12. Grey bars represent the mean values. Paired *t*-Test P-values are indicated on the graphs.

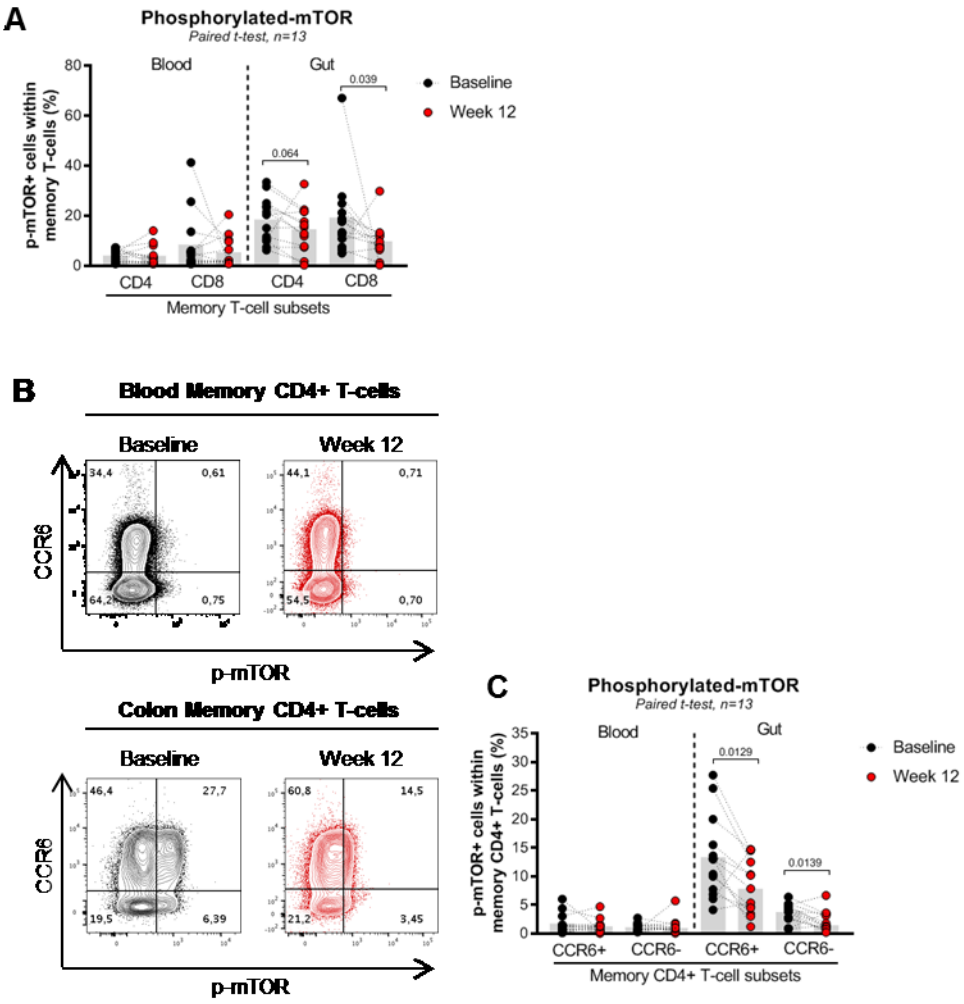


Figure 5: Metformin reduces mTOR phosphorylation in colon-infiltrating T-cells, most robustly in CCR6$^+$CD4$^+$ T-cells. The gating strategy for flow cytometry identification of memory CD4$^+$ and CD8$^+$ T-cells in matched blood and sigmoid colon biopsies of n=13 study participants (Supplemental Table 1) is depicted/explained in Figure 4 legend. **(A)** Shown are statistical analysis of intracellular expression of phosphorylated mTOR in memory CD4$^+$ and CD8$^+$ T-cells from blood and colon at baseline (**black circles**) and week 12 post-metformin treatment (**red circles**). **(B)** Shown are dot plots of intracellular phosphorylated mTOR (p-mTOR) and surface CCR6 expression in memory T-cells from blood and colon at baseline (**black dot plots**) and week 12 (**red dot plots**); dot plots are from one representative study participant (LILAC #2). **(C)** Shown are statistical analysis of the frequency of CCR6$^+$p-mTOR$^+$ from matched blood and colon memory CD4$^+$ T-cells before (**black circles**) and after 12 weeks of metformin (**red circles**). Grey bars represent the mean values. Paired *t*-Test P-values are indicated on the graphs.

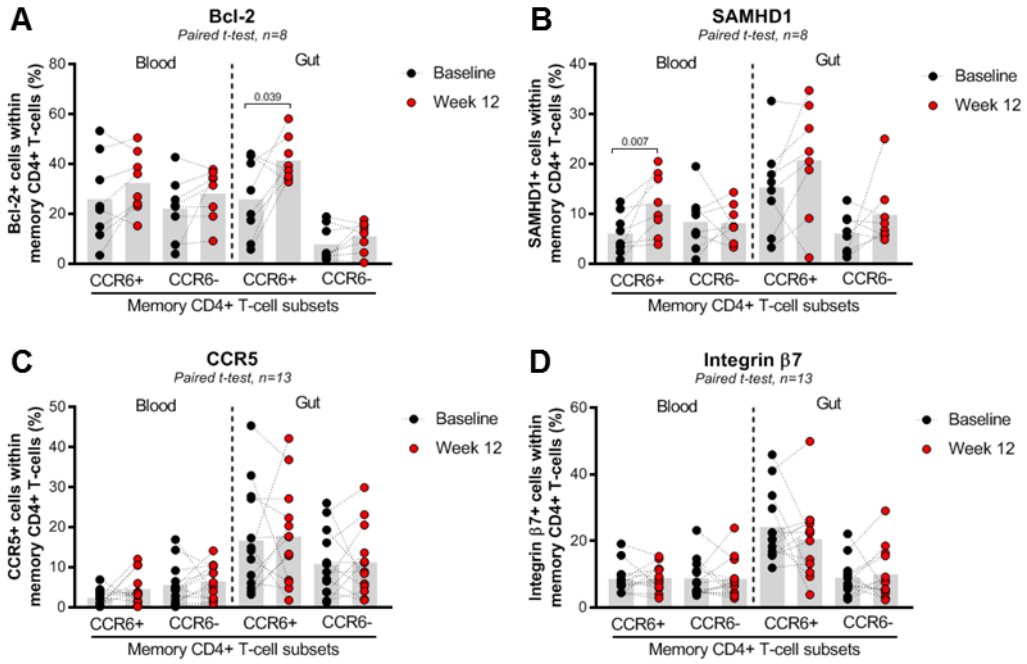


Figure 6: Effect of metformin treatment on markers of cell survival, HIV permissiveness, and gut-homing in colon-infiltrating CCR6$^+$CD4$^+$ T-cells. The gating strategy for flow cytometry identification of memory CCR6$^+$/CCR6$^-$ CD4$^+$ T-cells from matched blood and sigmoid colon biopsies is depicted in Figure 4A. **(A-D)** Shown are statistical analysis of intracellular Bcl-2 **(A)** and SAMHD1 **(B)** expression**,** as well as the surface expression of CCR5 **(C)** and integrin β7 **(D)** in blood and colon memory CCR6$^+$ *versus* CCR6$^-$ CD4$^+$ T-cells at baseline (**black circles**) and 12 weeks post-metformin treatment (**red circles**). Grey bars represent the mean values. Paired *t-T*est values are indicated on the graphs.

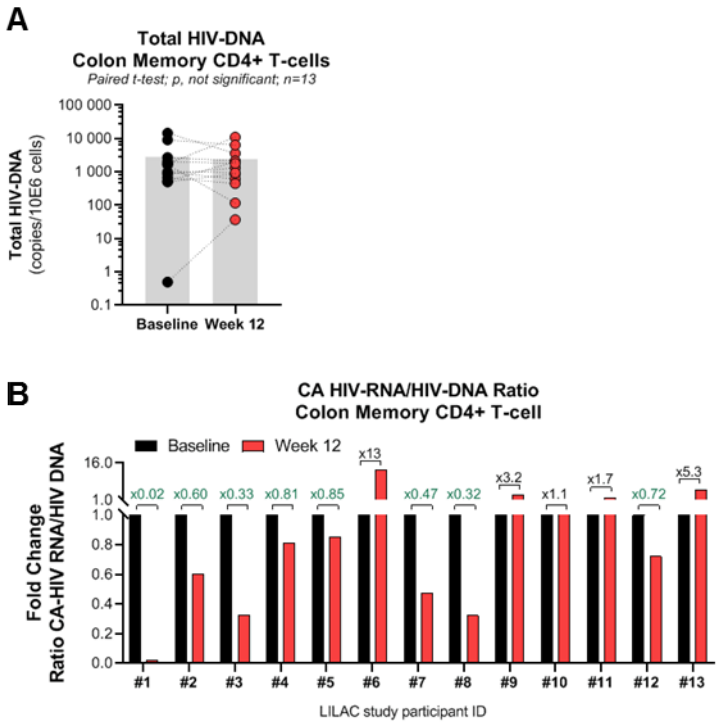


Figure 7: Effects of metformin treatment on CA HIV-DNA and HIV-RNA in colon-infiltrating memory CD4⁺ T-cells. Memory CD4⁺ T-cells were sorted by FACS from sigmoid colon biopsies. **(A)** Shown are total HIV-DNA (Gag primers) levels quantified by real time nested PCR in whole cell lysates. Grey bars represent the mean value. Paired *t-T*est values are indicated on the graphs. **(B)** In parallel, RNA and DNA were dually extracted from FACS-sorted colon memory CD4⁺ T-cells**.** CA total HIV-DNA (Gag primers) and HIV-RNA (Gag primers) levels were quantified by real time nested PCR. Shown are the CA HIV-RNA/HIV-DNA ratios in n=13 study participants (Supplemental Table 1), with baseline values considered at 1 and fold change values between baseline (**black bars**) and week 12 (**red bars**) depicted on the graph.

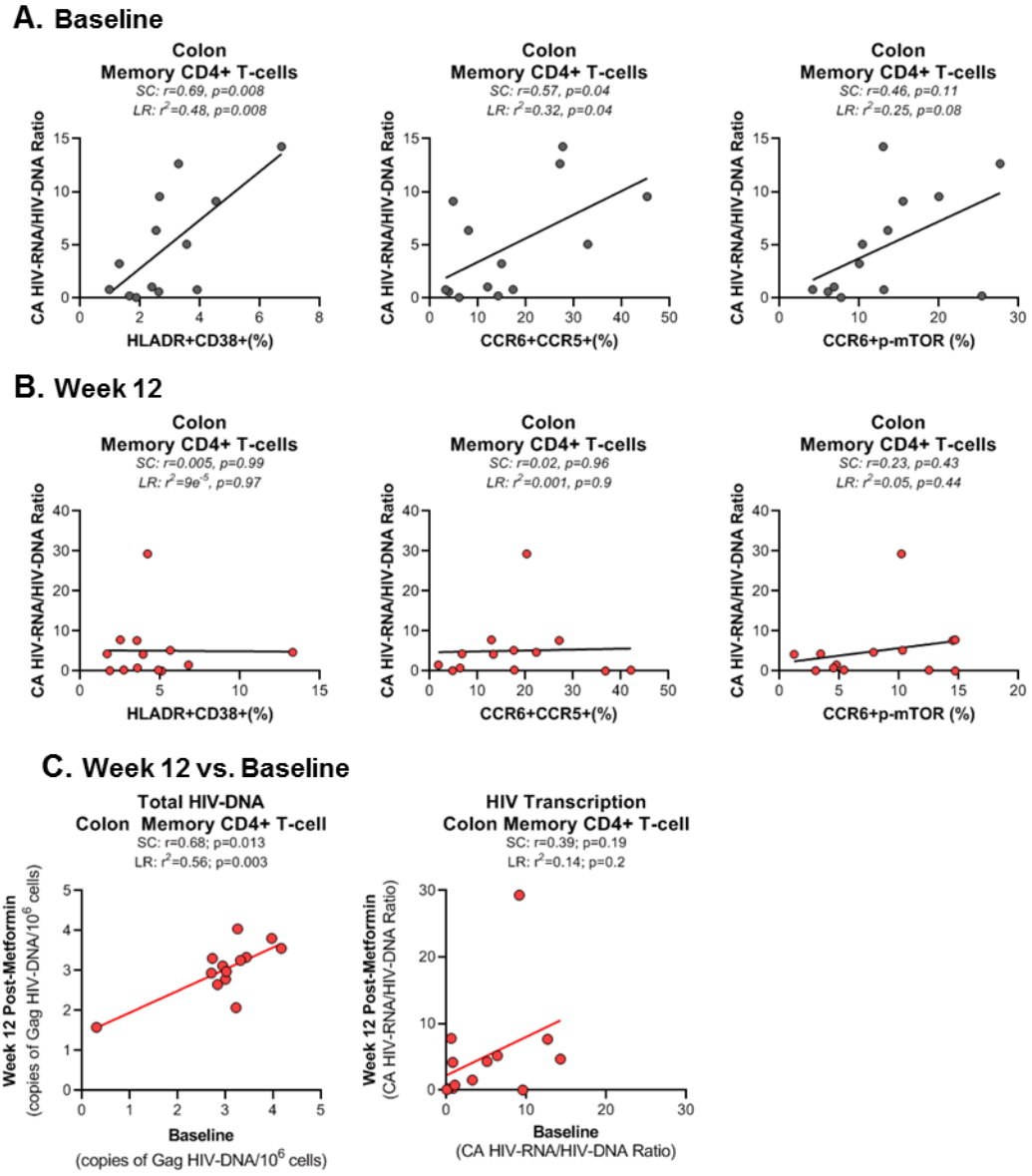
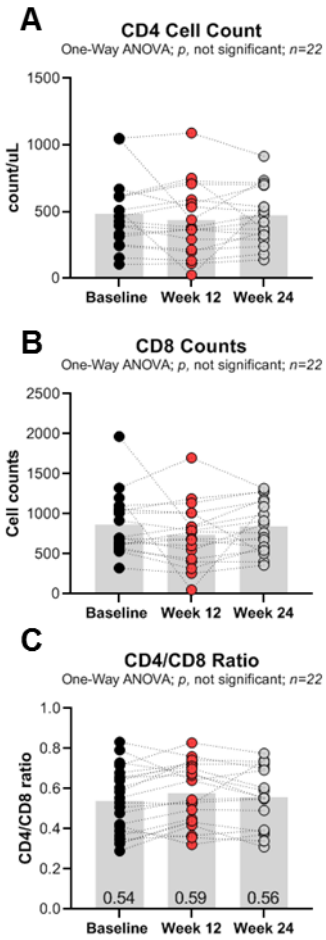
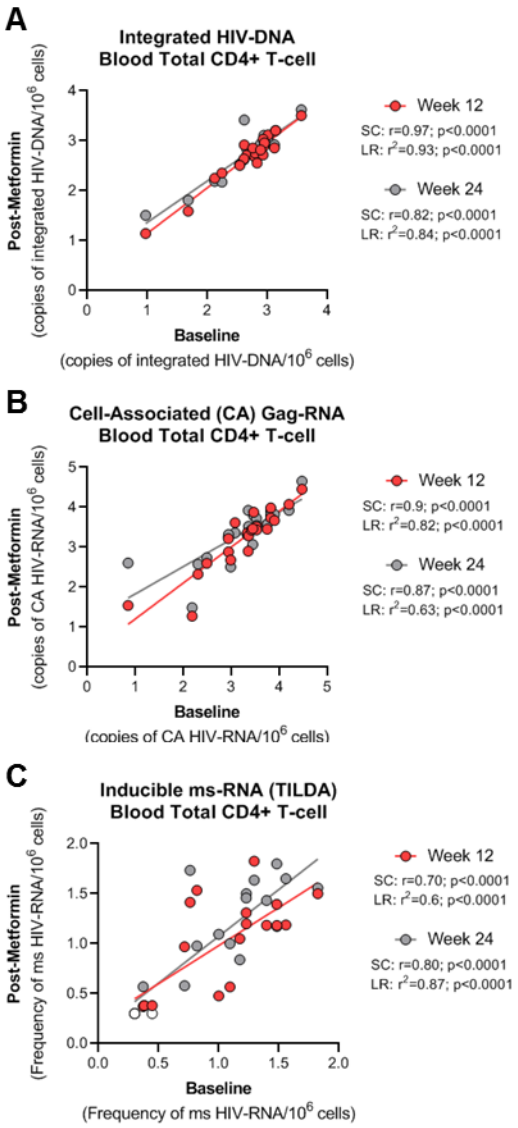


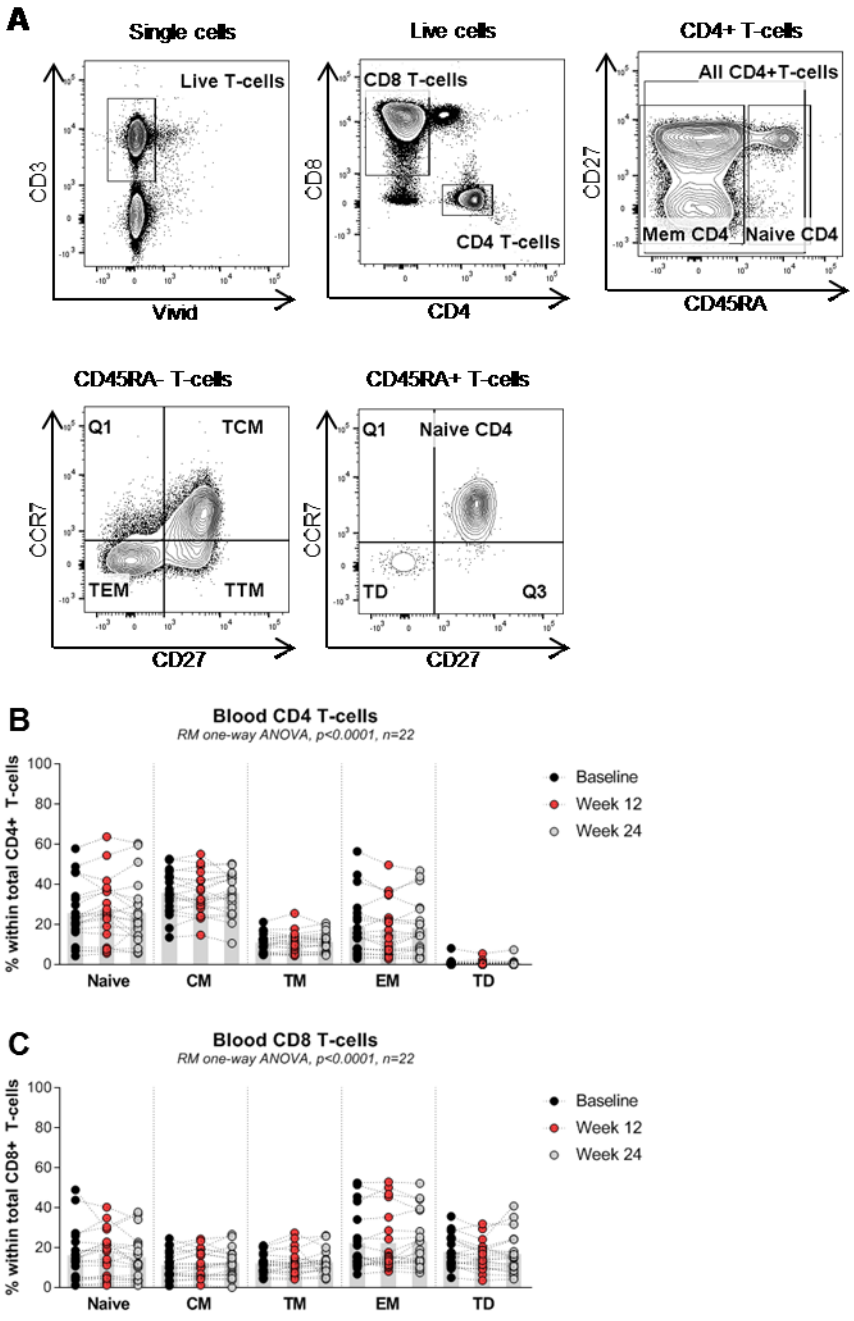
Figure 8: Baseline *vs.* Week 12 correlations of virological and immunological parameters in the colon-infiltrating memory CD4⁺ T-cells. The CA HIV-RNA/HIV-DNA ratios (measured in Figure 7B) were correlated with the co-expression of HLA-DR/CD38 (measured in Figure 4D), CCR6/p-mTOR (measured in Figure 5C), and CCR6/CCR5 (measured in Figure 6C) on memory CD4⁺ T-cells at Baseline (**grey circles**) **(A)** and Week 12 (**red circles**) **(B)** of metformin treatment. **(C)** Total HIV-DNA (Gag) (Log10 values) (left panel) and CA HIV-RNA/DNA ratios (right panels) were measured in FACS sorted memory CD4⁺ T-cells from n=13 participants (Supplemental Table 1), as depicted in Figure 7. Baseline values were correlated with values at Week 12 (**red circles**). Spearman correlation (SC) and linear regression (LR) models were applied and SC, r/P values, as well as LR r^2/P values, are depicted on the graphs.



Supplemental Figure 1: Effect of metformin treatment on CD4 and CD8 counts and CD4/CD8 ratios in the blood of ART-treated individuals. Clinical blood CD4 and CD8 counts were available for the majority of the participants (Supplemental Table 1). Shown are CD4 **(A)** and CD8 counts **(B)**, as well as CD4/CD8 ratios **(C)** at Baseline (**black circles**), Week 12 (**red circles**), and Week 24 (**grey circles**). One-way ANOVA (Friedman) Tukey's multiple comparisons test p-values are indicated on the graphs.

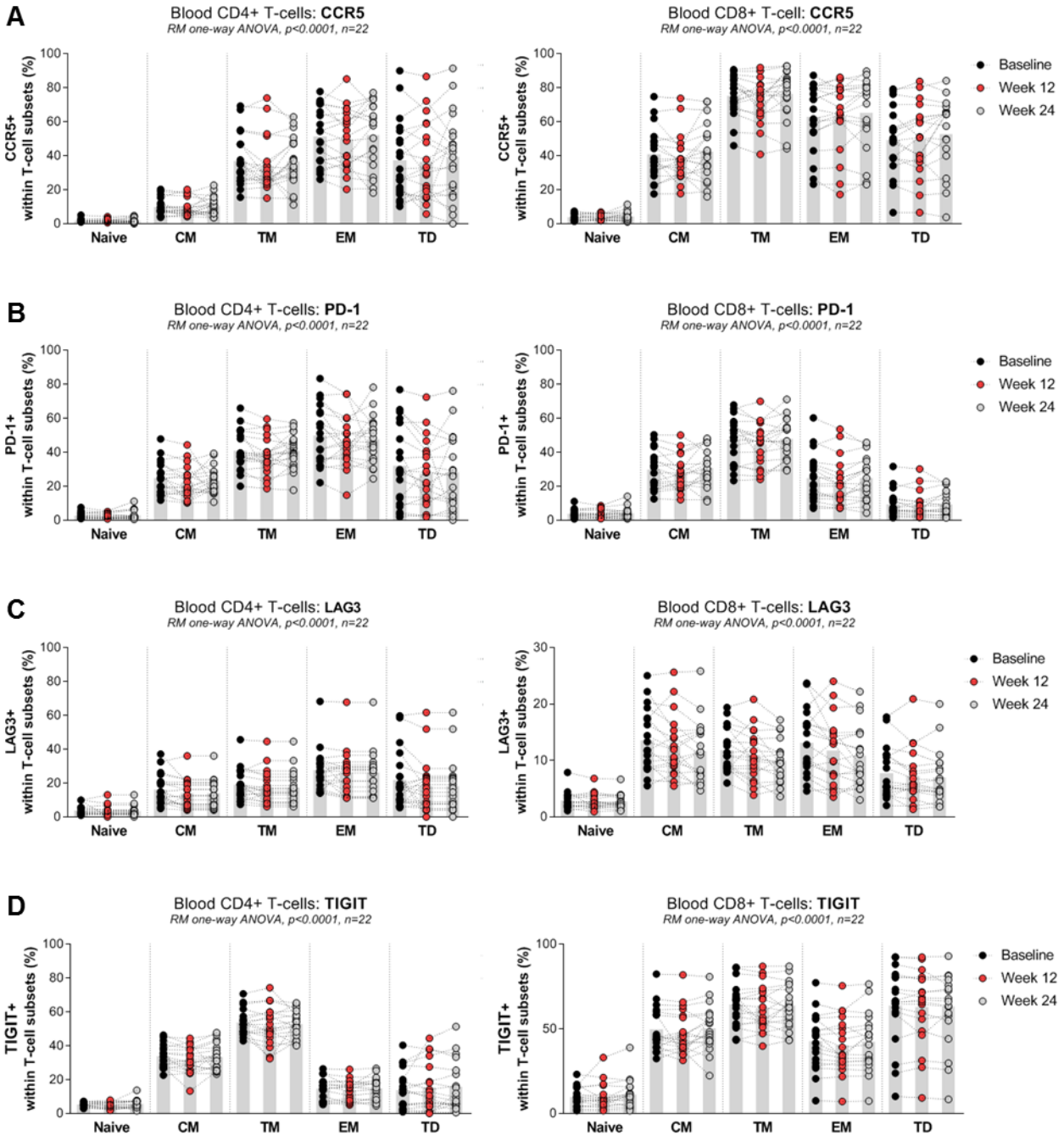


Supplemental Figure 2: Baseline *vs*. Week 12 correlations of virological parameters in blood memory CD4⁺ T-cells. Integrated HIV-DNA (Log10 values) **(A)**, CA HIV RNA (Log10 values) **(B)** and inducible MS HIV-RNA (TILDA) (Log10+1 values) **(C)** were measured in blood CD4⁺ T-cells from n=22 study participants (Supplemental Table 1), as depicted in Figure 3. Baseline values were correlated with values at Week 12 (**red circles**) and Week 24 (**grey circles**). Spearman correlation (SC) r and P-values as well as linear regression (LR) r2 and *P*-values are indicated on the graphs.

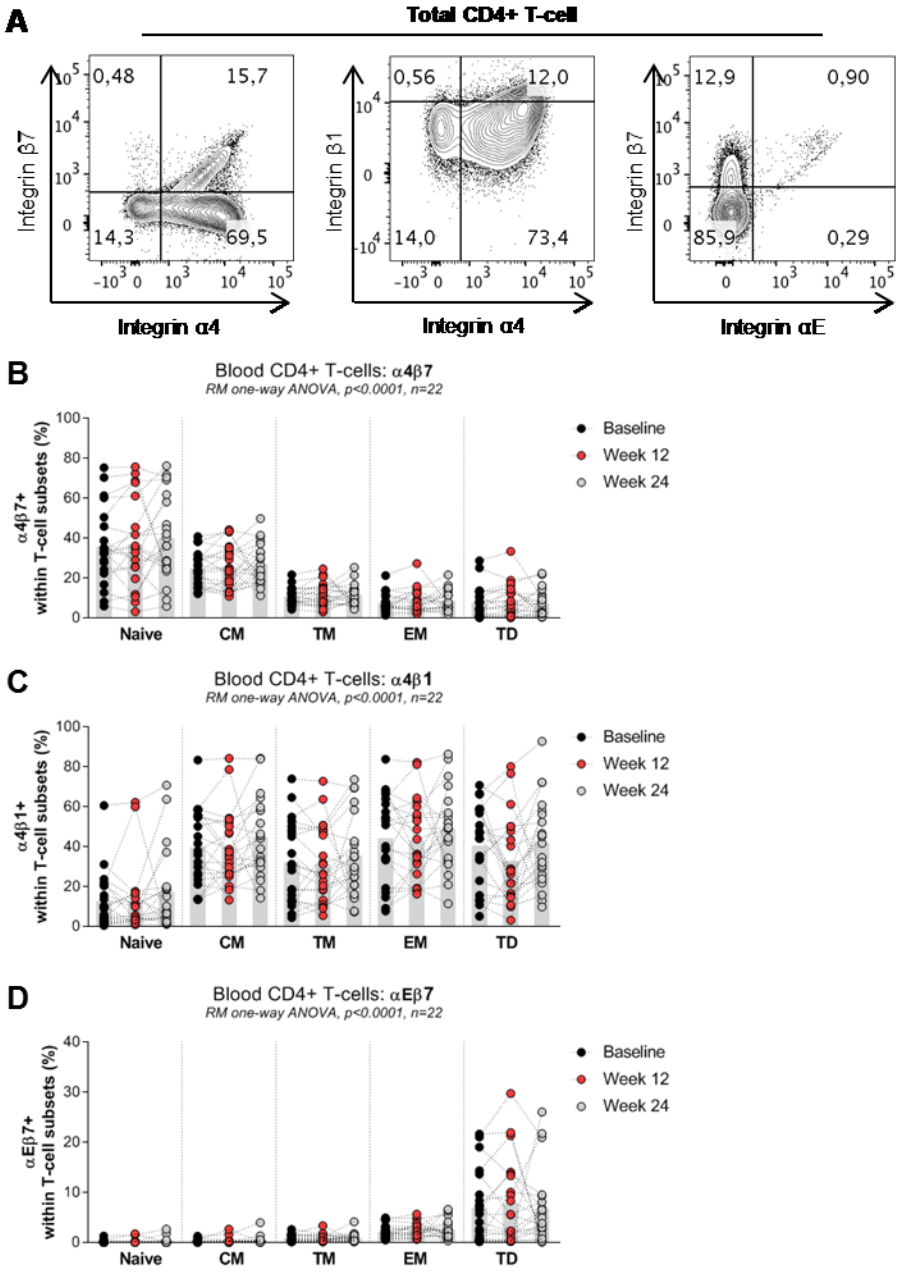


Supplemental Figure 3: Effects of metformin treatment on the frequency of blood naive and memory CD4$^+$ and CD8$^+$ T-cell subsets. Cryopreserved PBMCs from n=22 participants (Supplemental Table 1) at baseline , week 12 and week 24 were thawed and stained with a cocktail of specific Abs for flow cytometry analysis . **(A)** Shown is the gating strategy for flow cytometry identification of naive and memory CD4$^+$ and CD8$^+$ T-cell subsets. **(B-C)** Shown are statistical analysis of the frequencies of the following T-cell subsets: (naive (TN, CD45RA$^+$CD27$^+$CCR7$^+$), central memory (TCM, CD45RA$^-$ CD27$^+$CCR7$^+$), transitional memory (TTM, CD45RA$^-$CD27$^+$CCR7$^-$), effector memory (TEM, CD45RA$^-$CD27$^-$ CCR7$^-$) and terminally differentiated (TD, CD45RA$^+$CD27$^-$CCR7$^-$) within CD4$^+$ T-cells **(B)** and CD8$^+$ T-cells

(C) at baseline (**black circles**), week 12 (**red circles**) and week 24 (**grey circles**). Grey bars represent the mean value. One-way ANOVA (Friedman) Tukey's multiple comparisons test significant P-values are indicated on the graphs.



Supplemental Figure 4: Effects of metformin treatment on the frequency of blood CD4⁺ and CD8⁺ T-cell subsets expressing the HIV co-receptor CCR5 and the immune checkpoint inhibitors PD-1, LAG3, and TIGIT. Cryopreserved PBMCs from n=22 participants (Supplemental Table 1) at baseline, Week 12 and Week 24 were stained with a cocktail of specific Abs for flow cytometry analysis. The gating strategy for the identification of N, CM, TM, EM and TD CD4⁺ and CD8⁺ T-cell subsets was set depicted in Supplemental Figure 2A. **(A-J)** Shown are statistical analysis of the frequencies of CD4⁺ **(left panels)** and CD8⁺ **(right panels)** T-cell subsets expressing CCR5 **(A),** PD-1 **(B),** LAG-3 **(C**) or TIGIT **(D)** at baseline (**black circles**), week 12 (**red circles**) and week 24 (**grey circles**)**.** Grey bars represent the mean value. One-way ANOVA Tukey's multiple comparisons test significant P-values are indicated on the graphs.



Supplemental Figure 5: Effects of metformin treatment on the frequency of blood CD4⁺ and CD8⁺ T-cell subsets co-expressing the tissue-homing integrins $\alpha_4\beta_7$, $\alpha_4\beta_1$, $\alpha_E\beta_7$. Cryopreserved PBMCs from n=22 participants (Supplemental Table 1) at baseline, week 12 and week 24 were stained with a cocktail of specific Abs for flow cytometry analysis. The gating strategy for the identification of N, CM, TM, EM and TD CD4⁺ T-cell subsets was set depicted in Supplemental Figure 2A.**(A)** Shown is the gating strategy for flow cytometry identification of cells co-expressing the integrins $\alpha_4\beta_7$, $\alpha_4\beta_1$, $\alpha_E\beta_7$ in one representative participant (LILAC #5). **(B-E)** Shown are statistical analysis of the frequencies of N, CM, TM, EM, and TD CD4+ T-cell subsets co-expressing $\alpha_4\beta_7$ **(B)**, $\alpha_4\beta_1$ **(C)**, $\alpha_E\beta_7$ **(D)**, $\alpha_E\beta_1$ **(E)** at baseline (**black circles**), week 12 (**red circles**) and week 24 (**grey circles**). Grey bars represent the mean value. One-way ANOVA Tukey's multiple comparisons test significant P-values are depicted on the graphs.

Supplemental Table 1: **Clinical parameters of HIV-infected study participants receiving viral-suppressive antiretroviral therapy (ART).**

ID	COL	Age	Sex	Race	CD4[#]	CD8[#]	VL	ART	Time since aviremia *
LILAC #1	Yes	58	M	Cauc	428	1035	<40	Etravirine/Raltegravir/Norvir/Darunavir	9
LILAC #2	Yes	57	M	Cauc	395	581	<40	Truvada/Darunavir/Norvir	9
LILAC #3	Yes	53	M	Cauc	439	685	<40	Norvir/Raltegravir/Darunavir/Maraviroc	8
LILAC #4	Yes	61	M	Black	1045	1968	<40	Triumeq	4
LILAC #5	Yes	53	M	Cauc	106	324	<40	Tenofovir/ Ritonavir/Darunavir/Kivexa	9
LILAC #6	Yes	60	M	Cauc	244	701	<40	Dolutegravir/Etravirine/Darunavir/Norvir	7
LILAC #7	Yes	56	M	Cauc	512	615	ND	Raltegravir/Truvada	9
LILAC #8	Yes	53	F	Cauc	612	1104	<40	Reyataz/Complera/Raltegravir	10
LILAC #9	Yes	55	M	Cauc	155	533	<40	Tivicay/Truvada	7
LILAC #10	Yes	51	M	Black	618	1057	<40	Complera	6
LILAC #11	Yes	69	M	Cauc	465	708	ND	Dolutegravir/Ritonavir/Sarunavir	12
LILAC #12	Yes	56	M	Cauc	202	Unk	ND	Stribild	5
LILAC #13	Yes	54	M	Cauc	252	646	ND	Triumeq	3
LILAC #14	No	67	M	Cauc	616	940	ND	Edurant/Triumeq	12
LILAC #15	No	53	M	Black	670	918	ND	Kaletra/Kivexa	10
LILAC #16	No	65	M	Cauc	202	551	ND	Stribild	5
LILAC #17	No	61	M	Black	670	918	ND	Kivexa/Kaletra	7
LILAC #18	No	49	M	Cauc	252	646	<20	Triumeq	4
LILAC #19	No	51	F	Black	417	1271	<40	Stribild/Reyataz	unk
LILAC #20	No	61	M	Black	333	695	<40	Genvoya	unk
LILAC #21	No	41	M	Black	508	1200	<40	Atripla	unk
LILAC #22	No	57	M	Cauc	397	554	<40	Triumeq	unk

COL, colonoscopy; [#], cells/µl; [&], VL, viral load; HIV RNA copies per ml plasma; ND, not detected; ART, antiretroviral therapy; NA, information not available; *, years

Supplemental Table 2: Oligonucleotides sequences of primers and probes used for HIV-RNA and HIV-DNA quantification

Primers	Oligonucleotides sequences
ULF1	5’-ATGCCACGTAAGCGAAACTCTGGGTCTCTCTDGTTAGAC-3’
UR1	5’-CCATCTCTCTCCTTCTAGC-3’.
Lambda (λ) T	5’-ATGCCACGTAAGCGAAACT-3’
UR2	5’-CTGAGGGATCTCTAGTTACC-3’.
Alu 1	5’-TCCCAGCTACTGGGGAGGCTGAGG-3’
Alu 2	5’-GCCTCCCAAAGTGCTGGGATTACAG-3’
UHIV FamZen	5'-/56-FAM/CACTCAAGG/ZEN/CAAGCTTTATTGAGGC/3IABkFQ/-3'
HCD3OUT 5’	5’-ACTGACATGGAACAGGGGAAG-3’
HCD3OUT 3’	5’-CCAGCTCTGAAGTAGGGAACATAT-3’
HCD3IN 5’	GGCTATCATTCTTCTTCAAGGT
HCD3IN3’	CCTCTCTTCAGCCATTTAAGTA
CD3 FamZen	5'-/56FAM/AGCAGAGAA/ZEN/CAGTTAAGAGCCTCC AT/3IABkFQ/-3'
GusbF1	5’-GGTCGTGATGTGGTCTGTG-3’
GusbR1	5’-GTCGTGATACCAAGAGTAGTAGC-3’
GusbF2	5’-ACCTAGAATCTGCTGGCTACTA-3’
GusbR2	5’-GTTCAAACAGATCACATCCACATAC-3’
GUSB-HEX	5’-/5-HEX/TCGCTCACA/ZEN/CCAAATCCTTGGACC/3IABkFQ/-3’.
Tat1.4	5’-TGG CAG GAA GAA GCG GAG A-3’
Rev	5’-GGA TCT GTC TCT GTC TCT CTC TCC ACC-3’.
Tat2	5’-ACA GTC AGA CTC ATC AAG TTT CTC TAT CAA AGC A-3’
Rev	5’-GGA TCT GTC TCT GTC TCT CTC TCC ACC-3’.
msHIV FamZen	5’-/56-FAM/TTC CTT CGG /ZEN/GCC TGT CGG GTC CC/3IABkFQ/-3’.

Supplemental Table 3: Antibodies used for flow cytometry analysis

Antibody anti	Fluorochrome	Clone	Vendor
CCR4	Pe-Cy7	1G1	BD
CCR5	BV421	2D7/CCR5	BD
CCR6	BV650	11A9	BD
CCR7	PE-CF594	150503	BD
CD25	BV421	M-A251	BD
CD3	Alexa700	UCHT1	BD
CD38	PE	HIT2	BD
CD45RA	BV786	HIT100	BD
CD8	BUV395	RPA-T8	BD
CD8	PerCP-Cy5.5	RPA-T8	BD
CD95	Pe-Cy7	DX2	BD
CXCR3	APC	1C6/CXCR3	BD
CXCR5	BB515	RF8B2	BD
HLA-DR	APC	G46-6	BD
ITGB1	BB515	MAR4	BD
ITGB7	BUV737	FIB504	BD
PD-1	BUV737	EH12.1	BD
Bcl-2	FITC	6C8	BD
CCR5	FITC	2D7/CCR5	BD
CD38	APC	HIT2	BD
CCR6	PE	11A9	BD
CD127	PE-CF594	HIL-7R-M21	BD
ITGA4	PeCy7	9F10	Biolegend
ITGAE	BV605	Ber-ACT8	Biolegend
CD8	PerCP-Cy5.5	RPA-T8	Biolegend
CD19	PerCP-Cy5.5	HIB19	Biolegend
CD66b	PerCP-Cy5.5	CG10F5	Biolegend
CD3	Alexa700	UCHT1	Biolegend
CD4	Pe-Cy7	RPA-T4	Biolegend
CD326	BV650	9C4	Biolegend
HLA-DR	BV780	L243	Biolegend
CD45RA	APCeFluor780	HI100	Cederlane
FoxP3	PE	236A/E7	eBiociences
CD27	APC-eF480	O232	eBioscience
TIGIT	PerCP-e710	MBSA43	eBioscience
Integrin β7	FITC	FIB504	eBioscience
phospho-mTOR	eFLuor450	MRRBY	eBioscience
SAMHD1	FITC		From Dr O. Schwartz
Lag3	FITC	polyclonal	R&D

CHAPITRE 5 : PPARγ INHIBITION FOR HIV-1 RESERVOIR PURGING AND TH17 FUNCTIONAL (MANUSCRIT #3)

En révision au Journal of Clinical Investigation, juin 2019

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#, Contribution égale
**, Auteur de correspondance*

Contribution des auteurs:
DP a conçu et effectué les travaux de recherche surtout résultats dans la Figure 1 et les expériences sur des cellules T $CCR6^+/CCR6^-$ triés; elle a analysé les résultats, préparé les figures et a rédigé le manuscrit. YZ a initié le projet de recherche et généré des résultats préliminaires, en plus de ceux inclus dans les Figures 2-3 et les Figures supplémentaires 1-3, et a contribué à l'écriture du manuscrit. JPG a réalisé l'analysé des résultats de séquençage d'ARN et a généré des figures. MJR a réalisé la quantification de l'ADN/ARN viral dans la Figure 1. LRM, AF, HC, TRWS, and AG ont contribué à la collecte/préparation d'échantillons et/ou aux mesures de réservoir du VIH. JPR a organisé l'accès à des échantillons/informations cliniques, a mis en place des protocoles de recherche clinique et a réalisé le recrutement des participants. WWB and CG a fourni des protocoles pour les travaux sur mir29. EAC a fourni des protocoles et a contribué à l'écriture du manuscrit. NC a fourni des protocoles, a participé au design des expériences de mesure des réservoirs du VIH, et a contribué à l'écriture du manuscrit. PA a conçu le design de l'étude, dirigé la recherche, analysé les données, préparé des figures et a rédigé le manuscrit.

PPARγ Inhibition for HIV-1 Reservoir Purging and Th17 Functional Restoration

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Running title: PPARγ inhibition for HIV cure/remission
Abstract word count: 200 words
Text word count: 10,610
Number of figures: 8
Number of supplemental figures: 7
Number of Tables: 1
Number of Supplemental Tables: 4
Number of references: 125

ABSTRACT

The Th17-polarized $CCR6^+RORγt^+CD4^+$ T-cells, maintainers of mucosal barrier immunity, contribute to HIV reservoir persistence during antiretroviral therapy (ART). This raises the need for novel Th17-targeted remission/cure strategies. In addition to HIV-permissiveness factors, Th17-cells express PPARγ, a transcriptional repressor of multiple genes, including HIV and RORγt. Here, we investigated the effect of PPARγ pharmacological inhibition on HIV reactivation/replication and Th17 functions. As predicted, T-cell receptor triggering in the presence of the PPARγ antagonist T0070907 increased both cell-associated HIV-RNA levels and IL-17A expression in T-cells from ART-treated people living with HIV (PLWH). Unexpectedly, T0070907 inhibited HIV production *de novo* in viral outgrowth assay. Genome-wide RNA-sequencing analysis revealed pathways/transcripts modulated by T0070907 in $CCR6^+CD4^+$ T-cells, including down-regulated HIV-permissiveness (CCR5, furin) and up-regulated HIV transcription (CDK9, HTATIP2), HIV-restriction (Caveolin-1, TRIM22, TRIM5α, BST2, miR-29), and Th17-specific (RORγt, STAT3, BCL6 IL-17A/F, IL-21) transcripts. IL-21, a Th17 survival cytokine, exhibits miR-29-dependent antiviral properties. Functional validations using miR-29 antagomirs point to the existence of T0070907-mediated miR-29-independent mechanisms, likely located at the level of virion production and/or spread. Altogether, the PPARγ inhibition may represent a new promising therapeutic strategy to reactivate HIV reservoirs, while preventing *de novo* infection and boosting Th17-specific functions required for mucosal immunity restoration during ART.

Key words: HIV-1, ART, CD4+ T-cells, Th17, PPARG, T0070907, IL-21

INTRODUCTION

Combined antiretroviral therapies (ART) efficiently control HIV-1 replication to undetectable plasma levels and have improved the life expectancy of people living with HIV (PLWH) (1-4). However, ART does not cure HIV. Latent viral reservoirs persist in long-lived memory $CD4^+$ T-cells during ART, leading to viral rebound rapidly occurring upon treatment interruption (3, 5-12). In addition, immunological alterations, mainly located at mucosal barrier surfaces such as the gut-associated lymphoid tissues (GALT), persist and represent a cause of increased risk for non-AIDS co-morbidities in ART-treated PLWH (13). Alternative therapeutic interventions are therefore needed to purge viral reservoirs and restore immunological competence in PLWH.

HIV infects and persists in a small fraction of $CD4^+$ T-cells (4, 6, 14-16). Studies by our group and others demonstrated that Th17-polarized $CD4^+$ T-cells efficiently support HIV replication as they express multiple HIV permissiveness factors and lack anti-HIV defense mechanisms. Subsequently, Th17 cells are depleted from the GALT during chronic HIV/SIV infection and their frequency is not restored with ART. This leads to dramatic alterations in mucosal barrier integrity, increased microbial translocation from the gut, and systemic immune activation, all leading to non-AIDS co-morbidities [reviewed in (17, 18)]. Of note, the preservation of mucosal Th17 cells upon HIV infection is associated with slow disease progression (19-24). In addition to infection *per se*, several other mechanisms contribute to Th17 depletion/dysfunction in chronically infected PLWH and their non-restoration upon long-term ART [reviewed in (17, 18)]. These mechanisms include an alteration of Th17 cell gut-homing capacity (25, 26), a decreased frequency of Th17 precursors (27, 28), an impaired Th17-lineage differentiation potential (29-31); suboptimal drug concentration in Th17 cells (32); and a deficit in IL-21 (33, 34), a survival factor for Th17 cells (35). Although the depletion of mucosal Th17 cells is well-documented during HIV/SIV infection, a fraction of Th17 cells is long-lived and are enriched in HIV reservoir in the blood and colon of ART-treated PLWH [reviewed in (17, 18)]. Considering the key role played by Th17 cells in mucosal homeostasis, their contribution to HIV persistence,

as well as the deleterious consequence of their paucity in ART-treated PLWH, novel Th17-specific target strategies are needed for HIV remission (reviewed in (18)).

In addition to multiple HIV permissiveness factors (*e.g.,* CCR5, NF-κB, mTOR), Th17-polarized memory $CD4^+$ T-cells display a distinctive transcriptional signature that includes the *peroxisome proliferator-activated receptor gamma* (PPARγ) (36-39), an intrinsic negative regulator of NF-κB (40), as well as an inhibitor of HIV transcription (36, 41-43). PPARγ is a member of the PPAR subfamily of ligand-dependent non-steroid nuclear receptors; PPARγ forms an obligatory heterodimer with *retinoic X receptor* (RXR) and binds onto PPAR responsive elements (PPREs) expressed on the promoters or other regulatory regions of specific genes, thus functioning as transcriptional repressor or activator (40, 44-48). PPARγ is expressed by multiple immune and non-immune cells, acts as a lipid sensor that controls the expression of multiple genes involved in lipid/glucose metabolism. Natural/synthetic PPARγ agonists are documented to regulate metabolic/inflammatory processes (40, 47-52), in part *via* the mTOR activation pathway (53). Noteworthy, PPREs are present in the HIV long terminal repeat (LTR) region, indicative that PPARγ participates directly in the negative regulation of HIV transcription (54). Increasing evidence supports a role of PPARγ in the regulation of immunity by acting on T-cell proliferation and differentiation (50-52, 55-58). Of particular importance, it was reported that PPARγ triggering inhibits Th17 differentiation *via* the transcriptional repression of RORγt (55, 56), the master regulator of Th17 differentiation (59).

Although the PPARγ activation blocks HIV replication (36), studies in SIV-infected rhesus macaques demonstrated that hematopoietic alterations caused by Nef are dependent on the PPARγ activation and are mimicked by the PPARγ agonist rosiglitazone (RGZ) (60, 61). Based on these results, Prost *et al.* concluded that PPARγ inhibition may be more appropriate to counteract hematopoietic alterations caused by HIV/SIV infections (60, 61) and emphasized the need for the development of clinically advanced PPARγ antagonists for HIV remission/cure strategies.

In this study, we investigated the use of PPARγ pharmacological inhibition for Th17-specific immunological restoration and viral reservoir eradication strategies in ART-treated PLWH. Our results demonstrate that the PPARγ antagonist T0070907 combines three unique features linked to ***i)*** viral latency reactivation, ***ii)*** *de novo* infection blockade, and ***iii)*** Th17 effector function boosting. These results point to the beneficial use of PPARγ antagonism as a new therapeutic strategy toward HIV cure/remission.

RESULTS

T0070907 increases IL-17A and HIV transcription but reduces the release of virions in CD4$^+$ T-cells of ART-treated PLWH. We hypothesized that the pharmacological inhibition of PPARγ, a transcriptional repressor of RORγt and HIV, will boost RORγt-mediated Th17 effector functions and reactivate latent HIV reservoirs. To test this hypothesis, memory CD4$^+$ T-cells isolated from ART-treated PLWH (Table 1) were stimulated with CD3/CD28 Abs for 2 days to induce optimal PPARγ expression (36), and further cultured in the presence or in the absence of the PPARγ antagonist T0070907 for 2 additional days. To study the late steps of HIV replication at transcriptional and post-transcriptional level while preventing *de novo* infection in culture, T-cell receptor (TCR) triggering and T0070907 exposure were performed in the presence of the antiretroviral drugs (ARV) that inhibit virion maturation (Saquinavir) and HIV-DNA integration (Raltegravir) (Figure 1A). Preliminary experiments allowed us to identify the optimal T0070907 concentration (10 μM) that up-regulated IL-17A production without affecting cell viability/proliferation (data not shown). Cell-associated (CA) HIV-DNA levels remained similar in T-cells exposed or not to T0070907 (Figure 1B) and were used for the normalization of CA HIV-RNA levels. As predicted, T0070907 significantly increased CA Gag HIV-RNA levels (Figure 1C), as well as IL-17A mRNA expression in all donors tested (Figure 1D). Unexpectedly, viral production in cell-culture supernatants, measured as cell-free (CF) Gag HIV-RNA levels, was significantly reduced by T0070907 in 7/8 donors (Figure 1E). These results reveal the unique ability of T0070907 to reverse the PPARγ-mediated repression of HIV and RORγt transcription while decreasing *de novo* production of virions. These results point to a T0070907-mediated post-transcriptional block of the viral replication cycle, before and/or during the HIV budding/release steps.

T0070907 inhibits HIV outgrowth from CD4$^+$ T-cells of ART-treated PLWH

HIV replication is regulated at multiple transcriptional and post-transcriptional steps (1, 62). To further investigate the T0070907 mechanism of action, we used a viral outgrowth assay (VOA) established in our laboratory (38, 39). Briefly, memory CD4$^+$ T-cells isolated from ART-

treated PLWH (Table 1, n=4) were stimulated *via* CD3/CD28 (to induce viral reservoir reactivation and propagation in culture) in the presence or absence of T0070907 for 12 days (Figure 2A). Replication-competent HIV propagated in VOA, measured by the frequency of cells expressing HIV-p24, was detected upon CD3/CD28 triggering, but significantly reduced in the presence of T0070907 (Figure 2B-C). Of note, T0070907 had no significant impact on cell viability (Figure 2D). To determine whether T0070907 limits HIV propagation in IL-17A-producing cells, intracellular HIV-p24 expression was quantified in cells expressing IL-17A and/or IFN-γ identified as follows: IL-17^{+}IFNγ^{-} (Th17) and IL-17^{+}IFNγ^{+} (Th1Th17), IL-17^{-}IFNγ^{+} (Th1) and IL-17^{-}IFNγ^{-} (Th0) (Figure 2E-F). The frequency of IL-17A-producing cells remained stable at day 12 post-CD3/CD28 triggering in the presence/absence of T0070907 (data not shown). In the absence of T0070907, HIV-p24^{+} cells were enriched in Th17 and Th1Th17 compared to Th1 and Th0 subsets, consistent with previous studies [reviewed in (17, 18, 63)]. In the presence of T0070907 there was a significant decrease in the frequency of HIV-p24^{+} T-cells in all Th subsets, including Th17 and Th1Th17 (Figure 2F-G).

Considering the stochastic distribution of HIV reservoirs, to further strengthen our results, the viral outgrowth was further measured by ELISA in cell-culture supernatants from experimental quadruplicates (10^6 cells/well) collected at day 3, 6, 9 and 12 post-culture performed in the presence/absence of T0070907. Results generated with cells from n=2 additional donors (Table 1) confirmed that HIV outgrowth was significantly reduced when CD3/CD28 triggering was performed in the presence of T0070907 (Figure 2H).

The PPARγ agonist rosiglitazone (RGZ) inhibits HIV replication (36, 64) by repressing HIV transcription (54). Similar to T0070907, a significant but less robust inhibition of the viral outgrowth was observed in the presence of RGZ (50 μM, optimal dose previously identified (36)) in HIV+ART individuals (Table 1; n=4) (Supplemental Figure 1A-B, D), with no changes in cell viability (Supplemental Figure 1C). Nevertheless, in contrast to T0070907 that increased IL-17A expression (Figure 1B), RGZ blunted IL-17A production (Supplemental Figure 1E), consistent with the ability of PPARγ agonist to repress RORγt expression (55, 56). Thus, the

PPARγ antagonism inhibits viral outgrowth likely by acting on viral replication steps downstream of viral transcription, steps that are important for *de novo* viral particle production and/or propagation/spread.

T0070907 inhibits HIV replication *in vitro*

We further investigated the ability of T0070907 to modulate HIV replication in memory $CD4^+$ T-cells *in vitro*. For this, we used the transmitted/founder (T/F) HIV strain THRO, documented to exhibit high virulence and resistance to the antiviral effects of type I IFN (65-67). HIV_{THRO}-infected memory $CD4^+$ T-cells were treated with different concentrations of T0070907 (10 µM, 5 µM and 1 µM) for up to 9 days. Results demonstrate a dose-dependent effect of T0070907, with an optimal decrease in HIV replication and increase in IL-17A production observed at 10 µM, and no significant effects on cell viability and proliferation (Supplemental Figure 2A-D).

To get further insights into the mechanisms of action of T0070907, we investigated its effect on the expression of the HIV receptor CD4 and co-receptors CCR5 and CXCR4. Although T0070907 does not significantly change CD4 and CXCR4 expression, a significant decrease in CCR5 expression was observed on memory $CD4^+$ T-cells activated *via* the TCR and cultivated in the presence and the absence of T0070907 (Supplemental Figure 3A-D). Thus, T0070907 may limit the propagation of newly produced virions in culture, in part, by limiting CCR5-mediated HIV entry in bystander target T-cells.

T0070907 induces the expression of the antiviral factor cholesterol-25-hydroxylase. The interferon-inducible cholesterol-25-hydroxylase (CH25H), an enzyme over-expressed in PPARγ-deficient macrophages (68), has been previously identified for its ability to inhibit HIV replication *via* the conversion of cholesterol into 25-hydroxycholesterol (25HC) (Figure 3A) (69). The CH25H/25HC-mediated mechanism of action involves blocking the early steps of HIV replication (between fusion and reverse transcription) but not HIV transcription (69), with the effects of CH25H/25HC on the late steps of HIV replication (*e.g.,* virion production) remaining unexplored. This raised the possibility that T0070907 blocks HIV replication through a

CH25H/25HC-dependent mechanism. To test this possibility, CH25H mRNA levels were quantified in TCR-activated memory $CD4^+$ T-cells cultured in the presence/absence of T0070907. Result in Figure 3B show that T0070907 induces CH25H mRNA expression in $CD4^+$ T-cells from 3/3 donors (fold changes: 3.3, 3.3 and 1.6). Then, TCR-activated $CD4^+$ T-cells exposed to HIV infection *in vitro* were cultured in the presence or absence of exogenous 25HC (50 nM, optimal dose identified by titration to avoid cell toxicity) up to 9 days post-infection. In this model of multiple-round of HIV replication, 25HC, similar to T0070907, reduced levels of integrated HIV-DNA (Figure 3C) and HIV-p24 in cell culture supernatants (Figure 3D). Finally, the impact of 25HC on HIV outgrowth was assessed in memory $CD4^+$ T-cells of ART-treated PLWH (Figure 3E-F), using the experimental design illustrated in Figure 2A. Consistently, 25HC decreased HIV-p24 expression in cell-culture supernatants (Figure 3E) and in all Th subsets, notably Th17 and Th1Th17 cells (Figure 3F). Together, these results confirm the antiviral potential of 25HC and point to CH25H/25HC as a potential mechanism of action for T0070907.

T0070907 inhibits HIV replication in $CCR6^+CD4^+$ T-cells *via* CH25H-independent mechanisms

Because IL-17A production and HIV replication/persistence mainly occurs in $CCR6^+CD4^+$ T-cells (39, 70-73), we further tested the immune-regulatory and antiviral effects of T0070907 in flow cytometry-sorted memory $CCR6^+$ and $CCR6^-$ T-cells. To this end, we used a model of multiple-round HIV infection *in vitro*, in which TCR-activated T-cells were exposed to HIV for 3 hours and then cultured in the presence or in the absence of T0070907. HIV replication was evaluated by integrated HIV-DNA quantification. In the absence of T0070907, integrated HIV-DNA levels were significantly higher in $CCR6^+$ compared to $CCR6^-$ T-cells (2 $\log_{10}$ difference) (Figure 4A), consistent with their reported superior permissiveness to infection (39, 70-73). T0070907 robustly reduced HIV replication in $CCR6^+$ T-cells, without significant effects on basal levels of HIV-DNA integration in $CCR6^-$ T-cells (Figure 4A). As predicted, T0070907 increased IL-17A expression/production preferentially in sorted $CCR6^+$ T-cells (Figure 4B-C). Further RT-PCR quantifications demonstrated the highest levels of CCR5 mRNA expression in $CCR6^+$ *versus* $CCR6^-$ T-cells, with exposure to T0070907 resulting in decreased CCR5 mRNA expression in $CCR6^+$ T-cells (Figure 4D). Surprisingly, while CH25H mRNA expression was up-regulated by

T0070907 in CCR6$^-$ T-cells, this expression remained low/undetectable in CCR6$^+$ T-cells (Figure 4E). These results demonstrate that T0070907 exerts its antiviral effects in CCR6$^+$ T-cells *via* CH25H/25HC-independent mechanisms.

RNA-Seq reveals T0070907-mediated transcriptional reprogramming in memory CCR6$^+$CD4$^+$ T-cells

To identify mechanisms by which T0070907 exerts its virological and immunological effects, we performed genome-wide transcriptional profiling using the Illumina RNA-Seq technology in CCR6$^+$ T-cells stimulated *via* the TCR for 3 days and cultured in the presence or absence of T0070907 for additional 18 hours (Figure 5A). This experimental strategy was selected based on preliminary experiments demonstrating optimal detection of CH25H mRNA in T-cells at this specific time point (Supplemental Figure 4A-B), assuming that other HIV restriction factors will be expressed in CCR6$^+$ T-cells at the same time point.

To extract meaning from the large sets of differentially expressed genes generated by RNA-Seq, we employed an intelligent search and multiple systematic analysis methods. First, **Differentially Expressed Genes (DEG)** were classified based on *P-values* (P) or *adjusted P-values* (adj. P) and fold change (FC) gene expression. Profound transcriptional changes were induced by T0070907 in CCR6$^+$ T-cells, with 4,002 transcripts up-regulated and 1,249 transcripts down-regulated (adj. P<0.05; FC cutoff, 1.3) (Figure 5B), with top 50 up-regulated (adj. P<0.05; FC>8) and down-regulated (adj. P<0.05; FC<-3.2) transcripts listed in Supplemental Tables 1 and 2, respectively.

An intelligent search performed among the top modulated DEG revealed the regulation of transcripts linked to the regulation of HIV replication (up-regulated: FMOD, CAV1, SERINC5; down-regulated: RARRES3, IL-16, furin), T-cell activation *via* the TCR (up-regulated: NR4A3, FASLG; down-regulated: LIME1); circadian clock machinery (up-regulated: ARNTL/BMAL1, NFIL3); immune check-point negative regulators (up-regulated: CD160, CTLA4, CD152, PD-1/PDCD1, TIGIT, LAG3); and Th17 differentiation (up-regulated: IL-21, IL-17A, CSF2, IL-22,

CCL20), and follicular helper T cell (Tfh) differentiation (up-regulated: IL-21, CXCR5, ICOS, BCL6, PD-1, CD40L, IL-10, IL-4) (Supplemental Tables 1-2).

Gene Set Variation Analysis (GSVA) was then used for the identification of Gene ontology (GO) Biological processes (false discovery rate (FDR) <0.05) using the Broad Institute data base (MSigDB C2, V6.2). This analysis identified 71 pathways modulated by T0070907 in $CCR6^+$ T-cells (Figure 5C), including top modulated pathways linked to the following GO terms: *i)* lipid/phospholipid and glucose metabolism (Supplemental Figure 5A-C), *ii)* inflammation/immune response to type I interferon (Supplemental Figure 5D), *iii)* and cytokines, chemokines and adhesion molecules (Supplemental Figure 5E-H). Among DEG linked to the GO term lipid/phospholipid metabolism, one notes the up-regulation of the transcription factors PPARγ, PPARα, KLF4 and NR4A3; the pattern recognition receptor NOD2; the tetraspanin CD81; the signaling molecules PTK2, PLA2G6, FGF2 and FLT1; the guanine nucleotide exchange factor VAV3; the hormone ADIPOQ/adiponectin; the cytokines TNF and IFNG; as well as the down-regulation of the ATP transporter ABCG1; the G protein RAC1; and the cell cycle regulator CDC42 (Supplemental Figure 5A-B). Among DEG linked to the GO term glucose metabolism, one notes the up-regulation of the glycosylphosphatidylinositol (GPI) degrading enzyme GPLD1, the insulin-like growth factors (IGF) 1 and IGF2, and the phorbol-12-myristate-13-acetate-induced protein 1 (PMAIP1); as well as the down-regulation of the enzymes tyrosine-protein phosphatase non-receptor type 2 (PTPN2) and diglyceride acyltransferase (DGAT2) (Supplemental Figure 5C). Moreover, DEG linked to the GO term inflammation/immune response to type I interferon were mainly down-regulated by T0070907 and included genes documented to play a positive/negative regulatory role in HIV replication such as ADAR, MX2, MX1, OAS1, RNASEL, SAMHD1, ISG15, ISG20, IFITM2, IFITM3, and TRIM56; of note, transcripts coding for the restriction factor BST2 were up-regulated (Supplemental Figure 5D). Furthermore, DEG related to the GO terms cytokines, chemokines and adhesion molecules included up-regulated transcripts for chemokine receptors (CXCR5, CXCR4, CX3CR1, CCR8), chemokines (CCL20, CCL1, XCL1, XCL2), cell-to-cell adhesion molecules/immune checkpoints (CD276/B7-H3, LAG3, CTLA4, TIGIT), and cytokines/cytokine regulators (IL-4, IL-

10, CD28, BCL10, STAT5B, CD3E, CCL20, CD80, IL-21, KLF4, IFNG, TLR9, TNF, TNFAIP3, IRAK3, AXL, PTPN22); as well as down-regulated transcripts for chemokine receptors (CCR1-3, CCR5, CCR7, CCR9, CCR10, CXCR3, CXCR6), chemokines (CCRL2), cell-to-cell adhesion molecules (CD274/PD-L1, LGALS9, CD300A, CD74, CEACAM1, TNFSF14, LGALS3, TNFSF4), and cytokine biosynthesis (TLR1, NFKB1, LTB, TLR6, NLRC3, RARA) (Supplemental Figure 5E-H).

Finally, Ingenuity Pathway Analysis (IPA) revealed the modulation of transcripts previously linked to the negative (*e.g.,* IL-21, CAV1, BST2) *versus* positive (*e.g.,* furin) control of HIV replication (Figure 5D). Considering the well-documented role of IL-21 in modulating Th17 and Tfh survival (35, 74), as well as its antiviral properties (75-77), we pursued to the validation of IL-21 at the protein level. Results generated with memory CCR6^{+} T-cells from five different individuals confirmed the significant up-regulation of IL-21 production by T0070907 (Figure 5E). These results point to IL-21 as a key transcript modulated by T0070907, thus providing one potential molecular explanation for the immunological/virological consequences of PPARγ inhibition.

T0070907 exerts its antiviral activity *via* miR-29-independent mechanisms

IL-21 is documented to exert its antiviral functions *via* the induction of miR-29 (75), a non-coding RNA that reduces HIV replication by interfering with Nef (78). Consistently, IPA of T0070907-modulated transcripts in CCR6^{+} T-cells (Supplemental Figure 7) revealed the interactome linked to IL-21 and the connection with miR-29 *via* the up-regulation of STAT3 (75). Thus, we hypothesized that T0070907-mediated post-transcriptional antiviral effects are mediated at least in part *via* the IL-21/miR-29 axis. To test this hypothesis, TCR-activated memory CD4^{+} T-cells from 2 different donors were nucleofected with synthetic miR-29 antagomir locked nucleic acids or a control non-targeting antagomir, infected with HIV_{THRO}, and cultured in the presence/absence of T0070907. Consistent with previous reports (75), sequestration of miR-29 from their target mRNA with specific antagomirs boosted HIV replication in the absence of T0070907; however, this effect was not observed in the presence of T0070907 (Figure 7). In conclusion, although these results do not exclude the contribution

of miR-29 to the antiviral effects of T0070907, they support the existence of other miR-29-independent mechanisms by which T0070907 blocks *de novo* HIV production and/or viral spread.

Multiple HIV-dependency factors are modulated by T0070907

A meta-analysis using the NCBI HIV-1 interactions database allowed the identification of human genes previously described to be involved in HIV-1 infection that were modulated by T0070907 in $CCR6^{+}$ T-cells. Specifically, the up-regulated transcripts included TRIM5, TNF, TRIM22, BST2, IL-2, IL-3, LIF, IL-10, CXCR4, SERP1, and CD4; while the down-regulated transcripts included VIM, CCR5, IFITM1, OASL, NFKB1, ISG15, IFIT2, OAS2, OAS1, IFIT35, STAT1, IL15, MX2, MX1, and USP18 (Figure 7). In addition, among transcripts documented to mediate HIV transcription, T0070907 up-regulated the expression of the nuclear receptor co-activators NCOA1-3, the nuclear factor of activated T-cells, cytoplasmic 1 (NFATC1), the HIV-1 Tat Interactive Protein 2 (HTATIP2), CD3E, CD3D, IKBKB, and CDK9; while down-regulating the expression of MAPK1, NOX1, and the DNA-directed RNA polymerases POLR2C, POLR2H, POLR2D, POLR2E, POLR2F, and POLR2L (Supplemental Figure 5).

Together, these results reveal that the T0070907-mediated transcriptional reprogramming negatively regulates multiple steps of the viral replication cycle such as CCR5-mediated entry, the uncoating (*e.g.,* TRIM5, TRIM22), reverse transcription (*e.g.,* SAMHD1), release (*e.g.,* BST2), Env processing (*e.g.,* furin); while promoting HIV transcription (*e.g.,* HTATIP2, CDK9) and Th17 effector functions (*e.g.,* IL-17A, IL-21).

DISCUSSION

Study findings demonstrate the unique features combined by the PPARγ antagonist T0070907 in reversing HIV latency in memory $CD4^+$ T-cells of ART-treated PLWH, as well as boosting Th17/Tfh-specific effector functions, while limiting *de novo* virion production and/or spread from HIV reservoir cells. By using a genome-wide transcriptional profiling in Th17-polarized $CCR6^+CD4^+$ T-cells, we revealed the complex transcriptional reprogramming mediated by T0070907, with antiviral mechanisms located at multiple steps of the HIV replication cycle, including CCR5-mediated entry, reverse transcription, budding and/or HIV envelope processing (Figure 8).

According to the knowledge that PPARγ acts as a repressor of HIV (54) and RORγt transcription (55, 56), we demonstrate that the pharmacological inhibition of PPARγ by T0070907 (79) in TCR-activated $CD4^+$ T-cells boosted HIV transcription and the RORγt-mediated transcription of Th17-specific genes. In contrast, there was an unpredicted block in viral production and/or spread observed during viral outgrowth *ex vivo* and infection *in vitro*. Upon ligand binding, the PPARγ/RXR heterodimer binds to PPREs within the promoter of target genes (45), including genes involved in lipid metabolism such as cholesterol and fatty acids (40, 44-48, 80). Cholesterol and its derivatives influence multiple aspects of immunity, including antiviral immunity. Among oxysterols presenting antiviral properties, 25HC, metabolized from cholesterol by the enzyme CH25H, blocks the replication of HIV by acting on the viral entry but not transcription (69, 81); the effects of 25HC on the post-transcriptional steps of the HIV replication cycle remain unexplored. Of note, PPARγ deficiency was previously linked to CH25H overexpression (68). In addition to its antiviral properties, 25HC was identified as a natural ligand for RORγt (82, 83). Altogether, this knowledge prompted our initial hypothesis that T0070907 blocks HIV outgrowth *ex vivo* and infection *in vitro via* the induction of CH25H expression, and subsequently 25HC production. This hypothesis was in line with the possibility that 25HC may also contribute to up-regulated RORγt-mediated IL-17A expression/production and reduced viral spread. In agreement with this hypothesis, T0070907 indeed up-regulated

the expression of CH25H mRNA in TCR-activated memory $CD4^+$ T-cells. Nevertheless, despite the ability of T0070907 to strongly inhibit HIV replication in flow-cytometry sorted $CCR6^+$ Th17 cells, our results demonstrated CH25H mRNA expression in $CCR6^-$ but not $CCR6^+$ T-cells. Thus, in $CCR6^+$ T-cells the T0070907-mediated block in HIV production relies on CH25H/25HC-independent mechanisms.

A genome-wide transcriptional profiling provided valuable insights into the T0070907-mediated mechanisms of action in $CCR6^+$ T-cells. GSVA identified activation of pathways linked to lipid/phospholipid and glucose metabolism. Metabolic reprogramming during TCR triggering train T-cells to integrate immunological and metabolic information required for the subsequent acquisition of specific effector functions (84, 85). Glucose metabolism has been identified to play a central role in HIV replication including a preferential infection of T-cells that highly express the glucose transporter GLUT1 (86, 87). In addition, metabolism disruption is associated with HIV disease progression, with higher glucose uptake being observed $CD4^+$ T-cells of HIV-infected compared to non-infected individuals (88). Importantly, recent studies described a positive correlation between the metabolic activity of different stages of $CD4^+$ T-cell differentiation and their susceptibility to HIV infection (89). Of note, changes in metabolic programs of $CD4^+$ T-cells are controlled by the mTORC1/PPARγ axis (84, 90). In line, T0070907 up-regulated genes associated with the PI3K/Akt, a pathway leading to mTOR activation (18). Indeed, several groups including ours, identified mTOR as a positive regulator of HIV replication (39) by acting at the level of viral entry (91) and transcription (92, 93). Therefore, the activation of the Akt/PI3K pathway in presence of T0070907 might be in part responsible of the increase in HIV transcription, likely *via* mTOR phosphorylation.

GSVA of GO pathways modulated by T0070907 in $CCR6^+$ T-cells revealed that PPARγ antagonism produces profound transcriptional modifications linked to the metabolism of cellular membrane components, including glycosaminoglycan, glycosphingolipid and sphingolipid. These components of the cellular membrane play a key role in membrane organization and membrane raft formations (52). Membrane receptors such as the HIV co-

receptors CCR5 and CXCR4 are recruited to the membrane raft and the clustering of theses receptors promotes HIV entry into target cells (94). In addition to their roles in virus entry, membrane rafts play a crucial role in HIV-1 assembly and release (95, 96). Therefore, modification of the cellular composition and membrane raft formation by T0070907 may contribute to the decreased HIV entry/release; additional investigation are needed in this sense.

The GSVA of GO pathways revealed the down-regulation of pathways/transcripts linked to interferon responses, with multiple interferon-stimulated genes, documented to restrict HIV replication, being down-regulated by T0070907 in $CCR6^{+}$ T-cells. Among these transcripts, we noted a decreased expression of SAMHD1, which limits HIV reverse transcription and promotes HIV-RNA degradation (97-100); MX2, which limits viral decapsidation, pre-integration complex formation and nuclear import (101, 102); IFITM2 and IFITM3, known to interact with HIV-1 Env in infected cells and impair Env processing and incorporation into virions (103-105); and ISG15, known to induces ISGylation of viral Gag proteins and impeded HIV release (106). These results uncover the capacity of T0070907 to interfere with HIV outgrowth/replication likely *via* mechanisms independent of the above listed HIV restriction factors. These results also point to a potential direct implication of PPARγ in the positive direct/indirect transcriptional regulation of specific HIV-restriction factors, including SAMHD1, MX2, IFITM2, IFITM3, BST2, and ISG15, in line with the antiviral program promoted by PPARγ activation (36).

Our RNA-Seq data also demonstrated that T0070907 increases the expression of transcripts that represent classical Tfh markers, including CXCR5, ICOS, BCL6, PD-1, CD40L, IL-10 and IL-21. Our results are in line with previous studies demonstrating that PPARγ activation prevents Tfh differentiation (58). By boosting IL-21 production T0070907 may improve Th17/Tfh survival and their effector functions. Indeed, in a model of SIV infection, the IL-21 supplementation of ART reduced inflammation, restored mucosal Th17 frequency, decreased the size of viral reservoir (34, 76, 107, 108), and also delayed viral rebound upon ART interruption (76). In addition to its immune-regulatory properties, IL-21 exhibits antiviral functions *via* the

induction of miR-29 (75), with the IL-21/miR-29 axis being linked to slow HIV disease progression (77). MiR-29 targets HIV-Nef for degradation and decreases viral replication in Jurkat cell line (78). In the absence of T0070907, we validated the capacity of antagomirs against miR-29 family to significantly reduce HIV replication *in vitro*. However, the miR-29 antagomirs did not counteract the antiviral effects of T0070907, indicative that the pleiotropic effects of T0070907 on $CCR6^+$ T-cells involve most likely miR-29-dependent and -independent mechanisms.

Finally, the meta-analysis we performed using the NCBI HIV-1 interaction data-base pointed to alternative mechanisms by which T0070907 likely interfere with HIV outgrowth/replication in $CCR6^+$ T-cells. Specifically, T0070907 up-regulated expression of CAV1, reported to inhibit HIV particle production in macrophages (109); SERINC5, which is incorporated into virions and prevents the fusion of the virion with the cellular membrane of a new target cell (110); TRIM22, which blocks Gag migration to the plasma membrane and inhibits HIV particle production (111); and BST2, which limits viral particle release (112). A T0070907-mediated up-regulation of the HIV restriction factor TRIM5α, which interacts with the HIV capsid and induce its proteasomal degradation leading to premature decapsidation (113), was also observed. In contrast, T0070907 down-regulated furin, a protease preferentially expressed in Th17 cells (36, 37) involved HIV protein Env maturation and virion infectivity (114). Thus, the antiviral features of T0070907 are likely mediated by mechanisms dependent on CAV1, SERINC5, TRIM22, and BST2 over-expression, as well as furin down-regulation, thus explaining a post-transcriptional block in HIV virion production and/or release. At this point, it remains unclear whether T-cells carrying HIV reservoir express viral proteins upon T0070907 exposure. Addressing this question will be essential for the recognition of HIV reservoir cells by the immune system in view of their elimination in "*shock and kill*" strategies. Future studies should clarify these aspects using ultrasensitive HIV-p24 quantification methods at intracellular levels (Quanterix technology (115)), as well as single-cell level strategies for HIV RNA and protein quantification (HIV FlowFISH: (116)).

In conclusion, our results support a model in which the PPARγ antagonist T0070907 limits productive HIV infection in $CCR6^+$ Th17 cells by mechanisms independent of CH25H, a cholesterol metabolizing enzyme uniquely expressed by $CCR6^-$ non-Th17 cells, and miR-29, involved in antiviral mechanisms promoted by IL-21 (Figure 8). T0070907 abrogates the PPARγ-mediated repression of HIV transcription, while negatively interfering with multiple early/late steps of the HIV replication cycle, including CCR5-mediated entry, reverse transcription, budding and/or HIV envelope processing. This unique combination of HIV latency reversing and antiviral features deserves to be further explored in HIV cure strategies. Finally, the ability of T0070907 to boost expression of Th17/Tfh-specific transcripts, including IL-21, supports the benefits of PPARγ pharmacological inhibition in mediating mucosal immunity restoration in ART-treated PLWH.

MATERIALS AND METHODS

Study participants

PLWH receiving viral suppressive ART study participants (Table 1) and HIV- individuals (n=15 males; n=2 females) were recruited at the Montreal Chest Institute, McGill University Health Centre and Centre Hospitalier de l'Université de Montréal (CHUM) in Montreal, Quebec, Canada. Large quantities of PBMCs (10^9–10^{10} cells) were collected by leukapheresis from these study participants as previously described (117).

Ethics statement

This study, using PBMC samples from healthy HIV-uninfected subjects as well as HIV-infected study participants receiving ART, was conducted in compliance with the principles included in the Declaration of Helsinki. This study received approval from the Institutional Review Board (IRB) of the McGill University Health Centre and the IRB of the CHUM-Research Centre, Montreal, Quebec, Canada. All human leukapheresis donors provided written informed consent for their participation to the study. All human study participants agreed with the publication of the subsequent results generated using the biological samples.

Drugs

The following drugs were used in this study: T0070907 (T007; 2-Chloro-5-nitro-*N*-4-pyridinylbenzamide; Tocris, Cayman Chemical, Michigan, USA); rosiglitazone (RGZ; Cayman Chemical, Michigan, USA), antiviral drugs (ARVs; Saquinavir and Raltegravir) (NIH AIDS Reagent Program, Maryland, USA).

Flow cytometry analysis

The following fluorochrome-conjugated Abs were used for polychromatic flow cytometry analysis: HIV-p24-FITC, IL-17A-PE, IFN-γ-Alexa700, CD4-Pacific Blue, CCR5-PE, and CXCR4-PE (Supplemental Table 3). A viability dye (Molecular Probes® LIVE/DEAD® Fixable Dead Cell Stain Kits, Invitrogen) was used to exclude dead cells. Intracellular staining was performed using

Fixation/Permeabilization Solution Kit (BD). Cells were analyzed using a LSRII cytometer, Diva version 6 (BD Biosciences, San Jose, CA), and FlowJo version 10.0.6 (Tree Star, Inc). Flow cytometry gates were defined using the *fluorescence minus one* (FMO) strategy (70, 118).

Cell sorting

Total $CD4^+$ T cells were enriched from PBMCs by negative selection using magnetic beads (magnetic-activated cell sorting [MACS], Miltenyi), with a purity of >95%, as previously described (39, 72, 73). Cells were then stained with CD45RA-APC-Cy7 and CCR6-PE Abs and a cocktail of FITC-conjugated Abs to exclude $CD8^+$ T-cells (CD8), NK cells (CD56), and B cells (CD19) (Supplemental Table 3). The sorting gates were set on lineage-$FITC^{neg}$ memory ($CD45RA^{neg}$) T-cells and then cells expressing or not CCR6 were subsequently sorted by flow cytometry (BDAria III). The viability dye was used to exclude dead cells. Post-sort quality control analysis demonstrated >97% $CCR6^+/CCR6^-$ T-cell purity, as previously reported (39, 72, 73).

Viral outgrow procedure *ex vivo*

Viral outgrowth was measured using a protocol previously established by our group (38, 39). Briefly, memory $CD4^+$ T-cells were isolated by MACS from PBMCs of PLWH receiving viral-suppressive ART (PLWH+ART) (Table 1). Cells were cultured in complete medium (RPMI1640, 10% FBS, 1% antibiotics) at $1x10^6$ cells/ml per well of 48-well plates in the presence of immobilized CD3 and soluble CD28 Abs (1 µg/ml) up to 12 days at 37 ºC. At day 3, cells from each well were harvested, washed, split in two new wells, and cultured in fresh complete media containing IL-2 (5 ng/ml). At days 6 and 9, cells from each well were split in two new wells and media was refreshed. Supernatants were collected at days 3, 6, 9 and 12 for HIV-p24 and cytokine quantification by ELISA (experimental triplicates). At day 12, cells were stimulated with PMA (50 ng/ml) and Ionomycin (1ug/ml) in the presence of Brefeldin A (5 ug/ml) for 5 hours and then harvested for the intracellular detection of HIV-p24, IL-17A, and IFN-γ by flow cytometry upon staining with specific Abs (Supplemental Table 3).

Quantification of cell-associated unspliced HIV-DNA and RNA

Cell-associated (CA) RNA and DNA was dually extracted from cell pellets (polled 5-6 replicates of 10e6 cells/experimental condition) using the AllPrep DNA/RNA Mini Kit (Qiagen), according to the manufacturer's instructions. Briefly, the quality (260nm/280nm ratio) and quantity of RNA/DNA collected were evaluated by spectrophotometry on a Nanodrop instrument.

The quantification of CA LTR-Gag HIV-RNA (CA HIV-RNA) was performed by one-step reel-time RT-PCR. The sequence of the external/internal primers and the taqman probe used are detailed in Supplemental Table 4a. The amplifications conditions for the pre-amplification were: denaturation for 4 min at 95°C, 45 cycles of amplification (reverse transcription at 50 °C for 30 min, denaturation at 94°C for 2 min; 16 cycles of amplification (94 °C for 15 s, 55 °C for 30 s, 68 °C for 1 min), and elongation at 68 °C for 5 min. The amplified products from the first PCR were diluted 10x in molecular grade water and subsequently used as templates in second nested real-time PCR amplifications. The amplification condition for the second amplification were as follows: denaturation at 95°C for 4 min; 45 cycles of amplification (95°C for 3 s, 60°C 10 s, and 68 °C for 1 min); and a final elongation at 68 °C for 5 min. The first amplification was performed on a conventional PCR instrument (ProFlex PCR System 9700; Applied Biosystems), while the second amplification was performed on the RotorGene instrument (Qiagen). For the standard curve, CA LTR-Gag HIV-RNA (unspliced) standards were generated using plasmids and transcription *in vitro* (MEGAscript™ T7 Transcription Kit, ThermoFisher). Serial dilutions of the transcripts were used to generate standard curves to measure the absolute copy numbers of each transcript.

To normalize HIV-RNA to HIV-DNA in matched samples, levels of CA LTR-Gag HIV-DNA were quantified by ultrasensitive nested real-time PCR using the same primers and taqman probe used for the CA HIV-RNA quantification (Table 4a). To normalize the HIV-DNA levels per number of cells, the CD3 gene was concomitantly amplified with HIV-DNA in the pre-amplification reaction and then amplified separately in the second amplification reaction. The amplifications conditions were: denaturation for 8 min at 95°C, 12 cycles of amplification

(denaturation: 1 min at 95°C; annealing: 40 sec at 55°C; elongation: 1 min at 72°C), and a final elongation for 15 min at 72°C. The sequence of external/internal primers and the taqman probe used are indicated in Supplemental Table 4b. The ACH2 cells carrying one copy of integrated HIV-DNA per cell (The National Institutes of Health AIDS Reagent Program) were used as a standard curve. The numbers of copies of CA HIV-RNA was normalized to the levels of CA HIV-DNA/10^6 cells. RT-PCR/PCR reactions were performed on RNA/DNA extracted from 6-8.10^6 cells/samples.

The quantification of cell-free (CF) HIV-RNA was performed as previously reported (119). Cell-culture supernatants from polled 5-6 replicates of 10e6 cells/well/1 ml/experimental condition were used to pellet viral particles (in final 100 µl/experimental condition) by centrifugation at 25,000g for 60 min. Viral RNA was extracted according to manufacturer's instructions using the QIAamp Viral RNA mini kit (Qiagen). The extracted RNA was first subjected to DNase (Invitrogen) treatment. Real-time nested-RT-PCR was then performed using the same primers and amplification protocol, as for the CA HIV-RNA quantification (Supplemental Table 4a). HIV-RNA extracted from ACH-2 cell culture supernatant was used to generate a standard curve. All measures were performed in triplicate. Results are expressed as HIV-RNA copies per reaction.

HIV infection *in vitro*

T-cells were stimulated with immobilized CD3 and soluble CD28 Abs (1 µg/ml) for 3 days and then exposed to the replication-competent transmitted/founder (T/F) HIV_{THRO} strain (NIH AIDS Reagent Program) (120) for 3 hours at 37 °C. Unbound virus was removed by extensive washing. HIV-exposed cells were cultured at 10^6 cells/ml in complete media (RPMI1640, 10% FBS, 1% antibiotics) in the presence of IL-2 (5 ng/ml), in the presence/absence of T0070907 (1 µM, 5 µM, 10 µM) or RGZ (50 µM) or 25HC (50 ng). HIV stocks were produced, titrated, and used to infect cells (25-50 ng HIV-p24/10^6 cells), as previously described (36-38, 70, 72, 73). HIV-p24 levels were quantified in cell culture supernatants using a homemade ELISA, as described (36-38, 70, 72, 73).

Integrated HIV-DNA was quantified by ultrasensitive nested real-time PCR in cell lysates (10^5 cells/test in triplicates; detection limit: 3 HIV-DNA copies/test), with normalization relative to CD3 copy numbers (two CD3 copies per one cell), as previously described (36-38, 70, 72, 73, 121). The sequence of the primers and the FRET probes used for integrated HIV-DNA and CD3-DNA quantification are detailed in Supplemental Tables c-d.

Real-time RT-PCR for quantification of cellular transcripts

One step SYBR Green real-time RT-PCR (Qiagen) was carried out in a LightCycler 480 II (Roche) according to manufacturer's recommendations, as we previously reported (36, 37). QuantiTect Primer Assays for human CH25H, CCR5, IL-17A, and IL-21were purchased from Qiagen. The expression of each gene was normalized relative to 28S rRNA levels (forward 5'-CGAGATTCCTGTCCCCACTA-3'; reverse 5'- GGGGCCACCTCCTTATTCTA-3', IDT). Melting curve analysis performed after real-time amplification revealed the uniformity of thermal dissociation profile for each amplification product. Amplifications were performed in triplicates on 70 ng (C25H), 70 ng (CCR5), 70 ng (IL-17A), 70 ng (PPARγ) or 2 ng (28S rRNA) total RNA per test.

Genome-wide RNA-sequencing and analysis

Flow-cytometry sorted memory $CCR6^+CD4^+$ T-cells were stimulated *via* the TCR and cultured in the presence or absence of T0070907 (10 μM) for 18 hours (Figure 5A). Total RNA was isolated using the RNeasy Kit (Qiagen) and quantified using the Pearl nanophotometer (Implen). Genome-wide transcriptional profiling was performed in collaboration with Genome Québec (Montreal, Québec, Canada) using the Illumina RNA-Sequencing model HiSeq 4000 PE100. Briefly, the paired-end sequencing reads were aligned to coding and non-coding transcripts from Homo Sapiens database GRCh 37 version75 and quantified using the kallisto software version 0.44.0 (122). The entire RNA-Seq 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 **GSE128121**. Statistical analyses were

performed using R version 3.5.1. Differential expression analysis was performed using the limma Bioconductor package (123) (version 3.38.3) on the log2-counts per million (logCPM) transformed transcript-level data. Gene set enrichment analysis was performed using the GSVA method (124) (package version 1.30.0) on the logCPM data using a Gaussian cumulative distribution function.

MiR29 antagomir electroporation

The human miR-29 family includes miR-29a, b, and c (125). Antagomirs are small oligonucleotides with a sequence complementary to the target miR; they bind to miR and prevent its binding to target mRNA. Oligosequences of the active miR-29a, b and c were determined based on the mir data base (http://mirbase.org/). Antagomirs sequences were selected based on the oligosequences and 5'3'orientation of the active miR. MiR-29a, b and c and were used to counteract the antiviral effects of miR-29 (78). Briefly, memory $CD4^+$ T-cells were stimulated with CD3/CD28 Abs for 2 days and electroporated. For each electroporation reaction, $2x10^6$ activated T-cells were pelleted and re-suspended in Nucleofector solution (Amaxa, Lonza), and 30ng of miR-29a or b or c or pooled a/b/c miRCURRY LNA miRNA inhibitors (Qiagen) was added to the cells. Control A and B miRCURRY LNA miRNA were used as control (Qiagen). Electroporation with pmaxGFP™ Vector (Amaxa, Lonza) was used to control nucleofection efficacy. Cells were electroporated using the Nucleofector programT-020 (Lonza) and then transferred into 48-well plates containing prewarmed 1ml of RPMI1640, 10% FBS, 5 ng/ml IL-2, and cultured at 37°C for 24 hours. Cells were then exposed to HIV_{THRO} and cultured for in the presence of IL-2 and/or T0070907 up to 6 days.

Statistics

All statistical analyses were performed using the Prism 8 (GraphPad software). Specifications on the statistical test used are included on the graphs and Figure legends. P-values are indicated on the graphs as follows: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; $****P < 0.0001$

COMPETING INTEREST STATEMENT

DP, YZ, JPG, MJR, LRM, AF, HC, TRWS, and AG declare no financial or non-financial competing interests to disclose.

JPR performed contract research and/or served on Advisory Boards for Gilead Sciences Canada Inc., Merck Canada Inc., Abbvie Corp., ViiV Healthcare, Bristol Myers Squibb, Janssen Inc., Argos Pharmaceuticals from InnaVirVax, and Theravectys.

NC received research funding from Merck & Co. and from InnaVirVax and served on the Advisory Board of Theravectys.

PA's laboratory receives research funding from Glaxo Smith Klein/NeoMed for projects different from the present study. PA serves as a Consultant at Merck Canada Inc.

AUTHORS' CONTRIBUTIONS

DP and YZ designed and performed research, analyzed data, and wrote the manuscript. JPG performed RNA-Seq analysis and prepared figures. MJR, LRM, AF, HC, TRWS, and AG performed research related to study design, sample collection/preparation and/or HIV reservoir measurements. WWB and CG provided protocols and expertise for miR-29 research. JPR conceived the clinical study hypothesis and design, provided access to clinical samples/information, set up clinical research protocols and recruited participants. NC provided protocols and reagents, contributed to research design and manuscript writing. PA conceived the research study hypothesis, designed research, analyzed data, and wrote the manuscript.

ACKNOWLEDGEMENTS:

The authors thank Dr. Dominique Gauchat and Philippe St Onge (Flow Cytometry Core Facility, CHUM-Research Center, Montréal, QC, Canada) for expert technical support with polychromatic flow cytometry sorting. The authors thank Olfa Debbeche (NLC3 Core Facility CHUM-Research Center, Montréal, QC, Canada); Mario Legault for help with ethical approvals and informed consents, and Josée Girouard, Angie Massicotte, and Cynthia Dion, for their key contribution to study participant recruitment and access to blood samples and clinical information from HIV-infected and uninfected donors. The authors thank Dr. Robert Lodge for providing protocols and valuable input for antagomir experiments. The authors address a special thanks to all PLWH study participants for their key contribution to this work.

FUNDING

This study was supported by grants from the Canadian Institutes of Health Research (CIHR) (MOP-114957, PJT-153052 to PA) and the *Fonds de Recherche du Québec-Santé* (FRQ-S)/AIDS and Infectious Diseases Network, Québec, Canada (to PA and JPR). The study was also supported by The Canadian HIV Cure Enterprise Team Grant (CanCURE 1.0) funded by the CIHR in partnership with CANFAR and IAS (CanCURE 1.0; # HIG-133050 to PA, EAC and JPR), and The Canadian HIV Cure Enterprise Team Grant (CanCURE 2.0) funded by the CIHR (#HB2–164064 to PA, EAC, JPR, and NC). JPR holds a Louis Lowenstein Chair in Hematology and Oncology, McGill University. The funding institutions played no role in the design, collection, analysis, and interpretation of data. DP received a Doctoral award from the Université de Montréal and the FRQ-S. JPR holds a Louis Lowenstein Chair in Hematology and Oncology, McGill University. Core facilities and HIV-infected patients' cohorts were supported by the *Fondation du CHUM* and the FRQ-S Network.

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TABLE

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

Participant ID	Sex	CD4 count[#]	CD8 count[#]	Plasma viral load[&]	Time since infection*	ART	Time of aviremia*
ART #1	M	398	775	<40	154	Complera	23
ART #2	M	841	1322	<40	150	Truvada/Viramune	NA
ART #3	M	796	399	<40	8	Stribild	1
ART #4	M	581	1060	<40	96	Sustiva/Truvada	5
ART #5	M	391	620	<40	165	Kivexa/Delavirdine	54
ART #6	M	318	431	<40	149	Kivexa/Delavirdine	44
ART #7	M	514	568	<40	16	Tivicay/ Truvada	6
ART #8	M	775	1000	<40	74	Complera	19
ART #9	M	459	545	<40	189	Truvada/Raltegravir	>12
ART #10	M	616	330	<40	666	Viracept/Truvada	34
ART #11	M	542	803	<40	13	Stribild	10
ART #12	M	458	899	<40	227	Truvada/Viramune	NA
ART #13	M	908	854	<40	89	Stribild	70
ART #14	F	833	445	<40	212	Viracept/Truvada	60

*[#], cells/µl; [&], HIV-RNA copies per ml plasma; *, months; ART, antiretroviral therapy; M, male; F, female; [$], months; NA, information not available.*

FIGURES

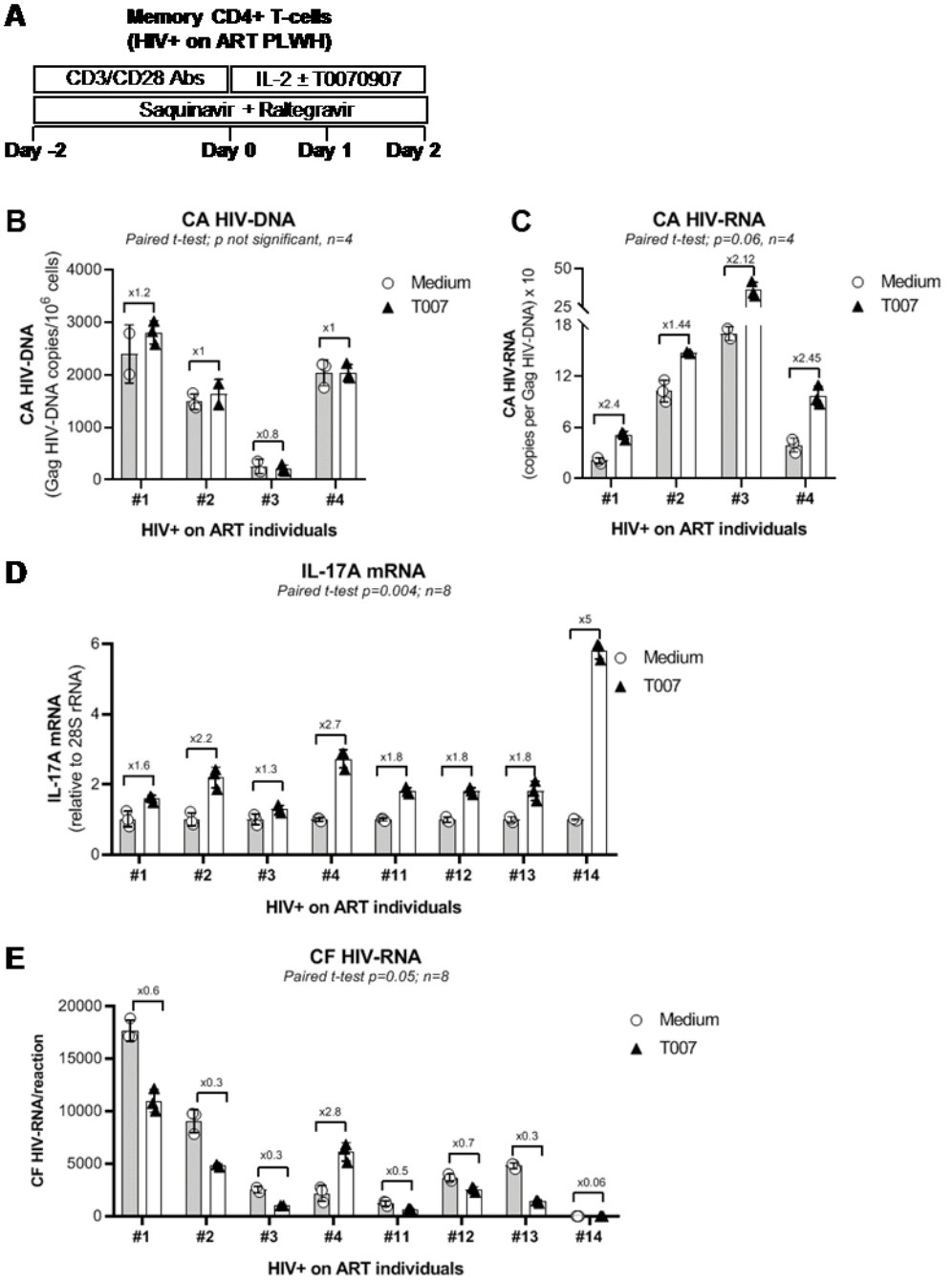


Figure 1: T0070907 increases HIV and IL-17A transcription but inhibits HIV production in CD4+ T-cells of ART-treated PLWH. (A) Shown is the experimental flow chart for a short-term viral outgrowth assay (VOA) performed with memory CD4$^+$ T-cells isolated from the PBMC of HIV+ART individuals (Table 1; ART#1-4 and ART#11-14). Briefly, cells were activated *via* CD3/CD28 for 2 days in the presence of ARV (Saquinavir 5 μM; Raltegravir 200 nM). Then, cells were washed to stop TCR triggering and further

cultured in the presence of the same ARV cocktail with or without the PPARγ antagonist T0070907 (10 μM) for an additional 48 hours. DMSO (1 μL/ml; identified as Medium) was used as a control for T0070907. Total RNA and DNA were dually extracted from the cells. Supernatants were collected and total RNA was extracted. **(B)** Levels of Gag HIV-DNA were quantified by nested real-time PCR and normalized per 10^6 cells, upon simultaneous CD3 DNA copy number quantification (two CD3 DNA copies per cell). **(C)** Levels of unspliced (Gag) cell-associated (CA) HIV-RNA were quantified by nested real-time RT-PCR and normalized to the HIV-DNA copy numbers/10e6 cells in panel B. **(D)** Levels of IL-17A transcripts were quantified by real-time PCR and normalized to 28S rRNA levels. Shown are fold-change (FC) ratios in IL-17A mRNA levels relative to the medium condition considered at 1. **(E)** Levels of unspliced (Gag) cell-free (CF) HIV-RNA were quantified by nested real-time RT-PCR in RNA extracted from cell culture supernatants. Each symbol represents one experimental replicate (mean±SD). Fold changes (FC) between medium and T0070907 conditions are indicated on the graphs.

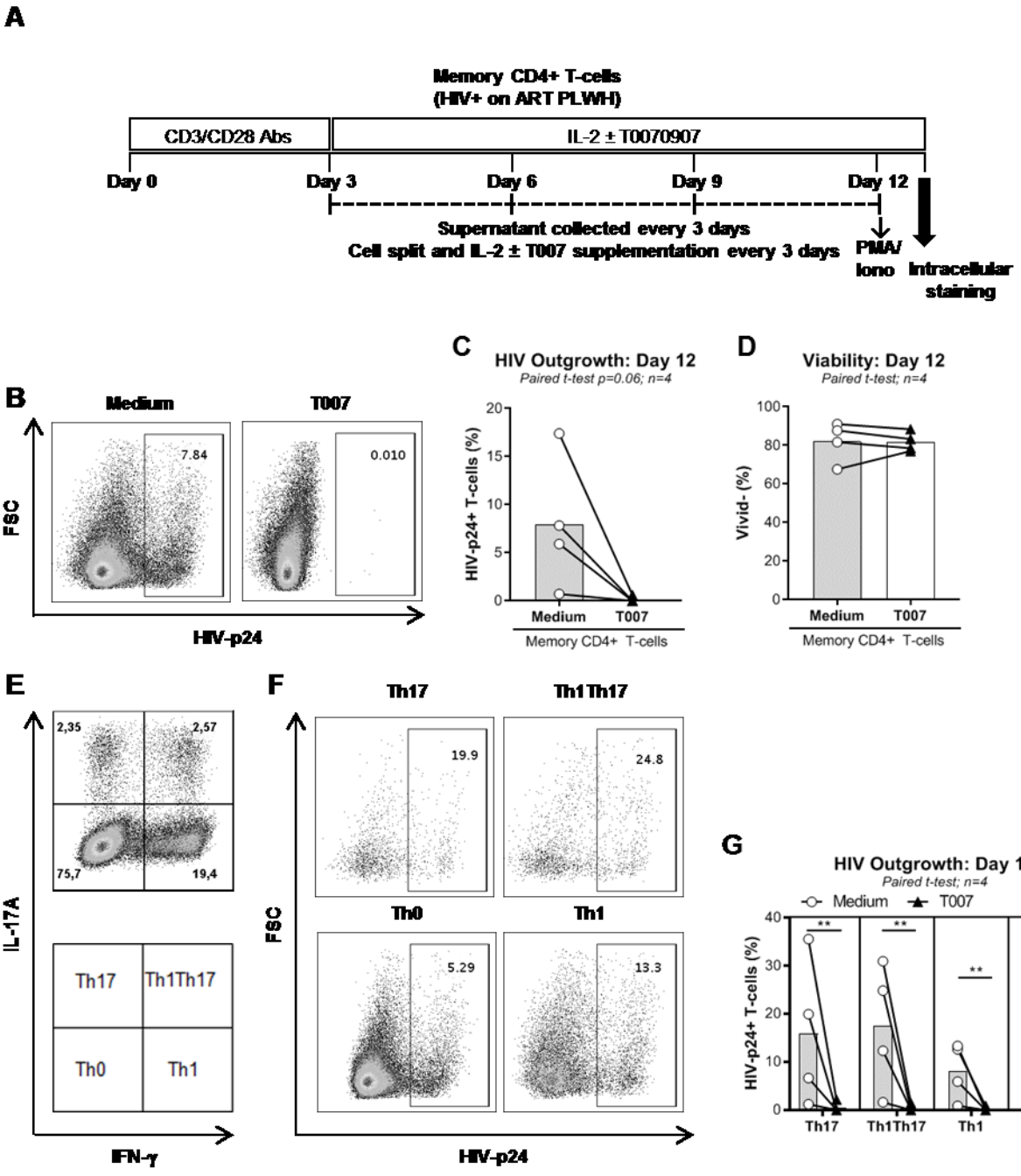

A
Memory CD4+ T-cells
(HIV+ on ART PLWH)
CD3/CD28 Abs
IL-2 ± T0070907
Day 0
Day 3
Day 6
Day 9
Day 12
Supernatant collected every 3 days
Cell split and IL-2 ± T007 supplementation every 3 days
PMA/
Iono
Intracellular
staining
B
Medium
T007
7.84
0.010
FSC
HIV-p24
C
HIV Outgrowth: Day 12
Paired t-test p=0.06; n=4
HIV-p24+ T-cells (%)
Medium
T007
Memory CD4+ T-cells
D
Viability: Day 12
Paired t-test; n=4
Vivid- (%)
Medium
T007
Memory CD4+ T-cells
E
2,35
2,57
75,7
19,4
Th17
Th1Th17
Th0
Th1
IL-17A
IFN-γ
F
Th17
Th1Th17
19.9
24.8
Th0
Th1
5.29
13.3
FSC
HIV-p24
G
HIV Outgrowth: Day 12
Paired t-test; n=4
Medium
T007
HIV-p24+ T-cells (%)
Th17
Th1Th17
Th1
Th0
H
HIV Outgrowth; ART #3
Med
T007
Day 3
Day 6
Day 9
Day 12
HIV-p24 (pg/mL)
Days post-reactivation
HIV Outgrowth; ART #4
Med
T007
Day 3
Day 6
Day 9
Day 12
HIV-p24 (pg/mL)
Days post-reactivation

Figure 2: T0070907 inhibits viral outgrowth in memory CD4$^+$ T-cells of ART-treated PLWH. (A) Shown is the experimental flow chart for the viral outgrowth assay (VOA) performed with memory CD4$^+$ T-cells isolated by MACS from PBMCs of ART-treated PLWH (Table 1; ART #3, 4, 5 and 10 for panels B-G; ART #3-4 for panel E). Briefly, cells were activated with CD3/CD28 Abs for 3 days to induce HIV reactivation (39) and optimal PPARγ expression (36). Then, cells were washed and cultured in the presence/absence of T0070907 (10 μM) up to 12 days. Cells were split in two wells every 3 days for optimal cell density. Supernatants were collected every 3 days for ELISA HIV-p24 quantification. To measure HIV replication in cytokine-expressing cells by flow-cytometry (38), at day 12 post-TCR triggering, cells were stimulated with PMA/Ionomycin in the presence of Brefeldin A for 5 hours. Cells were stained with a viability dye and HIV-p24, IL-17A and IFN-γ Abs. **(B-D)** Shown is the intracellular expression of HIV-p24 in memory CD4$^+$ T-cells treated or not with T0070907 in **(B)** one representative donor and **(C)** n=4 ART-treated PLWH, and **(D)** the effect of T0070907 on cellular viability (Vivid- cells; (n=4). **(E-G)** Shown are the gating strategies for **(E)** the identification of memory T-cells with Th17 (IL-17A$^+$IFN-γ$^-$), Th1Th17 (IL-17A$^+$IFN-γ$^+$), Th1 (IL-17A$^-$IFN-γ$^+$) and Th0 (IL-17A$^-$IFN-γ$^-$) cytokinic profiles and for **(F)** the HIV-p24 expression in the four Th cell subsets from one representative donor, and the **(G)** statistical analysis of results obtained from 3 different ART-treated PLWH. **(H)** Shown are HIV-p24 levels in cell culture supernatant quantified at days 3, 6, 9, and 12 by ELISA in a VOA performed in quadruplicate (each point represents one independent experimental replicate) using cells from n=2 additional ART-treated PLWH.. Paired t-Test P-values are indicated on the graphs.

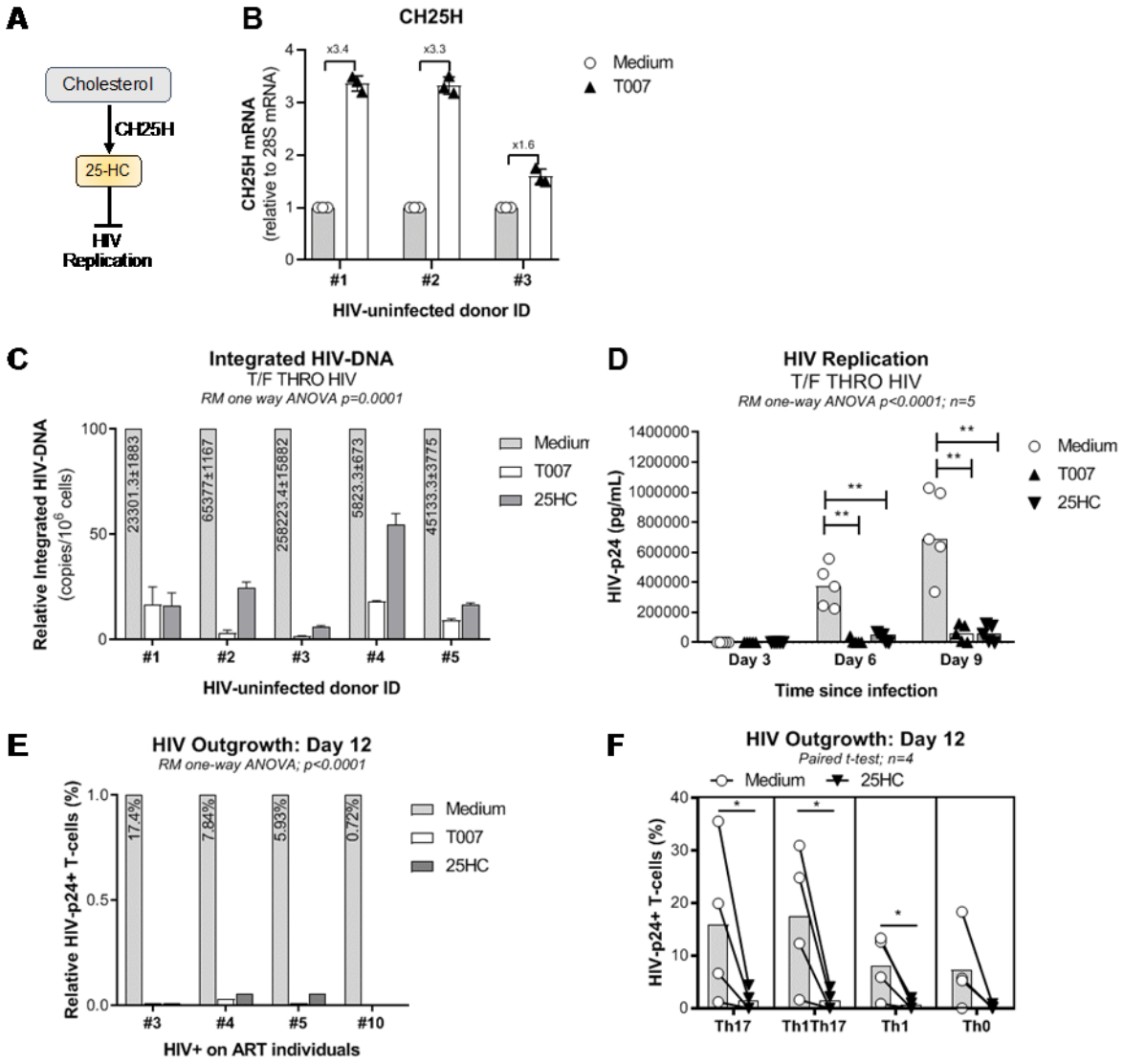


Figure 3: T0070907 induces CH25H mRNA expression and 25HC reduces HIV replication/outgrowth. **(A)** Depicted are the documented antiviral properties of CH25H/25HC (69) **(B-D)** Memory CD4$^+$ T-cells of HIV-uninfected individuals were stimulated with CD3/CD28 Abs for 3 days. **(B)** A fraction of TCR-activated cells was cultured in the presence/absence of T0070907 (10 μM) for 2 additional days. CH25H mRNA was quantified by SYBR Green real-time RT-PCR. Bar graphs depict mean±SD of triplicate values (each symbol represents one experimental triplicate; n=3). Shown are fold-change (FC) ratios in CH25H mRNA levels relative to the medium condition. **(C-D)** Another fraction of TCR-activated cells was exposed to HIV_{THRO} (25 ng/10e6 cells) and cultured with IL-2, in the presence/absence of 25HC (50 nM) or T0070907 (10 μM) for 9 days. Media was every 3 days. **(C)** Shown are relative levels of HIV-DNA integration at day 3 post-infection, with the medium condition considered 100%. Absolute HIV-DNA copies (mean±SD HIV-DNA copies/10e6 cells) are depicted on the graphs for the Medium condition. **(D)** Shown are absolute HIV-p24 levels quantified by ELISA at days 3, 6 and 9 post-infection. **(E-F)** In parallel, a VOA was performed as described in Figure 2A in the presence/absence of 25HC (0.05 μM) or T0070907 (10 μM) up to 12 days post-TCR triggering. Cells were split and media refreshed every 3 days. At day 12, cells were stimulated to identify HIV-p24+ cytokine-producing Th subsets, as in Figure 2. **(E)**

Shown are relative HIV-p24^{+} cell levels, with the medium condition being considered 100%. **(F)** Shown is HIV-p24 expression within Th17, Th1Th17, Th1 and Th0 cells (mean; n=4). **(C-E)** The RM one-way ANOVA p-values, with the significance of the Dunnetts's multiple comparisons test (relative to medium) is indicated on the figures. **(F)** Paired t-Test p-values are indicated on the graphs. Each symbol represents one different donor.

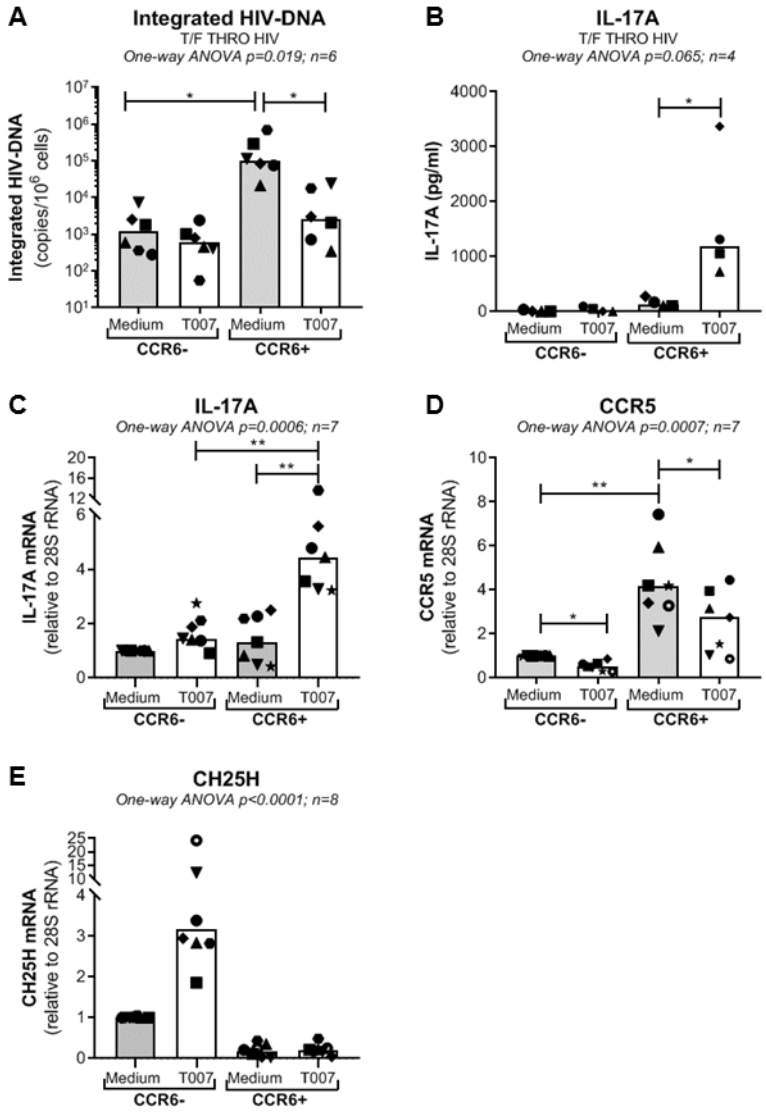


Figure 4: T0070907 reduces HIV replication and increases IL-17A production in CCR6$^+$ T-cells *via* CH25H/25HC-independent mechanisms. Memory CCR6$^+$ and CCR6$^-$ T-cells were isolated by MACS and FACS from PBMCs of HIV-uninfected individuals and stimulated via CD3/CD28 for 3 days. **(A-B)** A fraction of TCR-activated cells was exposed to the T/F HIV$_{THRO}$ strain (25 ng/10e6 cells) and cultured in the presence of IL-2 and/or T0070907 (10 μM). **(A)** Levels of integrated HIV-DNA in cells and **(B)** IL-17A in cell culture supernatants were quantified by nested real-time PCR and ELISA, respectively, at day 3 post-infection. **(C-E)** In parallel, TCR-activated CCR6- and CCR6+ T-cells were exposed to T0070907 (10 μM) for 18 hours in the absence of HIV, RNA was extracted for mRNA quantification by SYBR Green real-time RT-PCR. Shown are the statistical analyses of IL-17A **(C**, n=7**)**, CCR5 **(D**, n=8**)** and CH25H **(E**, n=7**)** mRNA levels relative to the medium condition in CCR6- T-cells considered at 1. Each symbol represents one different donor and bars represent median values. Repeated-measures (RM) 1-way ANOVA p-values with the significance of the Tukey's multiple comparisons test are indicated on the figures.

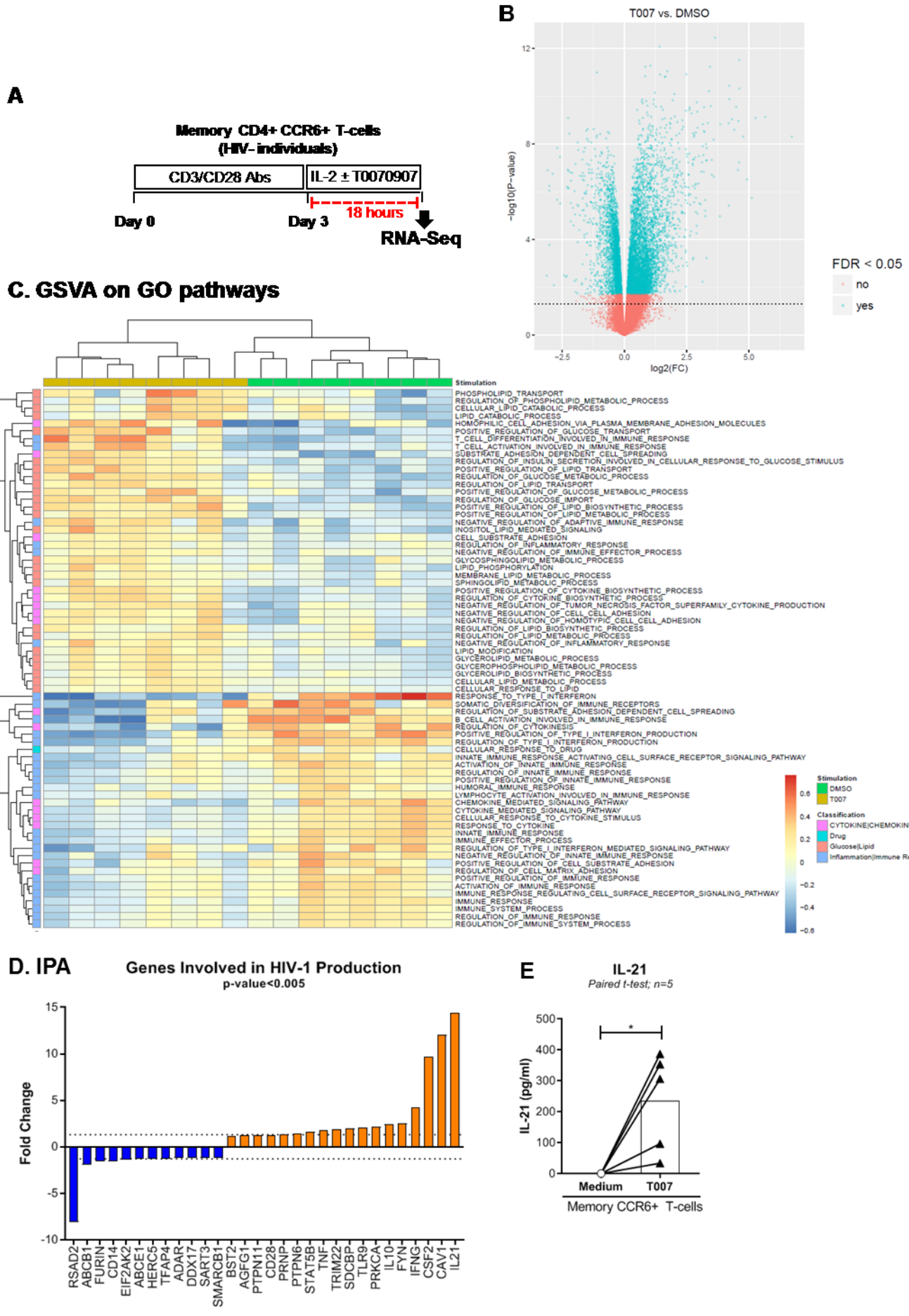
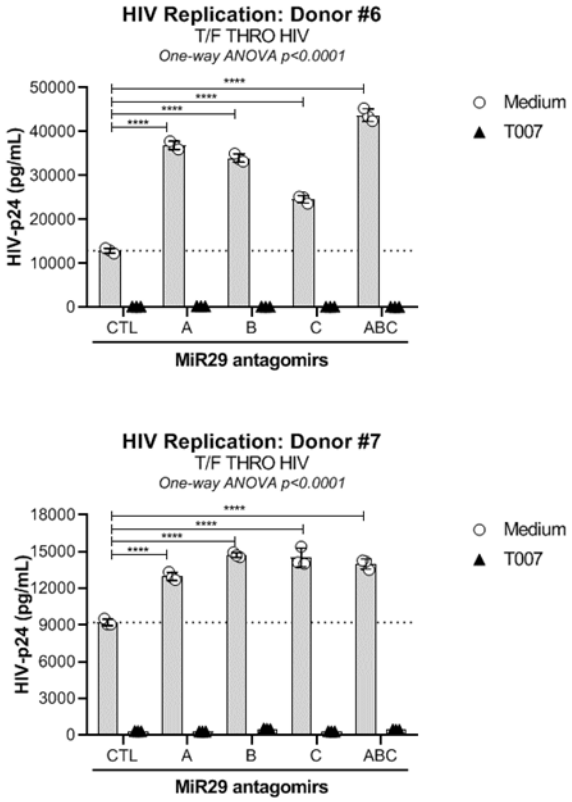

A
Memory CD4+ CCR6+ T-cells
(HIV- individuals)
CD3/CD28 Abs
IL-2 ± T0070907
Day 0
Day 3
18 hours
RNA-Seq
B
T007 vs. DMSO
−log10(P-value)
log2(FC)
FDR < 0.05
no
yes
C. GSVA on GO pathways
Stimulation
DMSO
T007
Classification
CYTOKINE|CHEMOKINE|ADHESION
Drug
Glucose|Lipid
Inflammation|Immune Response|Type I Inf
PHOSPHOLIPID_TRANSPORT
REGULATION_OF_PHOSPHOLIPID_METABOLIC_PROCESS
CELLULAR_LIPID_CATABOLIC_PROCESS
LIPID_CATABOLIC_PROCESS
HOMOPHILIC_CELL_ADHESION_VIA_PLASMA_MEMBRANE_ADHESION_MOLECULES
POSITIVE_REGULATION_OF_GLUCOSE_TRANSPORT
T_CELL_DIFFERENTIATION_INVOLVED_IN_IMMUNE_RESPONSE
T_CELL_ACTIVATION_INVOLVED_IN_IMMUNE_RESPONSE
SUBSTRATE_ADHESION_DEPENDENT_CELL_SPREADING
REGULATION_OF_INSULIN_SECRETION_INVOLVED_IN_CELLULAR_RESPONSE_TO_GLUCOSE_STIMULUS
POSITIVE_REGULATION_OF_LIPID_TRANSPORT
REGULATION_OF_GLUCOSE_METABOLIC_PROCESS
REGULATION_OF_LIPID_TRANSPORT
POSITIVE_REGULATION_OF_GLUCOSE_METABOLIC_PROCESS
REGULATION_OF_GLUCOSE_IMPORT
POSITIVE_REGULATION_OF_LIPID_BIOSYNTHETIC_PROCESS
POSITIVE_REGULATION_OF_LIPID_METABOLIC_PROCESS
NEGATIVE_REGULATION_OF_ADAPTIVE_IMMUNE_RESPONSE
INOSITOL_LIPID_MEDIATED_SIGNALING
CELL_SUBSTRATE_ADHESION
REGULATION_OF_INFLAMMATORY_RESPONSE
NEGATIVE_REGULATION_OF_IMMUNE_EFFECTOR_PROCESS
GLYCOSPHINGOLIPID_METABOLIC_PROCESS
LIPID_PHOSPHORYLATION
MEMBRANE_LIPID_METABOLIC_PROCESS
SPHINGOLIPID_METABOLIC_PROCESS
POSITIVE_REGULATION_OF_CYTOKINE_BIOSYNTHETIC_PROCESS
REGULATION_OF_CYTOKINE_BIOSYNTHETIC_PROCESS
NEGATIVE_REGULATION_OF_TUMOR_NECROSIS_FACTOR_SUPERFAMILY_CYTOKINE_PRODUCTION
NEGATIVE_REGULATION_OF_CELL_CELL_ADHESION
NEGATIVE_REGULATION_OF_HOMOTYPIC_CELL_CELL_ADHESION
REGULATION_OF_LIPID_BIOSYNTHETIC_PROCESS
REGULATION_OF_LIPID_METABOLIC_PROCESS
NEGATIVE_REGULATION_OF_INFLAMMATORY_RESPONSE
LIPID_MODIFICATION
GLYCEROLIPID_METABOLIC_PROCESS
GLYCEROPHOSPHOLIPID_METABOLIC_PROCESS
GLYCEROLIPID_BIOSYNTHETIC_PROCESS
CELLULAR_LIPID_METABOLIC_PROCESS
CELLULAR_RESPONSE_TO_LIPID
RESPONSE_TO_TYPE_I_INTERFERON
SOMATIC_DIVERSIFICATION_OF_IMMUNE_RECEPTORS
REGULATION_OF_SUBSTRATE_ADHESION_DEPENDENT_CELL_SPREADING
B_CELL_ACTIVATION_INVOLVED_IN_IMMUNE_RESPONSE
REGULATION_OF_CYTOKINESIS
POSITIVE_REGULATION_OF_TYPE_I_INTERFERON_PRODUCTION
REGULATION_OF_TYPE_I_INTERFERON_PRODUCTION
CELLULAR_RESPONSE_TO_DRUG
INNATE_IMMUNE_RESPONSE_ACTIVATING_CELL_SURFACE_RECEPTOR_SIGNALING_PATHWAY
ACTIVATION_OF_INNATE_IMMUNE_RESPONSE
REGULATION_OF_INNATE_IMMUNE_RESPONSE
POSITIVE_REGULATION_OF_INNATE_IMMUNE_RESPONSE
HUMORAL_IMMUNE_RESPONSE
LYMPHOCYTE_ACTIVATION_INVOLVED_IN_IMMUNE_RESPONSE
CHEMOKINE_MEDIATED_SIGNALING_PATHWAY
CYTOKINE_MEDIATED_SIGNALING_PATHWAY
CELLULAR_RESPONSE_TO_CYTOKINE_STIMULUS
RESPONSE_TO_CYTOKINE
INNATE_IMMUNE_RESPONSE
IMMUNE_EFFECTOR_PROCESS
REGULATION_OF_TYPE_I_INTERFERON_MEDIATED_SIGNALING_PATHWAY
NEGATIVE_REGULATION_OF_INNATE_IMMUNE_RESPONSE
POSITIVE_REGULATION_OF_CELL_SUBSTRATE_ADHESION
REGULATION_OF_CELL_MATRIX_ADHESION
POSITIVE_REGULATION_OF_IMMUNE_RESPONSE
ACTIVATION_OF_IMMUNE_RESPONSE
IMMUNE_RESPONSE_REGULATING_CELL_SURFACE_RECEPTOR_SIGNALING_PATHWAY
IMMUNE_RESPONSE
IMMUNE_SYSTEM_PROCESS
REGULATION_OF_IMMUNE_RESPONSE
REGULATION_OF_IMMUNE_SYSTEM_PROCESS
D. IPA
Genes Involved in HIV-1 Production
p-value<0.005
Fold Change
RSAD2
ABCB1
FURIN
CD14
EIF2AK2
ABCE1
HERC5
TFAP4
ADAR
DDX17
SART3
SMARCB1
BST2
AGFG1
PTPN11
CD28
PRNP
PTPN6
STAT5B
TNF
TRIM22
SDCBP
TLR9
PRKCA
IL10
FYN
IFNG
CSF2
CAV1
IL21
E
IL-21
Paired t-test; n=5
IL-21 (pg/ml)
Medium
T007
Memory CCR6+ T-cells

Figure 5: T0070907 reprograms CCR6$^+$CD4$^+$ T-cells toward an anti-viral transcriptional status. (A) Shown is the experimental flow chart for genome-wide transcriptional profiling in cells exposed to T0070907. Briefly, memory CCR6$^+$ T-cells were isolated from PBMCs of HIV-uninfected individuals (n=8) by MACS/FACS and stimulated via CD3/CD28 for 3 days. Then, cells were cultured in the presence of IL-2 and/or T0070907 (10 μM) for 18 hours. Total RNA was extracted and used for RNA sequencing. One-way ANOVA analysis identified differentially expressed genes based on P-values (P<0.05) or adjusted P-values (adj. P<0.05) and/or fold-change (FC, cutoff 1.3). **(B)** Volcano plots for all probes in each linear model with the log2 FC on the x axis and the negative logarithm of the adjusted P-values for false discovery rate (FDR) on the y axis. The red/green color code is based on the 5% FDR threshold. **(C)** Heatmap represents 71 pathways included in the gene ontology (GO) classification: cytokines/chemokines (pink), drug transporters (blue), glucose/lipid metabolism (orange) and inflammation/immune response to type I interferon (violet) based on the 5% FDR threshold. Heatmap cells are scaled by the expression level z-scores for each probe individually. **(D)** Ingenuity pathway analysis (IPA) identified genes involved in HIV-1 production and differentially modulated by T0070907 (P<0.05). The *y* axis represents the FC, with the 1.3 FC cut-off indicated by the dotted line. **(E)** Levels of IL-21 in cell culture supernatants were quantified by ELISA (n=5). Paired t-Test p-values are indicated on the graphs.



<u>Figure 6</u>: The miR29 antagomir increases HIV replication in the absence but not in the presence of T0070907. Memory $CD4^+$ T-cells isolated from HIV- subjects (n=2) were stimulated via CD3/CD28 for 2 days. Then, cells were electroporated with miR-29a, b, c or a/b/c antagomir-LNA or control antagomirs. 24 hours post-electroporation, cells were exposed to T/F HIV_{THRO} strain (25 ng/10e6 cells) and cultivated in the presence of IL-2 and/or T0070907 (10 μM) for up to 6 days. Levels of p24-HIV were quantified in cell culture supernatants by ELISA at day 6. Repeated-measures 1-way ANOVA p-values with the significance of the Tukey's multiple comparisons test are indicated on the figures.

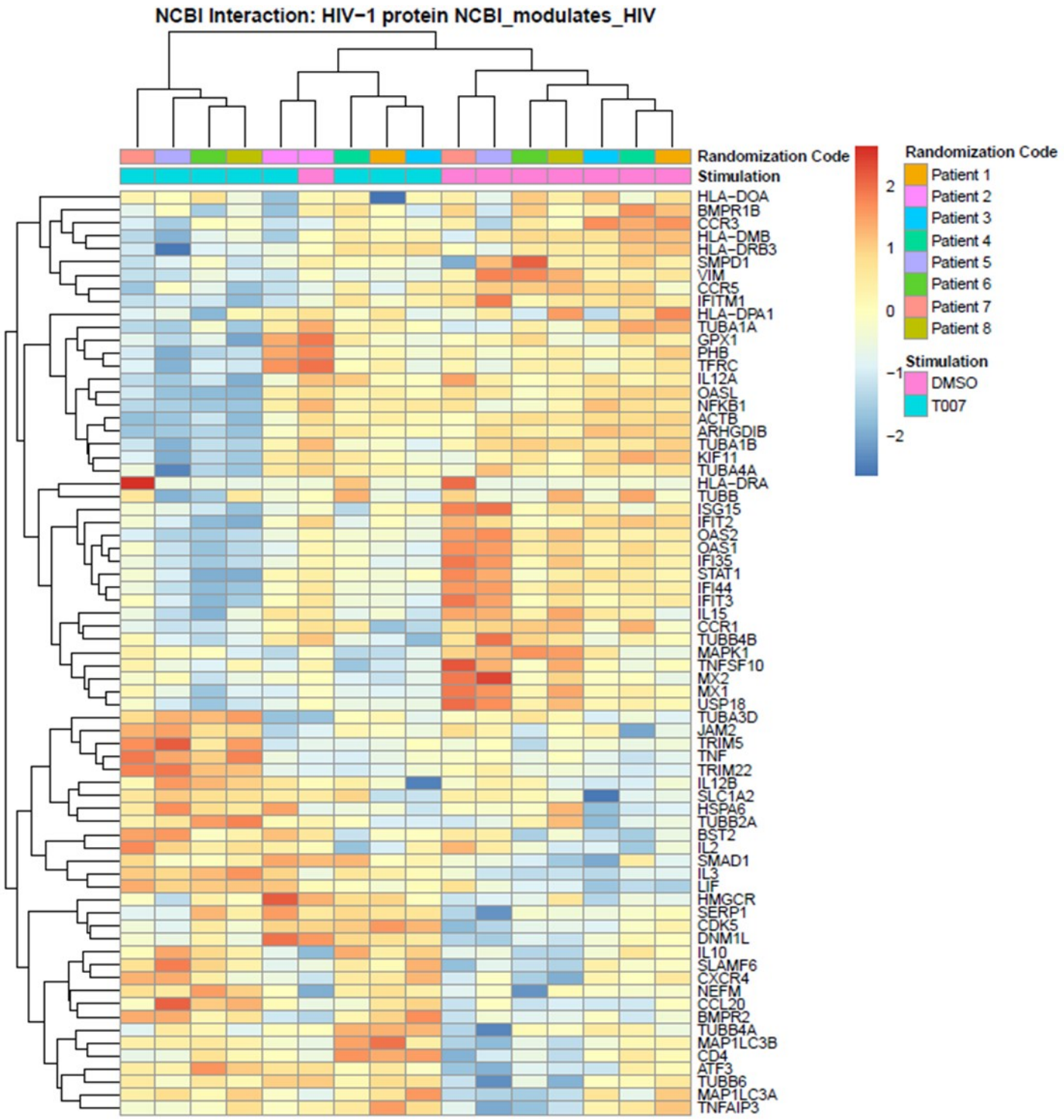


Figure 7: Meta-analysis using the NCBI HIV interaction database identifies HIV restriction/dependency factors modulated by T0070907 in CCR6^{+} T-cells. Genome-wide transcriptional profiles were generated as described in Figure 5. **(A)** Transcripts significantly modulated by T0070907 in CCR6^{+} T-cells ($p < 0.05$, FC cut-off 1.3) were matched to the lists of human genes included

on the NCBI HIV interaction database. Heatmap cells are scaled by the expression level z-scores for each probe individually. Results from each donor are indicated with a different color code (n=8).

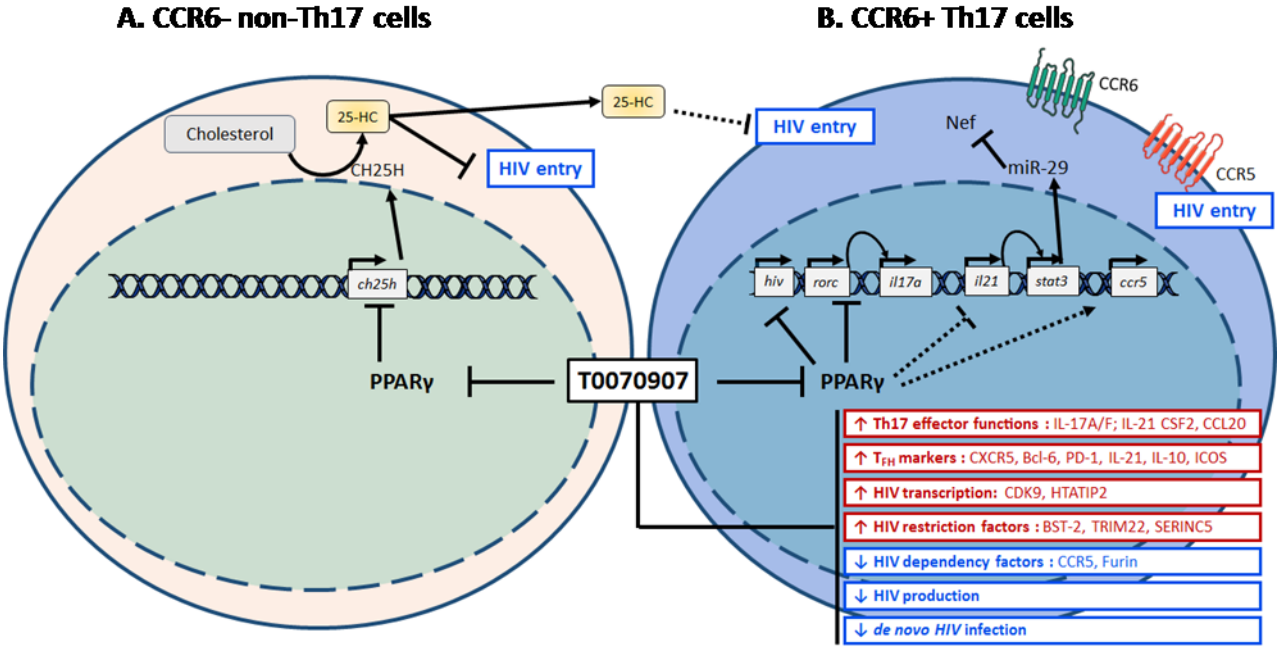
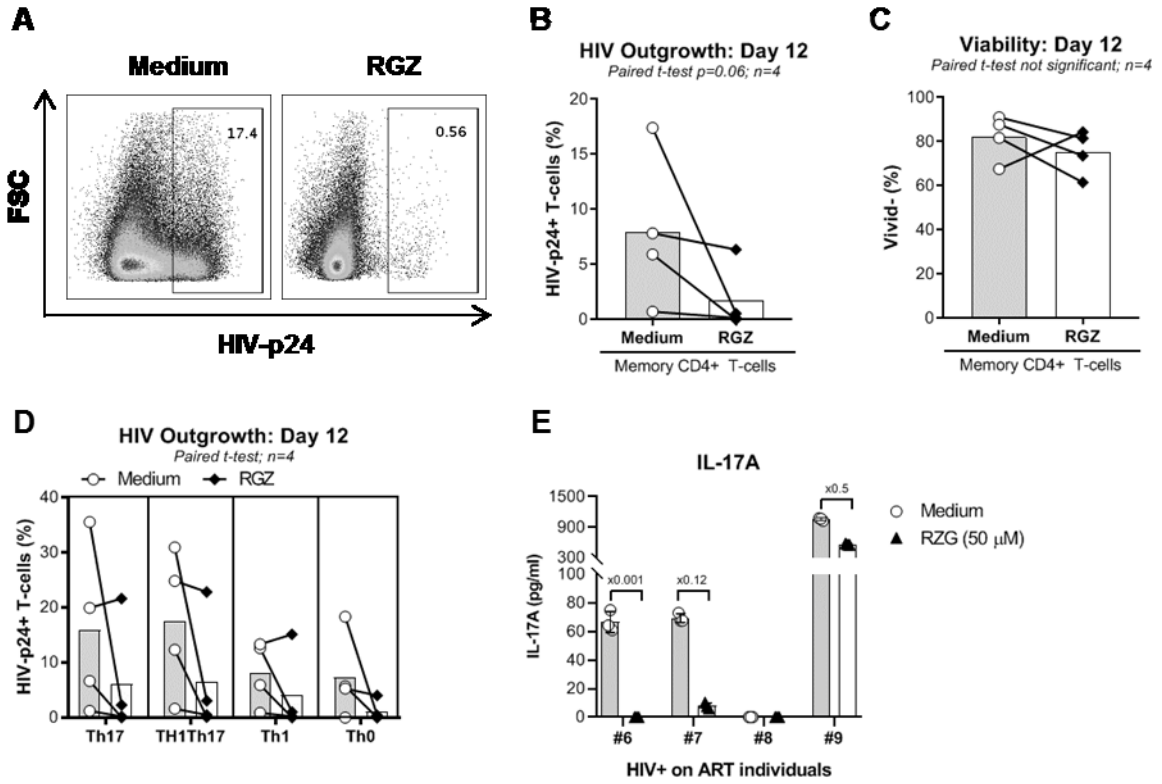
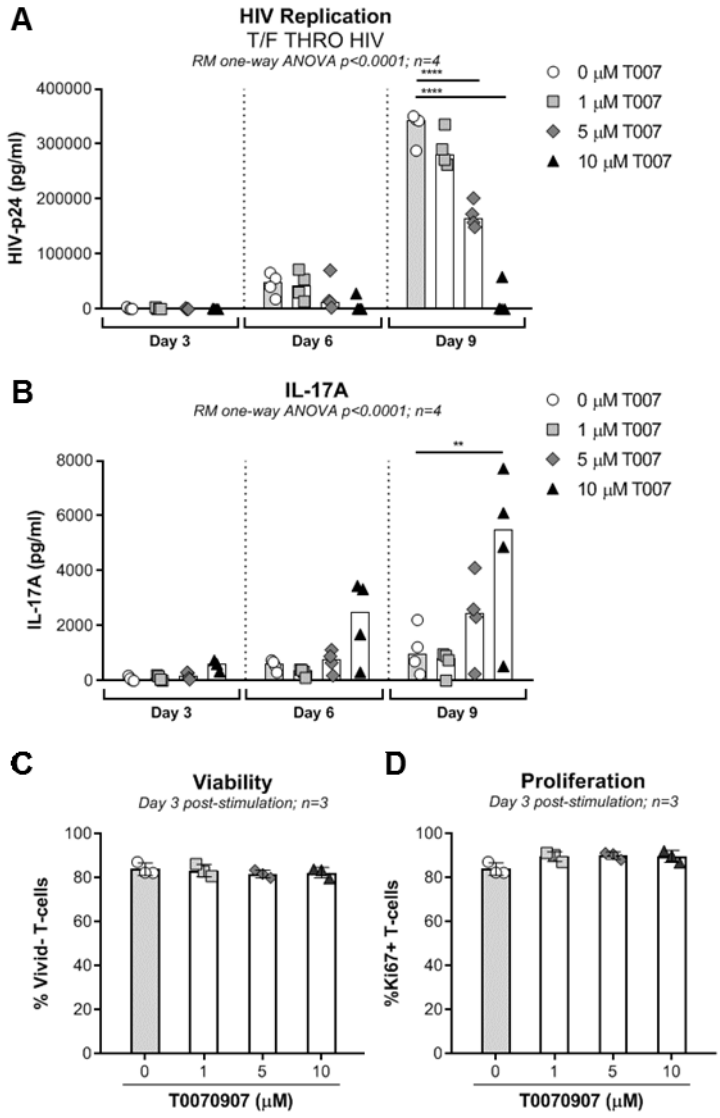


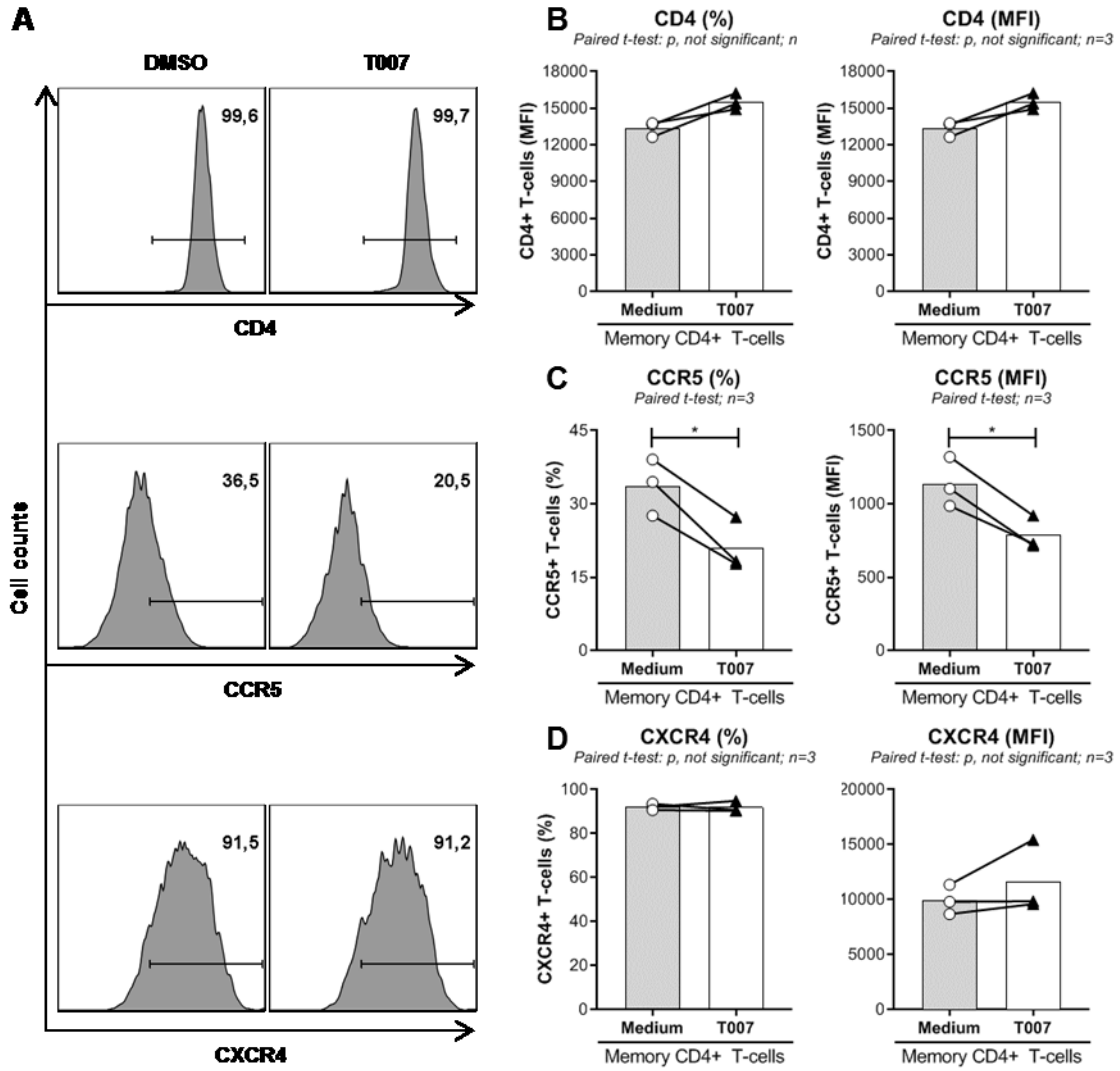
Figure 8: Prosed model for distinct antiviral and immune-modulatory mechanisms mediated by the PPARγ antagonist T0070907 in CCR6$^+$ *versus* CCR6$^-$ T-cells. In this manuscript, we provide evidence that the pharmacological inhibition of PPARG using T0070907 leads to distinct modulation of CCR6$^+$ and CCR6$^-$ T-cells features in the context of HIV infection. Briefly, in CCR6- non-Th17 cells T0070907 promotes an antiviral program governed by the CH25H (69) **(A)**. In contrast, T0070907 acts on CCR6$^+$ Th17 cells in a CH25H-independent manner and boosts HIV transcription and the expression of Th17/Tfh-specific transcripts (IL-17A, IL-21) but negatively interferes with multiple steps of the HIV replication cycle, from viral entry (CCR5) to virion maturation (furin) and budding (BST2). The up-regulation of miR-29 contributes to the antiviral status in T0070907-exposed CCR6$^+$ T-cells but other miR-29-independent mechanisms are induced by T0070907 in these CCR6$^+$ Th17 cells **(B)**.



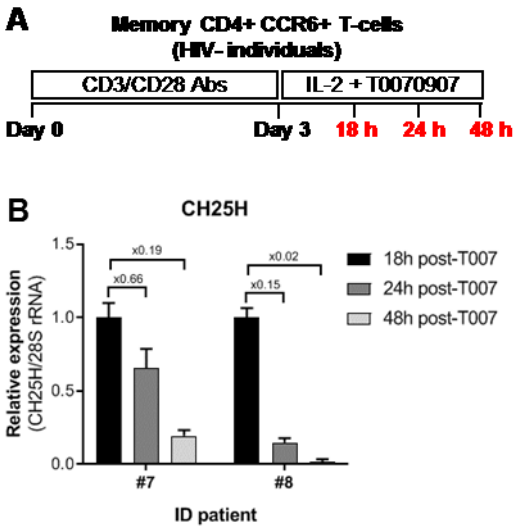
Supplemental Figure 1: Rosiglitazone limits HIV outgrowth and reduces IL-17A production in memory CD4⁺ T-cells of ART-treated PLWH. A VOA was performed with memory CD4⁺ T-cells of ART-treated PLWH (Table 1; ART #3, 4, 5 and 10 for panels A-D and ART #6-9 for panel E) in the presence of rosiglitazone (RGZ; 50 μM), as described in Figure 2. At day 12 post-culture, cells were stimulated and stained as in Figure 2. Shown are the effect of RGZ in the intracellular expression of HIV-p24 in one representative donor **(A)** and statistical analysis in n=4 different ART-treated PLWH (median values) **(B)**, as well as the effect of RGZ exposure on cell viability (median; n=4) **(C)**. By using a flow cytometry gate similar to that depicted in Figure 2E, the effect of RGZ on the frequencies of HIV-24⁺ cells was determined within Th17, Th1Th17, Th1 and Th0 subsets (median; n=4) **(D)**. Paired t-Test P-values are indicated on the graphs. Finally, levels of IL-17A in cell culture supernatant were measured by ELISA at day 6 post-stimulation (mean±SD of experimental triplicates; n=4), with the FC between medium and RGZ conditions being indicated on the graphs. **(B-C-D).**



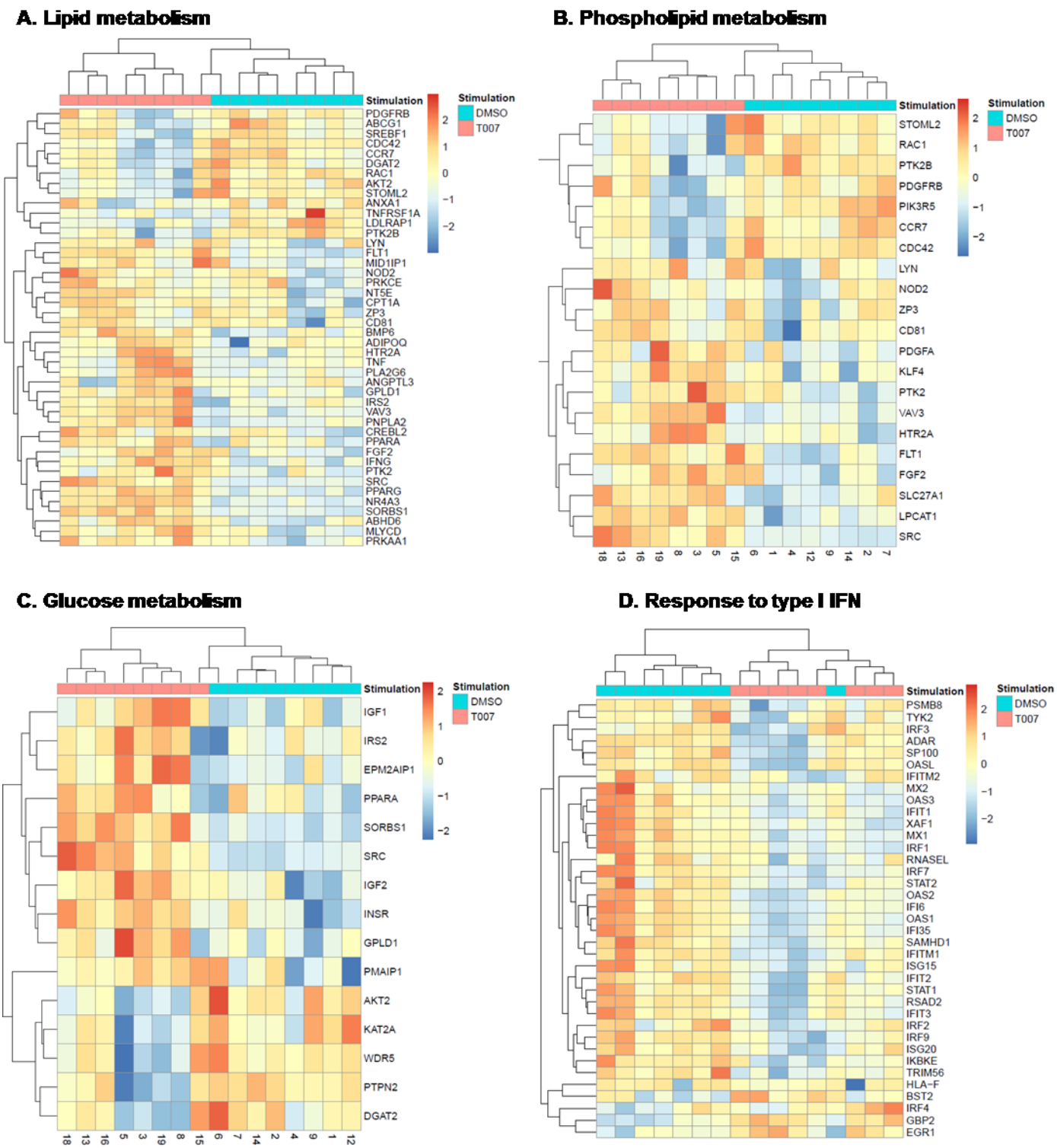
<u>Supplemental Figure 2</u>: T0070907 limits T/F HIV_{THRO} replication *in vitro* while boosting IL-17A production. Memory $CD4^+$ T-cells isolated from HIV-uninfected individuals were stimulated *via* CD3/CD28 for 3 days. Then, cells were exposed to T/F HIV_{THRO} strain (25 ng/10e6 cells) and cultured in the presence/absence of T0070907 (1 μM, 5 μM and 10 μM) for up to 9 days. Shown are HIV-p24 **(A)** and IL-17A **(B)** levels quantified by ELISA in cell culture supernatants at days 3, 6 and 9 post-infection (n=4, median. In separate experimental settings, cell viability (vivid- cells) **(C)** and proliferation ($Ki67^+$ cells) **(D)** were quantified by flow cytometry in cells stimulated *via* the TCR in the presence/absence of T0070907 for 3 days. Repeated-measures 1-way ANOVA p-values with the significance of the Tukey's multiple comparisons test are indicated on the figures.



Supplemental Figure 3: Effect of T0070907 on the expression of CD4, CCR5, and CXCR4 on memory CD4⁺ T-cells. Memory CD4⁺ T-cells from HIV-uninfected individuals were isolated, stimulated via CD3/CD28 for 3 days and then cultured in the presence/absence of T0070907 (10 µM) for 2 additional days. The expression of HIV-1 receptor CD4 and co-receptors CCR5 and CXCR4 was measured by flow cytometry. Shown are histograms from one representative donor **(A),** and the statistical analyses of CD4 **(B)**, CCR5 **(C)** and CXCR4 **(D)** expression (% and MFI) (median; n=3). Paired t-Test p-values are indicated on the graphs.



<u>Supplemental Figure 4</u>: Kinetics of CH25H expression induced by T0070907 in memory CD4$^+$ T-cells. Memory CD4$^+$ T-cells from HIV-uninfected individuals (n=2) were isolated, stimulated via CD3/CD28 for 3 days and then cultured in the presence/absence of T0070907 (10 µM) for up to 48 hours. Total RNA from cells was extracted 18, 24 and 48 hours post-T0070907 treatment. Level of CH25H transcripts was quantified by SYBR Green real-time PCR. FC values are indicated on the graph, with values at 18 hours being considered 1.


A. Lipid metabolism
Stimulation
DMSO
T007
PDGFRB
ABCG1
SREBF1
CDC42
CCR7
DGAT2
RAC1
AKT2
STOML2
ANXA1
TNFRSF1A
LDLRAP1
PTK2B
LYN
FLT1
MID1IP1
NOD2
PRKCE
NT5E
CPT1A
ZP3
CD81
BMP6
ADIPOQ
HTR2A
TNF
PLA2G6
ANGPTL3
GPLD1
IRS2
VAV3
PNPLA2
CREBL2
PPARA
FGF2
IFNG
PTK2
SRC
PPARG
NR4A3
SORBS1
ABHD6
MLYCD
PRKAA1
B. Phospholipid metabolism
Stimulation
DMSO
T007
STOML2
RAC1
PTK2B
PDGFRB
PIK3R5
CCR7
CDC42
LYN
NOD2
ZP3
CD81
PDGFA
KLF4
PTK2
VAV3
HTR2A
FLT1
FGF2
SLC27A1
LPCAT1
SRC
18 13 16 19 8 3 5 15 6 1 4 12 9 14 2 7
C. Glucose metabolism
Stimulation
DMSO
T007
IGF1
IRS2
EPM2AIP1
PPARA
SORBS1
SRC
IGF2
INSR
GPLD1
PMAIP1
AKT2
KAT2A
WDR5
PTPN2
DGAT2
18 13 16 5 3 19 8 15 6 7 14 2 4 9 1 12
D. Response to type I IFN
Stimulation
DMSO
T007
PSMB8
TYK2
IRF3
ADAR
SP100
OASL
IFITM2
MX2
OAS3
IFIT1
XAF1
MX1
IRF1
RNASEL
IRF7
STAT2
OAS2
IFI6
OAS1
IFI35
SAMHD1
IFITM1
ISG15
IFIT2
STAT1
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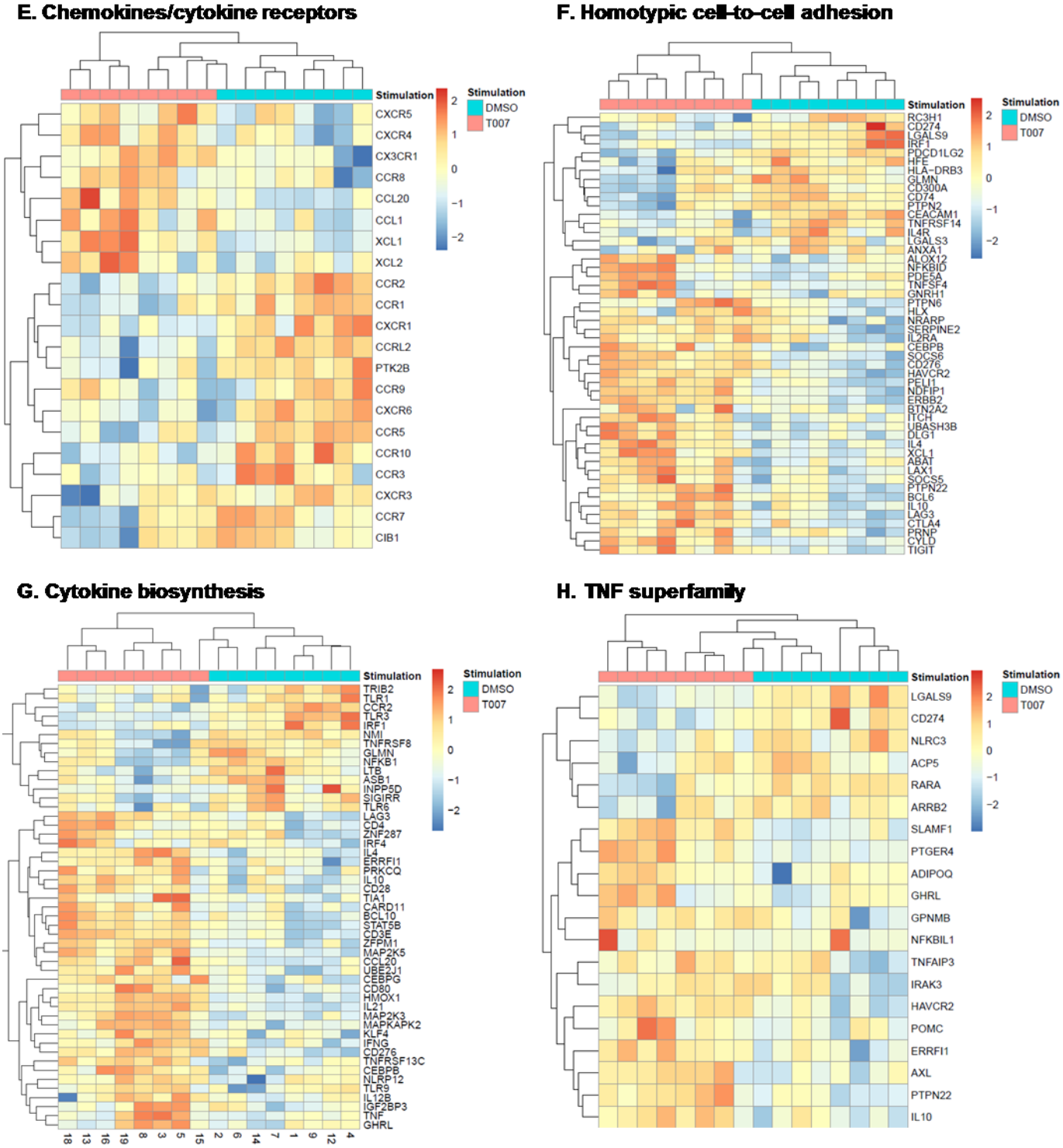
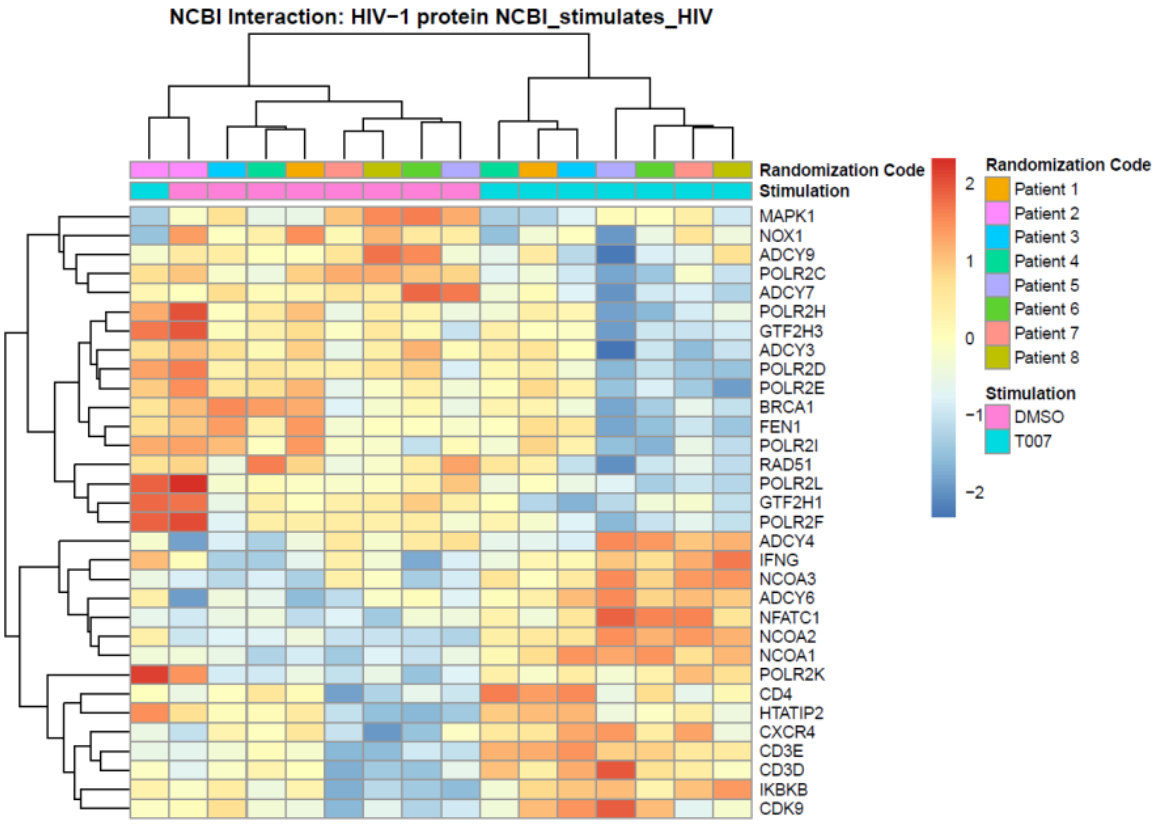
GBP2
EGR1

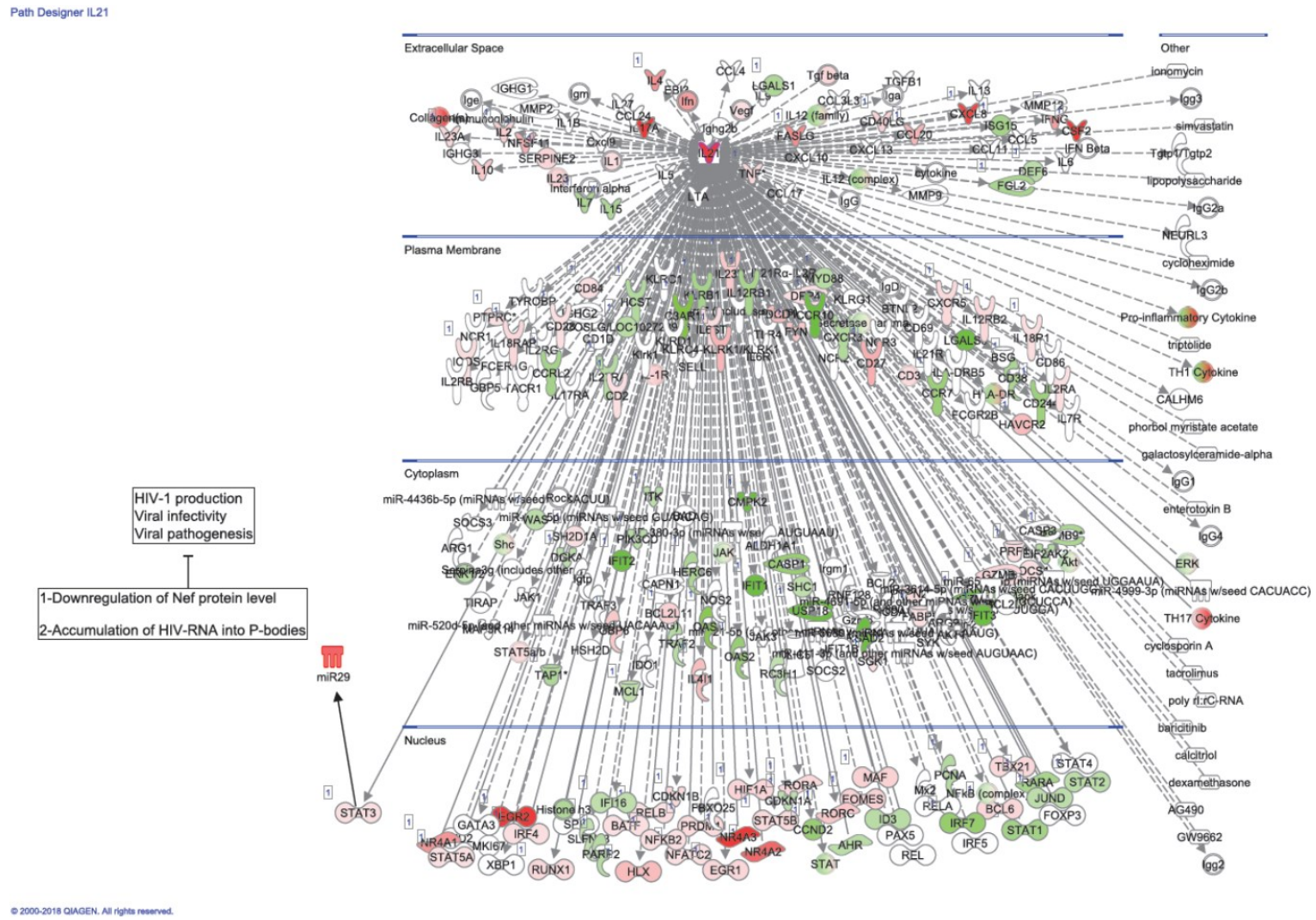


Supplemental Figure 5: Gene Ontology (GO) classification of differentially expressed genes in T0070907-treated *versus* untreated CCR6$^+$ T-cells. RNA-Seq transcriptional profiles were generated as described in Figure 5. Differentially expressed genes (p<0.05) were classified based on their biological functions using GO terms as follows: lipid metabolism **(A)**, phospholipid metabolism **(B)**, glucose metabolism **(C)**, response to type I IFN **(D)**, chemokines/cytokines receptors **(E)**, homotypic cell-to-cell

adhesion **(F)**, cytokine biosynthesis **(G)**, and TNF superfamily **(H)**. Heatmap cells are scaled by the expression level z-scores for each probe individually. For each heatmap, genes expression values are represented as a gradient from red (highest expression) to blue (lowest expression). Results from each donor are indicated with a different color code (n=8).



Supplemental Figure 6: Meta-analysis using the NCBI HIV interaction database. RNA-Seq transcriptional profiles were generated as described in Figure 5. Differentially expressed genes between T0070907-treated and untreated CCR6^{+} T-cells ($p < 0.05$, FC cut-off 1.3) were matched to the lists of human genes previously identified to interact with HIV-1 proteins. Heatmap cells are scaled by the expression level z-scores for each probe individually. For each heatmap, genes expression values are represented as a gradient from red (highest expression) to blue (lowest expression). Results from each donor are indicated with a different color code (n=8).



Supplemental Figure 7: Components of the IL-21 signaling pathway modulated by T0070907 in CCR6$^+$ T-cells. RNA-Seq transcriptional profiles were generated as described in Figure 5. Ingenuity pathway analysis (IPA) was used to illustrate gene networks associated with IL-21. The color code is based on the expression FC in T0070907-CCR6$^+$ *versus* untreated CCR6$^+$ T-cells (red and green for up-regulated and down-regulated transcripts, respectively). FC expression relative to IL-21, STAT3 and miR-29 in T0070907-treated *vs* untreated CCR6$^+$ T-cells for are illustrated. The miR-29 connection to the IL-21 network was added manually based on a recent publication demonstrating that IL-21 regulates its expression via STAT3 (75).

Supplemental Table 1: Transcripts up-regulated by T0070907 in memory CCR6+ T-cells				
Gene ID	FC	Adj. P	Gene symbol	Gene description
ENSG00000122176	105,93	9,47E-07	FMOD	fibromodulin [Source:HGNC Symbol;Acc:HGNC:3774]
ENSG00000253958	50,61	3,44E-07	CLDN23	claudin 23 [Source:HGNC Symbol;Acc:HGNC:17591]
ENSG00000167157	38,46	3,60E-07	PRRX2	paired related homeobox 2 [Source:HGNC Symbol;Acc:HGNC:21338]
ENSG00000117281	34,80	4,89E-05	CD160	CD160 molecule [Source:HGNC Symbol;Acc:HGNC:17013]
ENSG00000173404	30,42	1,42E-05	INSM1	INSM transcriptional repressor 1 [Source:HGNC Symbol;Acc:HGNC:6090]
ENSG00000109099	27,26	9,46E-07	PMP22	peripheral myelin protein 22 [Source:HGNC Symbol;Acc:HGNC:9118]
ENSG00000164932	24,52	6,72E-08	CTHRC1	collagen triple helix repeat containing 1 [Source:HGNC Symbol;Acc:HGNC:18831]
ENSG00000178947	24,50	2,05E-08	SMIM10L2A	small integral membrane protein 10 like 2A [Source:HGNC Symbol;Acc:HGNC:34499]
ENSG00000171724	24,38	5,02E-06	VAT1L	vesicle amine transport 1 like [Source:HGNC Symbol;Acc:HGNC:29315]
ENSG00000131831	23,92	4,20E-07	RAI2	retinoic acid induced 2 [Source:HGNC Symbol;Acc:HGNC:9835]
ENSG00000165895	23,62	7,73E-06	ARHGAP42	Rho GTPase activating protein 42 [Source:HGNC Symbol;Acc:HGNC:26545]
ENSG00000198535	22,56	1,84E-07	C2CD4A	C2 calcium dependent domain containing 4A [Source:HGNC Symbol;Acc:HGNC:33627]
ENSG00000164399	21,32	6,57E-07	IL3	interleukin 3 [Source:HGNC Symbol;Acc:HGNC:6011]
ENSG00000135960	20,08	3,78E-07	EDAR	ectodysplasin A receptor [Source:HGNC Symbol;Acc:HGNC:2895]
ENSG00000119508	18,63	1,41E-07	NR4A3	nuclear receptor subfamily 4 group A member 3 [Source:HGNC Symbol;Acc:HGNC:7982]
ENSG00000164684	18,58	5,54E-08	ZNF704	zinc finger protein 704 [Source:HGNC Symbol;Acc:HGNC:32291]
ENSG00000259422	18,38	8,48E-05		
ENSG00000255693	18,22	4,82E-07	LINC02389	long intergenic non-protein coding RNA 2389 [Source:HGNC Symbol;Acc:HGNC:53316]
ENSG00000138684	14,45	5,05E-07	IL21	interleukin 21 [Source:HGNC Symbol;Acc:HGNC:6005]
ENSG00000244457	14,29	1,78E-04	ENO1P1	enolase 1 pseudogene 1 [Source:HGNC Symbol;Acc:HGNC:3352]
ENSG00000150687	12,50	7,55E-09	PRSS23	serine protease 23 [Source:HGNC Symbol;Acc:HGNC:14370]
ENSG00000105974	12,06	1,18E-05	CAV1	caveolin 1 [Source:HGNC Symbol;Acc:HGNC:1527]
ENSG00000236320	11,95	1,11E-06	SLFN14	schlafen family member 14 [Source:HGNC Symbol;Acc:HGNC:32689]
ENSG00000248473	11,41	1,04E-05	LINC01962	long intergenic non-protein coding RNA 1962 [Source:HGNC Symbol;Acc:HGNC:52787]
ENSG00000106236	11,38	4,27E-06	NPTX2	neuronal pentraxin 2 [Source:HGNC Symbol;Acc:HGNC:7953]
ENSG00000122877	11,08	1,84E-07	EGR2	early growth response 2 [Source:HGNC Symbol;Acc:HGNC:3239]
ENSG00000080573	10,83	2,05E-08	COL5A3	collagen type V alpha 3 chain [Source:HGNC Symbol;Acc:HGNC:14864]
ENSG00000253304	10,53	8,21E-08	TMEM200B	transmembrane protein 200B [Source:HGNC Symbol;Acc:HGNC:33785]
ENSG00000205795	10,19	4,82E-07	CYS1	cystin 1 [Source:HGNC Symbol;Acc:HGNC:18525]
ENSG00000205502	9,89	2,01E-06	C2CD4B	C2 calcium dependent domain containing 4B [Source:HGNC Symbol;Acc:HGNC:33628]
ENSG00000235304	9,88	4,31E-06	LINC01281	long intergenic non-protein coding RNA 1281 [Source:HGNC Symbol;Acc:HGNC:50337]
ENSG00000196302	9,83	3,54E-03		
ENSG00000255026	9,82	4,31E-06		
ENSG00000164400	9,70	1,53E-06	CSF2	colony stimulating factor 2 [Source:HGNC Symbol;Acc:HGNC:2434]
ENSG00000112115	9,52	1,78E-04	IL17A	interleukin 17A [Source:HGNC Symbol;Acc:HGNC:5981]
ENSG00000106025	9,48	5,82E-05	TSPAN12	tetraspanin 12 [Source:HGNC Symbol;Acc:HGNC:21641]
ENSG00000204671	9,47	1,21E-04	IL31	interleukin 31 [Source:HGNC Symbol;Acc:HGNC:19372]

ENSG00000157680	9,33	7,61E-07	DGKI	diacylglycerol kinase iota [Source:HGNC Symbol;Acc:HGNC:2855]
ENSG00000105928	9,19	1,84E-07	GSDME	gasdermin E [Source:HGNC Symbol;Acc:HGNC:2810]
ENSG00000120549	9,13	7,24E-07	KIAA1217	KIAA1217 [Source:HGNC Symbol;Acc:HGNC:25428]
ENSG00000173210	9,09	8,78E-06	ABLIM3	actin binding LIM protein family member 3 [Source:HGNC Symbol;Acc:HGNC:29132]
ENSG00000153208	9,02	3,19E-06	MERTK	MER proto-oncogene, tyrosine kinase [Source:HGNC Symbol;Acc:HGNC:7027]
ENSG00000198734	8,90	5,05E-07	F5	coagulation factor V [Source:HGNC Symbol;Acc:HGNC:3542]
ENSG00000082781	8,85	1,76E-06	ITGB5	integrin subunit beta 5 [Source:HGNC Symbol;Acc:HGNC:6160]
ENSG00000262526	8,83	1,97E-02		
ENSG00000156535	8,68	5,02E-06	CD109	CD109 molecule [Source:HGNC Symbol;Acc:HGNC:21685]
ENSG00000134489	8,52	8,60E-06	HRH4	histamine receptor H4 [Source:HGNC Symbol;Acc:HGNC:17383]
ENSG00000169429	8,37	7,78E-05	CXCL8	C-X-C motif chemokine ligand 8 [Source:HGNC Symbol;Acc:HGNC:6025]
ENSG00000171658	8,11	7,17E-05	NMRAL2P	NmrA like redox sensor 2, pseudogene [Source:HGNC Symbol;Acc:HGNC:52332]
ENSG00000087494	7,96	3,39E-06	PTHLH	parathyroid hormone like hormone [Source:HGNC Symbol;Acc:HGNC:9607]
ENSG00000272273	7,84	1,88E-05	IER3-AS1	IER3 antisense RNA 1 [Source:HGNC Symbol;Acc:HGNC:53629]
ENSG00000141574	7,67	6,22E-07	SECTM1	secreted and transmembrane 1 [Source:HGNC Symbol;Acc:HGNC:10707]
ENSG00000250295	7,65	1,49E-04	RDH10-AS1	RDH10 antisense RNA 1 [Source:HGNC Symbol;Acc:HGNC:51658]
ENSG00000143878	7,62	2,41E-07	RHOB	ras homolog family member B [Source:HGNC Symbol;Acc:HGNC:668]
ENSG00000100292	7,52	1,52E-07	HMOX1	heme oxygenase 1 [Source:HGNC Symbol;Acc:HGNC:5013]
ENSG00000170647	7,51	3,46E-04	-	-
ENSG00000101134	7,43	5,93E-04	DOK5	docking protein 5 [Source:HGNC Symbol;Acc:HGNC:16173]
ENSG00000074410	7,32	4,89E-05	CA12	carbonic anhydrase 12 [Source:HGNC Symbol;Acc:HGNC:1371]
ENSG00000143184	7,32	1,91E-05	XCL1	X-C motif chemokine ligand 1 [Source:HGNC Symbol;Acc:HGNC:10645]
ENSG00000165457	7,31	1,32E-04	FOLR2	folate receptor beta [Source:HGNC Symbol;Acc:HGNC:3793]
ENSG00000259363	7,30	7,78E-07		
ENSG00000110675	7,23	2,06E-05	ELMOD1	ELMO domain containing 1 [Source:HGNC Symbol;Acc:HGNC:25334]
ENSG00000088826	7,17	3,65E-06	SMOX	spermine oxidase [Source:HGNC Symbol;Acc:HGNC:15862]
ENSG00000225125	7,13	2,57E-05	RANP4	RAN, member RAS oncogene family pseudogene 4 [Acc:HGNC:39859]
ENSG00000236833	7,00	8,82E-07		
ENSG00000235488	6,96	6,69E-05	JARID2-AS1	JARID2 antisense RNA 1 [Source:HGNC Symbol;Acc:HGNC:40314]
ENSG00000124212	6,95	9,46E-07	PTGIS	prostaglandin I2 synthase [Source:HGNC Symbol;Acc:HGNC:9603]
ENSG00000270164	6,91	4,25E-06	LINC01480	long intergenic non-protein coding RNA 1480 [Source:HGNC Symbol;Acc:HGNC:51124]
ENSG00000132329	6,81	8,12E-07	RAMP1	receptor activity modifying protein 1 [Source:HGNC Symbol;Acc:HGNC:9843]
ENSG00000116981	6,81	3,47E-03	NT5C1A	5'-nucleotidase, cytosolic IA [Source:HGNC Symbol;Acc:HGNC:17819]
ENSG00000108702	6,76	3,83E-03	CCL1	C-C motif chemokine ligand 1 [Source:HGNC Symbol;Acc:HGNC:10609]
ENSG00000243244	6,76	4,07E-06	STON1	stonin 1 [Source:HGNC Symbol;Acc:HGNC:17003]
ENSG00000124466	6,75	2,23E-05	LYPD3	LY6/PLAUR domain containing 3 [Source:HGNC Symbol;Acc:HGNC:24880]
ENSG00000163053	6,72	5,71E-06	SLC16A14	solute carrier family 16 member 14 [Source:HGNC Symbol;Acc:HGNC:26417]
ENSG00000153234	6,70	5,27E-06	NR4A2	nuclear receptor subfamily 4 group A member 2 [Source:HGNC Symbol;Acc:HGNC:7981]
ENSG00000121039	6,47	2,68E-07	RDH10	retinol dehydrogenase 10 [Source:HGNC Symbol;Acc:HGNC:19975]

ENSG00000132465	6,42	5,19E-04	JCHAIN	joining chain of multimeric IgA and IgM [Source:HGNC Symbol;Acc:HGNC:5713]
ENSG00000118257	6,29	3,90E-05	NRP2	neuropilin 2 [Source:HGNC Symbol;Acc:HGNC:8005]
ENSG00000088882	6,24	7,92E-05	CPXM1	carboxypeptidase X, M14 family member 1 [Source:HGNC Symbol;Acc:HGNC:15771]
ENSG00000248176	6,21	6,96E-04		
ENSG00000156804	6,15	1,87E-07	FBXO32	F-box protein 32 [Source:HGNC Symbol;Acc:HGNC:16731]
ENSG00000244242	6,13	2,17E-07	IFITM10	interferon induced transmembrane protein 10 [Source:HGNC Symbol;Acc:HGNC:40022]
ENSG00000145685	6,11	2,93E-06	LHFPL2	LHFPL tetraspan subfamily member 2 [Source:HGNC Symbol;Acc:HGNC:6588]
ENSG00000085733	6,03	3,71E-05	CTTN	cortactin [Source:HGNC Symbol;Acc:HGNC:3338]
ENSG00000109943	6,01	1,04E-05	CRTAM	cytotoxic and regulatory T cell molecule [Source:HGNC Symbol;Acc:HGNC:24313]
ENSG00000236324	5,99	2,45E-05		
ENSG00000137441	5,96	4,23E-05	FGFBP2	fibroblast growth factor binding protein 2 [Source:HGNC Symbol;Acc:HGNC:29451]
ENSG00000143869	5,95	1,04E-05	GDF7	growth differentiation factor 7 [Source:HGNC Symbol;Acc:HGNC:4222]
ENSG00000145113	5,91	5,02E-06	MUC4	mucin 4, cell surface associated [Source:HGNC Symbol;Acc:HGNC:7514]
ENSG00000160883	5,77	7,08E-05	HK3	hexokinase 3 [Source:HGNC Symbol;Acc:HGNC:4925]
ENSG00000120278	5,74	6,14E-03	PLEKHG1	pleckstrin homology and RhoGEF domain containing G1 [Acc:HGNC:20884]
ENSG00000113555	5,73	3,58E-06	PCDH12	protocadherin 12 [Source:HGNC Symbol;Acc:HGNC:8657]
ENSG00000174885	5,71	2,96E-06	NLRP6	NLR family pyrin domain containing 6 [Source:HGNC Symbol;Acc:HGNC:22944]
ENSG00000261707	5,64	4,80E-03		
ENSG00000244265	5,63	2,38E-05	SIAH2-AS1	SIAH2 antisense RNA 1 [Source:HGNC Symbol;Acc:HGNC:40526]
ENSG00000116299	5,61	2,54E-08	KIAA1324	KIAA1324 [Source:HGNC Symbol;Acc:HGNC:29618]
ENSG00000177426	5,57	2,26E-07	TGIF1	TGFB induced factor homeobox 1 [Source:HGNC Symbol;Acc:HGNC:11776]
ENSG00000185668	5,54	2,13E-06	POU3F1	POU class 3 homeobox 1 [Source:HGNC Symbol;Acc:HGNC:9214]
ENSG00000225899	5,54	2,02E-03	FRG2B	FSHD region gene 2 family member B [Source:HGNC Symbol;Acc:HGNC:33518]
ENSG00000235125	5,53	2,79E-02	NFKBIL1	NFKB inhibitor like 1 [Source:HGNC Symbol;Acc:HGNC:7800]
ENSG00000187479	5,52	5,82E-05	C11orf96	chromosome 11 open reading frame 96 [Source:HGNC Symbol;Acc:HGNC:38675]
ENSG00000230753	5,52	4,62E-04	ZNF341-AS1	ZNF341 antisense RNA 1 [Source:HGNC Symbol;Acc:HGNC:50736]
ENSG00000236528	5,52	2,32E-03		
ENSG00000272862	5,48	4,16E-05		
ENSG00000272486	5,39	7,72E-04		
ENSG00000174944	5,39	2,29E-05	P2RY14	purinergic receptor P2Y14 [Source:HGNC Symbol;Acc:HGNC:16442]
ENSG00000259251	5,37	1,47E-04		
ENSG00000102174	5,33	1,83E-05	PHEX	phosphate regulating endopeptidase homolog X-linked [Acc:HGNC:8918]
ENSG00000143185	5,29	7,57E-04	XCL2	X-C motif chemokine ligand 2 [Source:HGNC Symbol;Acc:HGNC:10646]
ENSG00000142102	5,15	1,43E-05	PGGHG	protein-glucosylgalactosylhydroxylysine glucosidase [Acc:HGNC:26210]
ENSG00000177494	5,14	5,78E-07	ZBED2	zinc finger BED-type containing 2 [Source:HGNC Symbol;Acc:HGNC:20710]
ENSG00000259948	5,13	6,08E-03		
ENSG00000188051	5,08	3,50E-05	TMEM221	transmembrane protein 221 [Source:HGNC Symbol;Acc:HGNC:21943]
ENSG00000127318	5,06	1,42E-04	IL22	interleukin 22 [Source:HGNC Symbol;Acc:HGNC:14900]
ENSG00000180834	5,04	1,33E-04	MAP6D1	MAP6 domain containing 1 [Source:HGNC Symbol;Acc:HGNC:25753]

ENSG00000231621	5,04	9,65E-06		
ENSG00000225400	5,02	2,45E-05	RAB28P5	RAB28, member RAS oncogene family pseudogene 5 [Acc:HGNC:51547]
ENSG00000117560	5,01	4,03E-07	FASLG	Fas ligand [Source:HGNC Symbol;Acc:HGNC:11936]
ENSG00000164938	4,99	6,57E-07	TP53INP1	tumor protein p53 inducible nuclear protein 1 [Source:HGNC Symbol;Acc:HGNC:18022]
ENSG00000228216	4,95	1,45E-04		
ENSG00000068781	4,91	3,56E-03	STON1-GTF2A1L	STON1-GTF2A1L readthrough [Source:HGNC Symbol;Acc:HGNC:30651]
ENSG00000244405	4,90	1,29E-04	ETV5	ETS variant 5 [Source:HGNC Symbol;Acc:HGNC:3494]
ENSG00000167244	4,88	1,50E-03	IGF2	insulin like growth factor 2 [Source:HGNC Symbol;Acc:HGNC:5466]
ENSG00000182580	4,86	9,73E-06	EPHB3	EPH receptor B3 [Source:HGNC Symbol;Acc:HGNC:3394]
ENSG00000229502	4,86	8,49E-05		
ENSG00000113520	4,84	1,10E-05	IL4	interleukin 4 [Source:HGNC Symbol;Acc:HGNC:6014]
ENSG00000049249	4,82	3,26E-05	TNFRSF9	TNF receptor superfamily member 9 [Source:HGNC Symbol;Acc:HGNC:11924]
ENSG00000198794	4,76	2,21E-05	SCAMP5	secretory carrier membrane protein 5 [Source:HGNC Symbol;Acc:HGNC:30386]
ENSG00000265787	4,69	4,21E-04	CYP4F35P	cytochrome P450 family 4 subfamily F member 35, pseudogene [Acc:HGNC:39954]
ENSG00000135862	4,69	2,57E-04	LAMC1	laminin subunit gamma 1 [Source:HGNC Symbol;Acc:HGNC:6492]
ENSG00000198574	4,68	3,86E-02	SH2D1B	SH2 domain containing 1B [Source:HGNC Symbol;Acc:HGNC:30416]
ENSG00000103196	4,67	8,25E-06	CRISPLD2	cysteine rich secretory protein LCCL domain containing 2 [Acc:HGNC:25248]
ENSG00000123358	4,63	4,98E-06	NR4A1	nuclear receptor subfamily 4 group A member 1 [Source:HGNC Symbol;Acc:HGNC:7980]
ENSG00000185634	4,61	8,12E-07	SHC4	SHC adaptor protein 4 [Source:HGNC Symbol;Acc:HGNC:16743]
ENSG00000206013	4,60	7,38E-04	IFITM5	interferon induced transmembrane protein 5 [Source:HGNC Symbol;Acc:HGNC:16644]
ENSG00000182397	4,60	2,03E-06	DNM1P46	dynamin 1 pseudogene 46 [Source:HGNC Symbol;Acc:HGNC:35199]
ENSG00000172380	4,55	2,27E-04	GNG12	G protein subunit gamma 12 [Source:HGNC Symbol;Acc:HGNC:19663]
ENSG00000204172	4,54	6,33E-03	AGAP9	ArfGAP with GTPase domain, ankyrin repeat and PH domain 9 [Acc:HGNC:23463]
ENSG00000266642	4,49	8,52E-03		
ENSG00000135318	4,48	5,82E-05	NT5E	5'-nucleotidase ecto [Source:HGNC Symbol;Acc:HGNC:8021]
ENSG00000165152	4,48	2,08E-04	TMEM246	transmembrane protein 246 [Source:HGNC Symbol;Acc:HGNC:28180]
ENSG00000234261	4,46	1,08E-05		
ENSG00000132170	4,46	1,39E-06	PPARG	peroxisome proliferator activated receptor gamma [Source:HGNC Symbol;Acc:HGNC:9236]
ENSG00000234361	4,45	3,35E-04		
ENSG00000168243	4,45	1,04E-05	GNG4	G protein subunit gamma 4 [Source:HGNC Symbol;Acc:HGNC:4407]
ENSG00000116991	4,43	3,16E-05	SIPA1L2	signal induced proliferation associated 1 like 2 [Source:HGNC Symbol;Acc:HGNC:23800]
ENSG00000186197	4,41	6,97E-06	EDARADD	EDAR associated death domain [Source:HGNC Symbol;Acc:HGNC:14341]
ENSG00000108511	4,39	1,81E-05	HOXB6	homeobox B6 [Source:HGNC Symbol;Acc:HGNC:5117]
ENSG00000089692	4,36	4,51E-07	LAG3	lymphocyte activating 3 [Source:HGNC Symbol;Acc:HGNC:6476]
ENSG00000119411	4,35	1,15E-05	BSPRY	B-box and SPRY domain containing [Source:HGNC Symbol;Acc:HGNC:18232]
ENSG00000249626	4,31	1,26E-05		
ENSG00000231651	4,30	5,53E-06	DLG3-AS1	DLG3 antisense RNA 1 [Source:HGNC Symbol;Acc:HGNC:40182]
ENSG00000102445	4,28	6,12E-04	RUBCNL	RUN and cysteine rich domain containing beclin 1 interacting protein like [Acc:HGNC:20420]
ENSG00000136205	4,26	3,00E-03	TNS3	tensin 3 [Source:HGNC Symbol;Acc:HGNC:21616]

ENSG00000110777	4,26	5,93E-06	POU2AF1	POU class 2 associating factor 1 [Source:HGNC Symbol;Acc:HGNC:9211]
ENSG00000148426	4,26	7,75E-05	PROSER2	proline and serine rich 2 [Source:HGNC Symbol;Acc:HGNC:23728]
ENSG00000267650	4,25	1,67E-03		
ENSG00000140859	4,25	8,92E-07	KIFC3	kinesin family member C3 [Source:HGNC Symbol;Acc:HGNC:6326]
ENSG00000122367	4,24	6,82E-03	LDB3	LIM domain binding 3 [Source:HGNC Symbol;Acc:HGNC:15710]
ENSG00000111537	4,23	2,45E-05	IFNG	interferon gamma [Source:HGNC Symbol;Acc:HGNC:5438]
ENSG00000164949	4,22	1,61E-05	GEM	GTP binding protein overexpressed in skeletal muscle [Acc:HGNC:4234]
ENSG00000258212	4,22	3,36E-03	ZNF75BP	zinc finger protein 75B, pseudogene [Source:HGNC Symbol;Acc:HGNC:13147]
ENSG00000204103	4,21	4,24E-04	MAFB	MAF bZIP transcription factor B [Source:HGNC Symbol;Acc:HGNC:6408]
ENSG00000151276	4,21	4,16E-06	MAGI1	membrane associated guanylate kinase, WW and PDZ domain containing 1 [Acc:HGNC:946]
ENSG00000153563	4,19	1,41E-03	CD8A	CD8a molecule [Source:HGNC Symbol;Acc:HGNC:1706]
ENSG00000102755	4,19	6,97E-06	FLT1	fms related tyrosine kinase 1 [Source:HGNC Symbol;Acc:HGNC:3763]
ENSG00000128253	4,17	2,51E-03	RFPL2	ret finger protein like 2 [Source:HGNC Symbol;Acc:HGNC:9979]
ENSG00000275778	4,15	2,02E-02		
ENSG00000106537	4,15	1,62E-05	TSPAN13	tetraspanin 13 [Source:HGNC Symbol;Acc:HGNC:21643]
ENSG00000198125	4,14	1,13E-03	MB	myoglobin [Source:HGNC Symbol;Acc:HGNC:6915]
ENSG00000224769	4,13	2,29E-05	MUC20P1	mucin 20, cell surface associated pseudogene 1 [Source:HGNC Symbol;Acc:HGNC:51921]
ENSG00000226260	4,12	4,07E-02	HLA-DRA	major histocompatibility complex, class II, DR alpha [Source:HGNC Symbol;Acc:HGNC:4947]
ENSG00000104081	4,10	3,19E-04	BMF	Bcl2 modifying factor [Source:HGNC Symbol;Acc:HGNC:24132]
ENSG00000050820	4,09	1,77E-05	BCAR1	BCAR1, Cas family scaffolding protein [Source:HGNC Symbol;Acc:HGNC:971]
ENSG00000230968	4,09	4,21E-04		
ENSG00000104921	4,09	1,53E-03	FCER2	Fc fragment of IgE receptor II [Source:HGNC Symbol;Acc:HGNC:3612]
ENSG00000169758	4,08	1,35E-04	TMEM266	transmembrane protein 266 [Source:HGNC Symbol;Acc:HGNC:26763]
ENSG00000213626	4,06	3,44E-07	LBH	limb bud and heart development [Source:HGNC Symbol;Acc:HGNC:29532]
ENSG00000230623	4,05	7,97E-05		
ENSG00000224360	4,04	3,76E-02	DDR1-AS1	DDR1 antisense RNA 1 (head to head) [Source:HGNC Symbol;Acc:HGNC:28694]
ENSG00000121900	4,03	1,04E-05	TMEM54	transmembrane protein 54 [Source:HGNC Symbol;Acc:HGNC:24143]
ENSG00000176641	4,03	4,24E-04	RNF152	ring finger protein 152 [Source:HGNC Symbol;Acc:HGNC:26811]
ENSG00000155926	4,02	9,51E-08	SLA	Src like adaptor [Source:HGNC Symbol;Acc:HGNC:10902]
ENSG00000107242	4,01	1,18E-05	PIP5K1B	phosphatidylinositol-4-phosphate 5-kinase type 1 beta [Acc:HGNC:8995]
ENSG00000124191	3,98	4,07E-05	TOX2	TOX high mobility group box family member 2 [Source:HGNC Symbol;Acc:HGNC:16095]
ENSG00000147041	3,98	8,15E-04	SYTL5	synaptotagmin like 5 [Source:HGNC Symbol;Acc:HGNC:15589]
ENSG00000273275	3,97	1,32E-03		
ENSG00000091972	3,97	3,33E-04	CD200	CD200 molecule [Source:HGNC Symbol;Acc:HGNC:7203]
ENSG00000258760	3,96	2,01E-05		
ENSG00000020577	3,95	3,39E-05	SAMD4A	sterile alpha motif domain containing 4A [Source:HGNC Symbol;Acc:HGNC:23023]
ENSG00000164023	3,92	7,95E-06	SGMS2	sphingomyelin synthase 2 [Source:HGNC Symbol;Acc:HGNC:28395]
ENSG00000215045	3,92	4,31E-06	GRID2IP	Grid2 interacting protein [Source:HGNC Symbol;Acc:HGNC:18464]
ENSG00000223687	3,90	2,81E-02	ZNF311	zinc finger protein 311 [Source:HGNC Symbol;Acc:HGNC:13847]

Supplemental Table 2: Transcripts down-regulated by T0070907 in memory CCR6+ T-cells				
Gene ID	FC	Adj. P	Gene symbol	Gene description
ENSG00000203772	-8,71	1,25E-02	SPRN	shadow of prion protein [Source:HGNC Symbol;Acc:HGNC:16871]
ENSG00000134326	-8,51	7,83E-06	CMPK2	cytidine/uridine monophosphate kinase 2 [Source:HGNC Symbol;Acc:HGNC:27015]
ENSG00000163464	-8,10	2,92E-05	CXCR1	C-X-C motif chemokine receptor 1 [Source:HGNC Symbol;Acc:HGNC:6026]
ENSG00000134321	-8,09	2,20E-05	RSAD2	radical S-adenosyl methionine domain containing 2 [Source:HGNC Symbol;Acc:HGNC:30908]
ENSG00000133101	-8,07	1,35E-03	CCNA1	cyclin A1 [Source:HGNC Symbol;Acc:HGNC:1577]
ENSG00000145649	-7,39	9,98E-06	GZMA	granzyme A [Source:HGNC Symbol;Acc:HGNC:4708]
ENSG00000251349	-7,15	2,55E-02	MSANTD3-TMEFF1	MSANTD3-TMEFF1 readthrough [Source:HGNC Symbol;Acc:HGNC:38838]
ENSG00000262655	-6,52	5,63E-05	SPON1	spondin 1 [Source:HGNC Symbol;Acc:HGNC:11252]
ENSG00000144476	-6,50	2,01E-06	ACKR3	atypical chemokine receptor 3 [Source:HGNC Symbol;Acc:HGNC:23692]
ENSG00000132359	-6,02	3,59E-06	RAP1GAP2	RAP1 GTPase activating protein 2 [Source:HGNC Symbol;Acc:HGNC:29176]
ENSG00000137959	-5,98	7,24E-05	IFI44L	interferon induced protein 44 like [Source:HGNC Symbol;Acc:HGNC:17817]
ENSG00000188822	-5,40	1,22E-02	CNR2	cannabinoid receptor 2 [Source:HGNC Symbol;Acc:HGNC:2160]
ENSG00000273217	-5,14	1,39E-02		
ENSG00000227422	-4,96	1,28E-02	DDR1-AS1	DDR1 antisense RNA 1 (head to head) [Source:HGNC Symbol;Acc:HGNC:28694]
ENSG00000184979	-4,94	2,34E-05	USP18	ubiquitin specific peptidase 18 [Source:HGNC Symbol;Acc:HGNC:12616]
ENSG00000116574	-4,84	7,73E-06	RHOU	ras homolog family member U [Source:HGNC Symbol;Acc:HGNC:17794]
ENSG00000133321	-4,81	3,60E-07	RARRES3	retinoic acid receptor responder 3 [Source:HGNC Symbol;Acc:HGNC:9869]
ENSG00000203896	-4,80	2,61E-05	LIME1	Lck interacting transmembrane adaptor 1 [Source:HGNC Symbol;Acc:HGNC:26016]
ENSG00000164342	-4,76	8,32E-06	TLR3	toll like receptor 3 [Source:HGNC Symbol;Acc:HGNC:11849]
ENSG00000185745	-4,74	2,45E-05	IFIT1	interferon induced protein with tetratricopeptide repeats 1 [Source:HGNC Symbol;Acc:HGNC:5407]
ENSG00000079385	-4,63	8,16E-07	CEACAM1	carcinoembryonic antigen related cell adhesion molecule 1 [Source:HGNC Symbol;Acc:HGNC:1814]
ENSG00000114455	-4,49	9,13E-06	HHLA2	HERV-H LTR-associating 2 [Source:HGNC Symbol;Acc:HGNC:4905]
ENSG00000182585	-4,47	2,54E-04	EPGN	epithelial mitogen [Source:HGNC Symbol;Acc:HGNC:17470]
ENSG00000122043	-4,46	7,09E-05	LINC00544	long intergenic non-protein coding RNA 544 [Source:HGNC Symbol;Acc:HGNC:43679]
ENSG00000138722	-4,44	1,54E-05	MMRN1	multimerin 1 [Source:HGNC Symbol;Acc:HGNC:7178]
ENSG00000157601	-4,28	9,31E-06	MX1	MX dynamin like GTPase 1 [Source:HGNC Symbol;Acc:HGNC:7532]
ENSG00000271288	-4,19	9,89E-05	IGHV1OR15-3	immunoglobulin heavy variable 1/OR15-3 (pseudogene) [Source:HGNC Symbol;Acc:HGNC:5565]
ENSG00000132832	-4,13	3,36E-04		
ENSG00000119917	-4,01	3,02E-05	IFIT3	interferon induced protein with tetratricopeptide repeats 3 [Source:HGNC Symbol;Acc:HGNC:5411]
ENSG00000141161	-4,00	1,88E-02	UNC45B	unc-45 myosin chaperone B [Source:HGNC Symbol;Acc:HGNC:14304]
ENSG00000005102	-3,94	1,56E-04	MEOX1	mesenchyme homeobox 1 [Source:HGNC Symbol;Acc:HGNC:7013]
ENSG00000198133	-3,91	5,42E-06	TMEM229B	transmembrane protein 229B [Source:HGNC Symbol;Acc:HGNC:20130]
ENSG00000248871	-3,88	4,23E-02	TNFSF12-TNFSF13	TNFSF12-TNFSF13 readthrough [Source:HGNC Symbol;Acc:HGNC:33537]
ENSG00000278139	-3,83	1,70E-02		
ENSG00000171595	-3,73	7,95E-05	DNAI2	dynein axonemal intermediate chain 2 [Source:HGNC Symbol;Acc:HGNC:18744]
ENSG00000228913	-3,69	4,63E-02	UBD	ubiquitin D [Source:HGNC Symbol;Acc:HGNC:18795]
ENSG00000119922	-3,67	1,37E-06	IFIT2	interferon induced protein with tetratricopeptide repeats 2 [Source:HGNC Symbol;Acc:HGNC:5409]

ENSG00000183662	-3,67	1,12E-03	FAM19A1	family with sequence similarity 19 member A1, C-C motif chemokine like [Acc:HGNC:21587]
ENSG00000089127	-3,62	2,24E-06	OAS1	2'-5'-oligoadenylate synthetase 1 [Source:HGNC Symbol;Acc:HGNC:8086]
ENSG00000184451	-3,61	8,70E-05	CCR10	C-C motif chemokine receptor 10 [Source:HGNC Symbol;Acc:HGNC:4474]
ENSG00000145491	-3,52	4,70E-04	ROPN1L	rhophilin associated tail protein 1 like [Source:HGNC Symbol;Acc:HGNC:24060]
ENSG00000113088	-3,47	1,65E-04	GZMK	granzyme K [Source:HGNC Symbol;Acc:HGNC:4711]
ENSG00000168961	-3,45	1,14E-05	LGALS9	galectin 9 [Source:HGNC Symbol;Acc:HGNC:6570]
ENSG00000223405	-3,27	2,24E-02	DDR1-AS1	DDR1 antisense RNA 1 (head to head) [Source:HGNC Symbol;Acc:HGNC:28694]
ENSG00000186399	-3,27	2,43E-02	GOLGA8R	golgin A8 family member R [Source:HGNC Symbol;Acc:HGNC:44407]
ENSG00000105088	-3,26	1,15E-05	OLFM2	olfactomedin 2 [Source:HGNC Symbol;Acc:HGNC:17189]
ENSG00000121807	-3,23	4,19E-06	CCR2	C-C motif chemokine receptor 2 [Source:HGNC Symbol;Acc:HGNC:1603]
ENSG00000151490	-3,22	1,17E-04	PTPRO	protein tyrosine phosphatase, receptor type O [Source:HGNC Symbol;Acc:HGNC:9678]
ENSG00000114315	-3,22	5,50E-03	HES1	hes family bHLH transcription factor 1 [Source:HGNC Symbol;Acc:HGNC:5192]
ENSG00000137965	-3,21	1,42E-05	IFI44	interferon induced protein 44 [Source:HGNC Symbol;Acc:HGNC:16938]
ENSG00000141540	-3,20	5,09E-05	TTYH2	tweety family member 2 [Source:HGNC Symbol;Acc:HGNC:13877]
ENSG00000138792	-3,19	9,41E-06	ENPEP	glutamyl aminopeptidase [Source:HGNC Symbol;Acc:HGNC:3355]
ENSG00000136514	-3,11	2,55E-06	RTP4	receptor transporter protein 4 [Source:HGNC Symbol;Acc:HGNC:23992]
ENSG00000168461	-3,10	1,15E-04	RAB31	RAB31, member RAS oncogene family [Source:HGNC Symbol;Acc:HGNC:9771]
ENSG00000107438	-3,09	2,36E-04	PDLIM1	PDZ and LIM domain 1 [Source:HGNC Symbol;Acc:HGNC:2067]
ENSG00000255221	-3,08	1,53E-05	CARD17	caspase recruitment domain family member 17 [Source:HGNC Symbol;Acc:HGNC:33827]
ENSG00000163823	-3,05	1,15E-05	CCR1	C-C motif chemokine receptor 1 [Source:HGNC Symbol;Acc:HGNC:1602]
ENSG00000187808	-3,05	6,57E-04	SOWAHD	sosondowah ankyrin repeat domain family member D [Source:HGNC Symbol;Acc:HGNC:32960]
ENSG00000231550	-3,05	7,99E-04	PTCHD3P2	patched domain containing 3 pseudogene 2 [Source:HGNC Symbol;Acc:HGNC:44946]
ENSG00000139626	-3,04	3,35E-05	ITGB7	integrin subunit beta 7 [Source:HGNC Symbol;Acc:HGNC:6162]
ENSG00000128833	-3,00	2,01E-06	MYO5C	myosin VC [Source:HGNC Symbol;Acc:HGNC:7604]
ENSG00000112303	-2,98	3,70E-05	VNN2	vanin 2 [Source:HGNC Symbol;Acc:HGNC:12706]
ENSG00000169413	-2,97	1,72E-04	RNASE6	ribonuclease A family member k6 [Source:HGNC Symbol;Acc:HGNC:10048]
ENSG00000204267	-2,97	4,70E-02	TAP2	transporter 2, ATP binding cassette subfamily B member [Source:HGNC Symbol;Acc:HGNC:44]
ENSG00000105967	-2,94	1,61E-02	TFEC	transcription factor EC [Source:HGNC Symbol;Acc:HGNC:11754]
ENSG00000165272	-2,90	3,62E-05	AQP3	aquaporin 3 (Gill blood group) [Source:HGNC Symbol;Acc:HGNC:636]
ENSG00000113070	-2,90	7,48E-05	HBEGF	heparin binding EGF like growth factor [Source:HGNC Symbol;Acc:HGNC:3059]
ENSG00000177409	-2,89	6,97E-07	SAMD9L	sterile alpha motif domain containing 9 like [Source:HGNC Symbol;Acc:HGNC:1349]
ENSG00000132530	-2,88	3,00E-05	XAF1	XIAP associated factor 1 [Source:HGNC Symbol;Acc:HGNC:30932]
ENSG00000240888	-2,87	2,78E-03		
ENSG00000144847	-2,85	6,06E-04	IGSF11	immunoglobulin superfamily member 11 [Source:HGNC Symbol;Acc:HGNC:16669]
ENSG00000112195	-2,85	5,44E-06	TREML2	triggering receptor expressed on myeloid cells like 2 [Source:HGNC Symbol;Acc:HGNC:21092]
ENSG00000083454	-2,84	1,13E-03	P2RX5	purinergic receptor P2X 5 [Source:HGNC Symbol;Acc:HGNC:8536]
ENSG00000117228	-2,82	1,06E-06	GBP1	guanylate binding protein 1 [Source:HGNC Symbol;Acc:HGNC:4182]
ENSG00000166963	-2,82	8,66E-05	MAP1A	microtubule associated protein 1A [Source:HGNC Symbol;Acc:HGNC:6835]
ENSG00000171246	-2,81	1,12E-03	NPTX1	neuronal pentraxin 1 [Source:HGNC Symbol;Acc:HGNC:7952]

Supplemental Table 3: Antibodies used for flow cytometry analysis and sorting

	Fluorochrome	Clone	Vendor
CD4	AlexaFluor700	RPAT4	BD Pharmingen
CCR6	PE	11A9	(San Diego, CA, USA)
CCR5	PE	2D7/CCR5	
IFN-γ	AlexaFluor700	B27	
CXCR4	PE	12G5	
Ki67	FITC	B56	
B7 integrin	FITC	FIB504	eBioscience
phospho-mTOR	FITC	MRRBY	(San Diego, CA, USA)
CD56	FITC	MEM188	
IL-17A	PE	eBio64BEC17	
CD8	FITC	BW135/80	Miltenyi Biotech
CD19	FITC	LT19	(Auburn, CA, USA)
CD45RA	APCeFluor780	HI100	Invitrogen
			(Waltham, MA, USA)
HIV-p24	FITC	KC57	Beckman Coulter
			(Brea, CA, USA)

Supplemental Table 4: Oligonucleotides sequences of primers and probes used for HIV-RNA and HIV-DNA quantification

Primers/Probes	Oligonucleotides Sequences
Table 4a: External/internal primers and paqman probe used for CA HIV-RNA and DNA quantification	
ULF1	5'-ATGCCACGTAAGCGAAACTCTGGGTCTCTCTDGTTAGAC-3'
UR1	5'-CCATCTCTCTCCTTCTAGC-3'.
Lambda (λ) T	5'-ATGCCACGTAAGCGAAACT-3'
UR2 IP	5'-CTGAGGGATCTCTAGTTACC-3'.
UHIV FamZen	5'-/56-FAM/CACTCAAGG/ZEN/CAAGCTTTATTGAGGC/3IABkFQ/-3'
Table 4b: External/internal primers and taqman probe used for CD3 quantification (together with Gag HIV-DNA)	
HCD3OUT 5' E	5'-ACTGACATGGAACAGGGGAAG-3'
HCD3OUT 3'	5'-CCAGCTCTGAAGTAGGGAACATAT-3'
HCD3IN 5'	GGCTATCATTCTTCTTCAAGGT
HCD3IN3'	CCTCTCTTCAGCCATTTAAGTA
CD3 FamZen	5'-/56FAM/AGCAGAGAA/ZEN/CAGTTAAGAGCCTCCAT/3IABkFQ/-3'
Table 4c: External/internal primers and FRET probes used for integrated HIV-DNA	
Alu1	5'-TCCCAGCTACTGGGGAGGCTGAGG-3'
Alu2	5'-GCCTCCCAAAGTGCTGGGATTACAG-3'
Lambda(λ)T	5'-ATGCCACGTAAGCGAAACT-3'
AA55M	5'-GCTAGAGATTTTCCACACTGACTAA-3'
LTRFL	5'-CACAACAGACGGGCACACACTACTTGA-3'-Flurorescein
LTRLC	5'-CACTCAAGGCAAGCTTTATTGAGGC-3'-Phosphate
Table 4b: External/internal primers and FRET probes used for CD3 quantification (together with integrated HIV-DNA)	
HCD3OUT5'	5'-ACTGACATGGAACAGGGGAAG-3'
HCD3OUT3'	5'-CCAGCTCTGAAGTAGGGAACATAT-3'
HCD3IN5'	5'-GGCTATCATTCTTCTTCAAGGT-3'
HCD3IN3'	5'-CCTCTCTTCAGCCATTTAAGTA-3'
P1	5'-GGCTGAAGGTTAGGGATACCAATATTCCTGTCTC-3'-Fluorescein
P2	5'-CTAGTGATGGGCTCTTCCCTTGAGCCCTTC-3'-Phosphate

CHAPITRE 6 : DISCUSSION ET PERSPECTIVES

L'objectif général de mes travaux de doctorat vise à comprendre les mécanismes cellulaires impliqués dans l'établissement, la persistance et la latence du VIH afin de développer de nouvelles stratégies thérapeutiques ciblant les cellules T $CD4^+$ à profil de polarisation Th17 pour l'éradication et/ou la rémission du VIH.

Dans la première partie de cette thèse, les mécanismes moléculaires expliquant la susceptibilité à l'infection par le VIH des cellules T $CCR6^+$ à tropisme intestinal ont été explorés. Les cellules Th17 sont stratégiquement localisées au niveau de l'intestin, qui est un site majeur de réplication virale. L'intestin est un site enrichi en acide rétinoïque, un métabolite de la vitamine A, qui induit le tropisme intestinal des cellules T. Nos travaux ont permis de mettre en évidence que, *in vitro*, l'induction de cette domiciliation intestinale via l'ATRA augmente la réplication/réactivation du VIH spécifiquement dans les cellules T CD4 $CCR6^+$ à profil de polarisation Th17 via des mécanismes dépendants de mTOR. Les cellules T $CCR6^+$ infiltrant le colon, chez les sujets VIH+ sous TAR, expriment de hauts niveaux de mTOR. Il nous a été possible d'observer que l'inhibition de cette enzyme *in vitro* est associée à une diminution de la réplication/réactivation virale. De plus, ces études ont permis d'identifier les cellules T $CCR6^+$ comme étant transcriptionnellement programmée pour devenir des cibles du VIH, en particulier dans les tissus intestinaux. Ces résultats supportent le rôle bénéfique des inhibiteurs de mTOR comme nouvelle stratégie thérapeutique afin de limiter la réplication virale et la réactivation dans les cellules Th17 du colon. (Manuscrit #1) L'utilisation avec succès des inhibiteurs de mTOR dans le traitement des maladies auto-immunes et métaboliques ainsi que du cancer supporte l'impact positif que pourraient avoir ces nouvelles interventions chez les sujets infectés par le VIH.

Dans la deuxième partie de cette thèse, nous avons évalué dans le cadre d'un essai clinique pilote non-randomisé l'effet de la metformine, un inhibiteur indirect de mTOR [318], sur la réplication/persistance du VIH dans les cellules Th17 du colon et sur la restauration de l'immunité

mucosale chez les sujets VIH+ sous TAR. Dans ces travaux, nous avons observé une diminution importante de l'expression de mTOR dans les cellules infiltrant le colon, dont celles à profil de polarisation Th17. Pour 68 % des participants, une diminution de la transcription virale résiduelle dans les cellules du colon a été observée après le traitement à la metformine. Ces travaux indiquent donc que la metformine est efficace pour moduler le métabolisme cellulaire *via* mTOR et diminuer la transcription du VIH résiduelle dans les cellules infiltrant les tissus (Manuscrit #2).

Le rétablissement de l'homéostasie de l'immunité mucosale chez les sujets VIH+ sous TAR est d'une importance majeure en vue d'une guérison fonctionnelle. La restauration de la fréquence et des fonctions des lymphocytes Th17 est l'un des points clefs permettant d'aboutir à ce rétablissement. Ainsi, dans la troisième partie de cette thèse, nous avons examiné l'efficacité *in vitro* de l'inhibition pharmacologique de PPARγ comme stratégie de « shock and kill » afin de purger le réservoir du VIH et de restaurer les fonctions effectrices des cellules Th17. L'inhibition de PPARγ est associée à une augmentation de la production d'IL-17A, à une hausse de la transcription du VIH et étonnamment à un blocage efficace de la réplication du VIH. De ce fait, nos résultats indiquent que l'inhibition de PPARγ constitue une stratégie thérapeutique prometteuse pour réactiver les réservoirs du VIH, tout en empêchant la propagation virale et en renforçant les fonctions Th17 nécessaires au rétablissement de l'immunité mucosale pendant le traitement antirétroviral. (Manuscrit #3)

La TAR actuelle supprime efficacement la réplication du VIH, ce qui permet de contrôler la charge virale à des niveaux indétectables. Ces traitements ont permis de transformer la maladie causée par l'infection par le VIH d'un statut mortel à un statut chronique contrôlé à long terme avec succès. Cependant, plusieurs obstacles visant à la guérison fonctionnelle du VIH subsistent sous TAR. Elle ne permet pas d'éliminer les cellules infectées à longue durée de vie, ce qui résulte en la persistance des réservoirs viraux et un rebond viral lors d'interruption de traitement [325] [177] [179]. Chez les sujets vivant avec le VIH sous TAR, l'altération de l'homéostasie de l'immunité mucosale et l'activation immunitaire chronique constituent un dysfonctionnement immunitaire persistant associé à des événements chroniques non infectieux, dont des maladies cardiovasculaires, hépatiques et rénales, ainsi que des tumeurs malignes non liées au SIDA. [326] [327]. De plus, d'après plusieurs travaux, la concentration des ARVs est insuffisante dans certains tissus lymphoïdes à cause d'une pénétration sous-optimale des drogues, ce qui pourrait empêche un blocage complet et efficace de la réplication virale dans ces sites [328] [181]. De ce fait, il est probable qu'une réplication virale à bas niveau dans certains tissus lymphoïdes contribue au réapprovisionnement constant des réservoirs du VIH sous TAR.

6.1. RÉPLICATION VIRALE ET PERSISTANCE DU VIH

Le VIH n'infecte et ne persiste pas de manière égale dans les différentes sous-populations de cellules T $CD4^+$ [329]. De nombreux groupes travaillent pour l'identification d'un marqueur cellulaire du réservoir du VIH, mais, jusqu'à présent, aucun marqueur spécifique des cellules infectées de manière latente n'a été mis en évidence. Cependant, plusieurs travaux ont montré qu'au niveau du sang périphérique, les cellules T $CD4^+$ mémoires centrales contribuent largement à la persistance du virus ([53]. Parmi ces cellules T $CD4^+$ mémoires centrales, celles à profil de polarisation Th17 sont particulièrement enrichies en ADN viral [156].

6.1.1. Les cellules T CCR6$^+$ sont hautement susceptibles à l'infection *in vitro* et initient efficacement la réplication virale

Afin de confirmer l'importance des cellules T CD4$^+$CCR6$^+$ dans l'établissement d'une infection chronique par le VIH et de souligner l'intérêt de cibler ces cellules pour limiter la formation du réservoir, nous avons réalisé des expériences de co-culture entre les cellules T CCR6$^+$ et CCR6$^-$ (Annexe 1). Lorsque les cellules T CCR6$^-$ sont exposées au VIH *in vitro* et ensuite transférées dans un système de co-culture avec des cellules CCR6$^+$ non exposées au VIH (Annexe 1A; condition 1), la fréquence de cellules infectées est relativement faible dans les deux sous-populations (Annexe 1C). Par contre, lorsque ce sont les cellules CCR6$^+$ qui sont exposées au VIH *in vitro* et qu'on les transfère dans le système de co-culture avec des cellules CCR6$^-$ non exposées au VIH (Annexe 1A; condition 2), la fréquence de cellules infectées est deux fois plus importante que dans la condition 1. Ces résultats montrent qu'une fraction des cellules T CCR6$^-$ s'infectent *in vitro* et sont capables de transmettre le virus aux cellules T CCR6$^+$, mais que toutefois les cellules T CCR6$^+$, qui sont initialement davantage permissives à l'infection, sont capables d'initier l'infection plus efficacement.

Dans ces expériences, les cellules T isolées *ex vivo* n'exprimant pas CCR6 (CD4$^+$CCR6$^-$) ont été marquées avec un tracer violet (CTV). Pourtant après activation via CD3/CD28, une population ayant un niveau intermédiaire de CTV (initialement CCR6$^-$) exprime CCR6. De façon surprenante, cette population CCR6$^+$CTV^{medium} sont enrichies en p24 (Annexe 1C). Il est possible que cette population soit des cellules ex-Th17, qui, en proliférant, expriment de nouveau CCR6 et soit hautement permissive à l'infection *in vitro*. Pourtant dans nos expériences sans co-culture, la réplication virale dans les cellules T CCR6$^-$ est mineure comparativement à celle observée dans les cellules T CCR6$^+$ (Manuscrit #1, Figure 1). Il serait donc étonnant que ces cellules CCR6$^+$CTV^{medium} fassent partie de la fraction CCR6$^-$ isolée *ex vivo*. Une condition de contrôle sans co-culture où les cellules T CCR6$^-$ marquées avec CTV seraient exposées au VIH et cultivées seules permettrait de savoir si une fraction de ces cellules sont des ex-Th17 capables de ré-exprimer CCR6 après stimulation et de répliquer le VIH efficacement.

Une autre hypothèse est que le traceur CTV soit transféré au niveau des synapses virologiques entre une cellule T CCR6-CTV$^+$ et une cellule T CCR6$^+$. Les contacts entre deux cellules lors d'une infection au VIH, initiés via l'interaction entre la protéine CD4 d'une cellule non infectée et la protéine Env exprimée à la surface d'une cellule infectée, facilitent ainsi la propagation du VIH [330]. Ces contacts cellule-cellule, appelés synapses virologiques, sont maintenus grâce à des molécules d'adhésion cellulaires telles que LFA-1, ICAM-1 et ICAM-3 et Zap-70 [331] [332]. Au niveau de ces synapses, des nanotubules et des ponts filopodiaux entre la cellule infectée et celle non infectée permettent au virus d'être transféré efficacement et d'échapper au système de reconnaissance immunitaire [333] [334]. Ces ponts peuvent également faciliter le transfert de composants cytoplasmiques [335] [333]. Il a également été montré que la machinerie virale en formation est efficacement transférée au niveau des synapses virologiques par un processus d'échange de molécules membranaires entre les deux cellules, appelé trogocytose. [336]. Un échange du contenu cytoplasmique via la trogocytose a également déjà été observé [337]. Il est ainsi possible d'imaginer qu'au niveau des synapses virologiques entre une cellule CCR6$^-$ CTV$^+$ et une cellule CCR6$^+$, le transfert de particules virales soit important et accompagné d'un transfert du contenu cytoplasmique incluant le marqueur CTV.

Des investigations supplémentaires seront toutefois nécessaires afin de confirmer ces hypothèses. Il serait également intéressant de déterminer si les cellules T CCR6$^+$ et CCR6$^-$ infectées diffèrent dans leur capacité à former ou maintenir les synapses virologiques. Il est à noter que la kinase ZAP-70, impliqué dans le modelage du cytosquelette d'actine et dans la formation des synapses, est hautement exprimée dans les cellules Th17 comparativement à celles à profil Th1 [322].

6.1.2. Les cellules T CD4$^+$CCR6$^+$ à profil de polarisation Th17 contribuent significativement au réservoir du VIH compétent pour la réplication

Les cellules Th17 peuvent être identifiées sur la base de plusieurs marqueurs de surface (CD25$^-$ CD4$^+$CCR6$^+$CD161$^+$) ou sur la base des cytokines qu'elles sécrètent (Th17 non pathogéniques: IL-17$^+$; Th17 pathogénique : IL17$^+$IFNγ$^+$). Dans nos travaux au laboratoire, nous utilisons le

marqueur de cellule Th17, CCR6, qui est un important médiateur de la migration des cellules T vers l'intestin [314]. Il est important de noter que les cellules T $CD4^+$ exprimant CCR6 incluent les répertoires Th17 pathogénique (Th1Th17) et non pathogénique (Th17), mais également des lymphocytes ex-Th17, T_{FH}.Th17 ainsi qu'une sous-population T_{REG} [338] [314] [339] [340] [341]. Cependant plusieurs études ont identifié CCR6 comme un marqueur de cellules hautement permissives à l'infection *in vitro* [342] [343] [316] [171].

Ce qui est bien défini dans la littérature sont l'ensemble des facteurs de transcription interconnectés entre eux et gouvernés par RORC dans la régulation transcriptionnelle des cellules Th17 chez la souris et l'homme. [344] [345] [207]. Ainsi, afin d'établir un consensus sur l'importante contribution des cellules Th17 à la persistance du VIH, nous avons isolé les cellules mémoires T $CD4^+CCR6^+$ exprimant (Th17 classique; $CCR6^+RORC^+$) ou non (T_{REG}/ex-Th17; $CCR6^+RORC^-$) RORC et les cellules T CCR6- (non-Th17) de cinq sujets VIH sous TAR (Annexe 2A). Les cellules T $CCR6^+RORC^+$ et, à un moindre niveau, les cellules T $CCR6^+RORC^-$ sont enrichies en ADN proviral comparativement aux cellules T $CCR6^-$ *(Fold Change (FC) $CCR6^+RORC^+$ vs $CCR6^-$:* 5.8; FC *$CCR6^+RORC^-$ vs $CCR6^-$: 1.8).* Parmi les cellules T $CCR6^+$, celles qui expriment RORC sont enrichies en ADN viral *(FC $CCR6^+RORC^+$ vs $CCR6^+RORC^-$: 3.04)* (Annexe 2B). Ces résultats confirment que malgré l'importante déplétion des cellules Th17 après infection par le VIH, une fraction d'entre elles persiste et contribue principalement au réservoir viral.

Nous avons également documenté la contribution des cellules T $CCR6^+$ à la persistance du VIH chez les sujets vivant avec le VIH et sous TAR. Nos études précédentes ont montré que les cellules T $CCR6^+$ du sang périphérique et du colon sont enrichies en ADN viral [317]. Cependant, la fréquence des cellules portant de l'ADN viral surestime largement la taille du réservoir, car cela inclut des cellules infectées avec des provirus défectueux qui sont incapables de produire des virions compétents pour la réplication. Il a été démontré que les cellules infectées qui produisent activement des transcrits multi-épissés du VIH, Tat et Rev, peuvent produire des virions compétents pour la réplication. Nous avons alors utilisé un essai qui mesure la fréquence de cellules portant les transcrits multi-épissés Tat et Rev après stimulation PMA/iono (TILDA; *Tat/rev Induced Limiting Dilution Assay*) [346]. Nous avons pu ainsi mettre en valeur que les cellules T $CD4^+$ $CCR6^+$ mémoires ont une teneur élevée en

transcrits viraux multi-épissés (Annexe 3A). En accord avec ces résultats, un nouvel essai, optimisé par le laboratoire du Dr Chomont et qui permet de détecter et caractériser les cellules infectées *ex vivo* capables de produire la protéine virale p24, a identifié un enrichissement en p24 dans les cellules T CCR6$^+$ de sujets VIH$^+$ sous TAR [347], confirmant que les cellules T CCR6$^+$ contribuent principalement à la persistance du VIH compétant pour la réplication sous TAR.

En parallèle à la mesure de TILDA, nous avons réalisé un essai de croissance virale en absence d'ARVs afin de permettre aux virus de se répliquer dans les sous-populations de cellules T CCR6$^+$ et CCR6$^-$ (Annexe 3B). Dans cet essai, la présence de cellules infectées capables d'être réactivées ainsi que la capacité des cellules bystander de répliquer le virus rentrent en compte dans la détection des virus compétents pour la réplication. En accord avec les résultats de TILDA, la détection de virus réplicatif est plus robuste dans les cellules T CCR6$^+$ comparé aux cellules CCR6$^-$ (Annexe 3B). Comme il était attendu, l'utilisation de l'ATRA durant cet essai mène à une amplification de la réactivation virale. Ceci est surtout vrai chez les sujets présentant une réactivation/réplication faible ou modérée en absence d'ATRA. La capacité de l'ATRA à promouvoir la réplication du VIH est expliqué par une augmentation de la transcription virale via une augmentation de l'expression de mTOR, et une augmentation de la permissivité à l'infection des cellules bystander via l'augmentation de l'expression de CCR5 (Manuscrit #1) [316] [171]. D'autre part, des éléments de réponses à l'acide rétinoïque (RARE), site de liaison à l'ADN du récepteur à l'ATRA (RARα), sont présents au niveau du LTR du VIH [348]. Il est ainsi probable que l'ATRA induise directement la réactivation du virus latent. D'autres groupes ont d'ailleurs démontré que des dérivés de l'acide rétinoïque tels que l'acitrétine favorisent directement la transcription du VIH [94].

Ensemble, ces résultats préliminaires montrent que les cellules T CD4$^+$CCR6$^+$ jouent un rôle important dans la persistance du réservoir viral. Les cellules T CCR6$^+$ sont hautement permissives au VIH, initient efficacement l'infection et sont enrichies en provirus compétents pour la réplication. De ce fait, les techniques utilisées en laboratoire afin de détecter la présence de virus compétents pour la réplication dans le cadre d'essais cliniques avec interruption de traitement devraient être réalisées préférentiellement sur les cellules T CD4$^+$CCR6$^+$, en présence d'ATRA ou d'autres agents favorisant

la réactivation virale, afin d'augmenter la probabilité de détecter un faible, mais existant réservoir viral.

Ces expériences seront répétées sur un plus grand nombre de sujet et des expériences complémentaires viendront compléter un futur manuscrit faisant l'emphase sur l'important rôle des cellules CCR6$^+$ à la persistance du réservoir compétent pour la réplication virale.

6.2. MÉCANISMES IMPLIQUÉS DANS LA PERMISSIVITÉ À L'INFECTION PAR LE VIH DES CELLULES T CCR6$^+$

6.2.1. Le profil transcriptionnel des lymphocytes Th17 médié par RORC augmente la réplication du VIH *in vitro*

Les cellules T CCR6$^+$RORC$^+$ sont principalement infectées par le VIH et persistent sous TAR (Annexe 2). Par contre, l'implication de RORC ou du programme transcriptionnel qu'il gouverne dans la régulation de la permissivité au VIH dans les cellules Th17 n'est pas connue. Nous avons donc examiné l'effet de l'interférence par ARN contre RORC sur la réplication virale *in vitro* (Annexe 4). Nos résultats ont montré que cette interférence contre RORC entraîne une diminution significative de l'intégration de l'ADN viral après exposition *in vitro* des cellules à une souche du VIH compétente pour la réplication (NL4.3Bal) (Annexe 4E). Cette diminution de l'intégration du VIH est également observée après exposition à une souche pseudotypée VSV-G, qui pénètre dans les cellules indépendamment du récepteur et co-récepteurs du VIH (Annexe 4F). Comme attendu, la diminution de l'intégration du VIH et de la réplication virale après inhibition de RORC est observée uniquement dans les cellules T CD4$^+$CCR6$^+$ (Annexe 4G-H). Nous apportons ainsi la preuve que le programme de transcription régi par RORC est favorable à la réplication du VIH.

Le mécanisme d'action de RORC favorisant la réplication virale n'est pas exactement connu, mais il semble que ce facteur de transcription régule positivement entre autres des mécanismes post-entrée et pré-intégration du cycle viral. Ceux-ci restent toutefois à être définis. Il est également nécessaire de déterminer si RORC agit directement ou indirectement sur la réplication du VIH. Étant un facteur de transcription, il est possible qu'il régule directement l'activité transcriptionnelle du VIH

ou bien que des gènes de l'hôte régulés positivement par RORC soient des facteurs de dépendance au VIH. Dans tous les cas, ces résultats encouragent la mise en place de futures stratégies thérapeutiques ciblées visant à interférer avec la réplication du VIH dans les cellules Th17.

6.2.2. Les cellules T CCR6$^+$ présentent un profil de migration vers l'intestin, site majeur de réplication virale

La migration des cellules Th17 vers l'intestin est médiée par les molécules d'adressage telles que CCR6, CCR9 et l'intégrine α4β7 [349]. Ces cellules qui sont donc hautement permissives à l'infection se retrouvent ainsi dans un environnement propice à la réplication virale [196] [350]. En raison de l'exposition constante à des antigènes, l'intestin contient une abondance de lymphocytes T CD4$^+$ mémoire effectrice qui expriment de hauts niveaux de CCR5 et sont donc très susceptibles à l'infection par le VIH [351]. De plus, les cellules présentes dans l'intestin expriment de hauts niveaux des marqueurs d'activation favorables pour la propagation de l'infection [251] (Manuscrit #2; Figure 4-5). La muqueuse intestinale est donc un site de réplication majeur du VIH qui sera rapidement altéré après infection [352]. Sous TAR, l'intestin représente un des plus importants réservoirs de VIH [249].

6.2.3. L'induction du tropisme intestinal augmente la permissivité des cellules T CCR6$^+$ à l'infection par le VIH *in vitro* (Manuscrit #1)

L'intestin est un site enrichi en acide rétinoïque, un métabolite de la vitamine A produit par les cellules dendritiques intestinales. L'acide rétinoïque induit l'expression de molécule importante pour le trafic des cellules T vers l'intestin, notamment l'intégrine α4β7 et CCR9. De ce fait, les cellules T se retrouvant dans un milieu riche en acide rétinoïque vont acquérir un code-barre qui va leur permettre de retourner vers l'intestin rapidement. Dans le manuscrit #1 de ma thèse, j'ai utilisé l'ATRA pour mimer partiellement l'environnement et l'induction du tropisme intestinal *in vitro*. L'ATRA augmente l'intégration et la réplication du VIH *in vitro* dans les cellules T CCR6$^+$ spécifiquement [316]. Ceci est expliqué par l'induction de l'expression des co-récepteurs du VIH (CCR5, CXCR6) (Manuscrit #1; Supplémental Figure 4) ainsi que des molécules associées à l'activation des cellules T (CD38, Lck, PTPN13, MAP3K4) en présence d'ATRA. L'étude transcriptionnelle au

niveau du génome entier par microarray, démontre que l'ATRA module différemment l'expression des gènes dans les cellules T CCR6$^+$ et CCR6- (Manuscrit #1; Figure 2). L'analyse des voies préférentiellement modulée par l'ATRA dans les cellules T CCR6$^+$ nous a permis d'identifier la voie AKT/PI3K qui est justement impliquée dans la polarisation Th17. L'illustration des composants de la voie canonique AKT/PI3K modulés par l'ATRA dans les cellules T CCR6$^+$ a ensuite mené à reconnaitre mTOR comme un transcrit régulé à la hausse. Compte tenu du rôle bien établi de ce dernier dans la régulation du métabolisme cellulaire et dans la réplication du VIH, nous avons utilisé deux inhibiteurs de mTOR pour tester leur capacité à contrecarrer l'effet de l'ATRA sur la permissivité au VIH des cellules T CCR6$^+$: la rapamycine, un inhibiteur du complexe mTORC1, et l'INK128, un inhibiteur des complexes mTORC1/2. La rapamycine et l'INK128 réduisent de manière significative les niveaux d'intégration du VIH ainsi que la réplication virale dans les cellules T CCR6$^+$ traitées à l'ATRA, avec INK128 ayant les effets inhibiteurs les plus robustes (Manuscrit #1; Figure 6). L'ATRA améliore significativement la réactivation et la croissance virale dans les cellules T CCR6$^+$ de sujets VIH+ sous TAR (Manuscrit #1; Figure 9 – Annexe 3). D'une importance particulière, l'inhibiteur de mTOR, INK128, inhibe complètement la réactivation du VIH en présence et en absence d'ATRA (Manuscrit #1; Figure 9). Ceci révèle que mTOR est un acteur clef de la réactivation du VIH induite par l'ATRA.

Chez les sujets VIH sous TAR, les niveaux d'expression les plus élevés de CCR5, d'intégrine β7 et de mTOR phosphorylé sont été observés dans les cellules T CCR6$^+$ infiltrant le colon comparativement au sang (Manuscrit #1, Figure 10). Considérant que l'intestin est enrichi en cellules dendritiques métabolisant la vitamine A en acide rétinoïque, nous confirmons ainsi que la « signature » moléculaire induite par l'ATRA *in vitro* peut également être observée sur des cellules infiltrant l'intestin. De plus, ceci fournit une explication possible de la permissivité et de la persistance préférentielle du VIH dans les cellules T CCR6$^+$ infiltrant le colon.

En conclusion, nous avons identifié CCR6 comme marqueur des cellules T transcriptionnellement programmées pour devenir des cibles du VIH, en particulier dans les tissus intestinaux. Nos résultats supportent le rôle bénéfique des inhibiteurs de mTOR pour limiter la réplication virale et la réactivation dans les cellules Th17 du colon.

6.2.4. Perspectives

D'autres équipes de recherche ont identifié mTOR comme un régulateur positif de la réplication du VIH régulant positivement l'entrée du VIH [353] [354] mais également la latence du VIH [168] [355]. Considérant les rôles importants que jouent les voies de signalisation mTORC1 et mTORC2 dans le métabolisme et la polarisation des cellules T, il est fort probable que mTOR régule la réplication du VIH en modulant différentes étapes du cycle cellulaire. En effet, les données de la littérature démontrent que mTOR régule ***i)*** la biosynthèse des nucléotides [356] [357], ***ii)*** le processus d'autophagie qui est impliqué dans la dégradation des protéines du VIH [358] [359] et ***iii)*** la translocation nucléaire de RORC *via* l'induction de la kinase S6K2 [360]. Il sera donc important de déterminer les mécanismes par lesquels mTOR régule positivement la permissivité du VIH dans les cellules Th17 à tropisme intestinal. Les pistes à explorer sont la régulation de ***i)*** la synthèse des nucléotides nécessaires à la transcription inverse du VIH, ***ii)*** la dégradation des protéines du VIH via le processus d'autophagie et ***iii)*** l'interférence du programme transcriptionnel médié par RORC dans les cellules Th17 [322].

mTOR comme un marqueur de cellules métaboliquement active. Il a été montré récemment que la susceptibilité à l'infection au VIH des sous-populations mémoires de cellules T $CD4^+$ est associé à leur programme métabolique [167]. Il serait ainsi important de comparer les niveaux de phosphorylation oxydative et de glycolyse dans les sous-populations de cellules T $CD4^+$ $CCR6^+$ versus $CCR6^-$ du sang périphérique et du colon.

6.3. ÉTUDE PILOTE VISANT À DIMINUER L'INFLAMMATION ET RÉDUIRE LA TAILLE DES RÉSERVOIRS VIRAUX DANS LES CELLULES TH17 INTESTINALES VIA UN INHIBITEUR INDIRECT DE MTOR, LA MERFORMINE (Manuscrit #2)

Sur la base des études précédentes (Manuscrit #1), nous avons évalué les effets de la metformine sur la persistance du réservoir viral dans les cellules Th17 et la restauration de l'immunité muqueuse chez les personnes traitées vivant avec le VIH sous TAR (Manuscrit #2*)*. La metformine est un immuno-modulateur et un inhibiteur indirect de mTOR. Cet essai clinique (Étude LILAC) a été mené sous la direction de Dr Jean-Pierre Routy à Montréal (Clinicien/Chercheur au IR-CUSM site Glen de Montréal) en collaboration avec Dr Jonathan Angel (Clinicien/Chercheur à l'institut de recherche de l'hôpital d'Ottawa). Une cohorte de 22 sujets vivant avec le VIH et non diabétiques a reçu un traitement de metformine durant 12 semaines en combinaison avec les ARVs.

Des modifications seulement très légères des marqueurs plasmatiques de l'inflammation systémique et du dysfonctionnement de la barrière intestinale ont été observées après les 12 semaines de traitement (Manuscrit #2 ; Figure 2). Également, la taille du réservoir mesuré dans les cellules T $CD4^+$ du sang périphérique montrent une parfaite stabilité tout au long de l'étude (Manuscrit #2 ; Figure 3).

De profonds changements ont cependant été notés dans les études réalisées sur les biopsies intestinale. D'abord, il est intéressant de voir une corrélation dans les échantillons du colon, entre la fréquence des cellules T $CCR6^+$ exprimant ces marqueurs (HLA-DR/CD38, CCR5 et mTOR phosphorylé) avec la transcription résiduelle VIH (Manuscrit #2 ; Figure 4-5-6). Ceci confirme que l'activation cellulaire élevée dans l'intestin est associée à la transcription résiduelle du VIH.

Comme attendu, les 12 semaines de traitement à la metformine ont permis de diminuer l'expression de mTOR phosphorylé dans les cellules T $CCR6^+$ du colon, et ainsi probablement le métabolisme cellulaire. Une tendance à l'augmentation de l'expression de SAMHD1 pourrait indiquer une diminution du bassin de nucléotides reliée également à une diminution du métabolisme cellulaire dans le colon (Manuscrit #2 ; Figure 5-6).

De plus, une diminution de la transcription résiduelle du VIH est observée chez 62% des participants (Manuscrit #2 ; Figure 7). Cette diminution de la transcription résiduelle du VIH devrait être associée à une diminution de l'inflammation et de l'activation immunitaire intestinale.

En conclusion, cette étude pilote révèle des avantages à utiliser la metformine en combinaison des ARVs dans le but maîtriser l'inflammation, en partie via la régulation mTOR chez des sujets VIH+ non diabétiques à haut risque d'inflammation ou de développement de maladies non liées au SIDA. Cependant, considérant que les participants qui ne répondent pas au traitement par une diminution de la transcription du VIH dans l'intestin sont sous dolutégravir et qu'il existe une interaction médicamenteuse entre cet ARV et la metformine, il serait probablement préférable de ne pas associer les deux médications [361].

6.3.1. Perspectives

Afin de confirmer que mTOR est associé à la transcription résiduelle du VIH, il est important de déterminer si les cellules T $CD4^+$ qui transcrivent activement le VIH co-expriment mTOR phosphorylé. Une méthode d'hybridation *in situ*, le RNAscope, détecte les ARN viraux et, combiné avec l'immunohistochimie, permet de détecter et de phénotyper les cellules infectées et transcriptionnellement actives dans la même coupe tissulaire [248]. Ces travaux sont d'ailleurs en cours dans le laboratoire du Dr Jake Estes (Oregon Health & Science University, Oregon, Etat-Unis).

Il est également important de confirmer par des techniques alternatives l'effet de la metformine dans le colon. L'étude du profil transcriptionnel des cellules isolées des biopsies intestinales avant et après le traitement metformine par séquençage à ARN est en cours. Il nous permettra d'identifier des voies de signalisation modulées par la metformine afin de déterminer ses effets sur le métabolisme cellulaire, l'activation et l'inflammation cellulaire dans le colon.

Par la suite, il serait intéressant de déterminer si la diminution de mTOR dans les cellules T $CD8^+$ du colon améliore la fréquence et la fonction des cellules T $CD8^+$ spécifiques au VIH. En effet, mTOR module également la différenciation des cellules T CD8 mémoires qui jouent un rôle essentiel dans

l'immunité protectrice et l'induction d'une réponse efficace des lymphocytes T mémoires. Il a été démontré, chez la souris, que la rapamycin, un immunosuppresseur et inhibiteur de mTOR a un effet bénéfique dans génération de cellules T mémoires $CD8^+$ [362].

Enfin, les effets du traitement à la metformine sont observés uniquement dans les échantillons provenant du colon. Il est connu que l'un des premiers sites d'action de la metformine soit l'intestin. Nos résultats nous incitent donc à étudier plus en détail les avantages immunologiques/virologiques de la supplémentation à long terme en metformine chez les personnes VIH+ sous ART. Une nouvelle étude clinique sur 6-12 mois de traitement avec metformine et incluant un groupe placebo devraient déterminer les effets plus globaux de la metformine.

6.4. L'INTESTIN, LA PIÈCE MAÎTRESSE POUR COMPRENDRE ET COMBATTRE LE VIH

L'intestin est un réservoir anatomique du VIH bien établit et les cellules Th17 y sont principalement localisées. Cependant, étant donné la difficulté de l'accès à des échantillons provenant de tissus lymphoïdes, la plupart des études les mécanismes impliqués dans la permissivité ou la latence du VIH sont effectuées dans les cellules T $CD4^+$ du sang périphérique. Même si les cellules T circulent en permanence entre la périphérie et les tissus, leur profil (activation, différenciation, fonction) est différent selon leur localisation. Il n'est donc pas clair, si ce que l'on examine dans les cellules T du sang est comparable à ce que l'on observerait dans les tissus. Alors que la fréquence des cellules T centrales mémoires majoritaire dans le sang périphérique, l'intestin renferme majoritairement des cellules T effectrices [363] [156]. Lorsque les cellules T pénètrent dans le tractus gastro-intestinal; elles se retrouvent au niveau de la plus grande interface avec l'environnement extérieur et donc en contact avec le microbiote, les pathogènes, les nutriments, etc [364]. Cet environnement particulier, incluant la concentration importante en acide rétinoïque, à un impact sur le profil transcriptionnel des cellules T.

Plusieurs marqueurs associés à l'activation et à l'infection par le VIH sont différemment exprimés dans les cellules du sang périphérique *versus* du colon. Dans le colon les fréquences des cellules T CCR6$^+$ exprimant HLADR et CD38 ou CCR5 ou mTOR phosphorylé sont nettement plus importantes que dans le sang (Manuscrit #2 ; Figure 4-5-6). Ceci peut être expliqué par l'environnement riche en acide rétinoïque (Manuscrit #1).

Considérant mTOR comme un régulateur important du métabolisme cellules, il serait intéressant de comparer le programme métabolique des cellules T CD4$^+$ du sang périphérique *versus* de l'intestin. Dans le contexte du VIH, l'altération de la composition du microbiote ainsi que le virus en lui-même modulent le profil transcriptionnel des cellules T intestinale [365]. Il faut continuer à explorer si ces modifications sont favorables à la réplication.

Si les mécanismes impliqués dans la permissivité, la réplication et la latence du VIH sont les mêmes dans les cellules T CD4$^+$ intestinales et du sang périphérique reste un sujet mal connu. Des études récentes par le groupe du Dr Yulk a démontré que les mécanismes gouvernant la latence du VIH dans les cellules T CD4$^+$ du sang périphérique *versus* de l'intestin sont différents [252]. Dans nos travaux, nous montrons que les effets de la metformine sont principalement retrouvés dans l'intestin. Il est donc d'une importance particulière, de considéré les cellules intestinales dans les études sur les mécanismes régissant la latence du VIH et également lors interventions médicamenteuses.

Il se peut également que l'accessibilité aux ARVs de certaines sous-populations cellulaires soit différente, de par leur localisation préférentielle dans certains tissus ou leur propre capacité à libérer les drogues. Dans le cadre de maladies inflammatoires, des études ont démontré que les cellules Th17 pathogéniques expriment de hauts niveaux de MDR1 («multidrug resistance type 1»), ce qui les rend résistantes à certains médicaments (par exemple, les glucocorticoïdes) [366] [367]. MDR1 ou ABCB1 (« ATP-binding cassette sub-family B member 1 »), est une protéine membranaire qui fonctionne comme une pompe d'efflux dépendant de l'ATP et permet le rejet à l'extérieur de la cellule de certains médicaments [368]. De manière particulièrement importante, l'expression de MDR1 est associée à la résistance de certains antirétroviraux [369] [370] [371]. Les médicaments anti-VIH connus pour être des substrats de MDR1 incluent certains inhibiteurs de

protéases (saquinavir, nelfinavir, amprénavir, indinavir) et inhibiteur de l'intégrase (dolutégravir). En revanche, l'inhibiteur de la protéase, le ritonavir, est un inhibiteur de MDR1 et est généralement inclus en thérapie combinée afin de renforcer l'action d'autres médicaments [372].

Les cellules T CCR6$^+$ expriment de hauts niveaux de MDR1 comparativement aux cellules T CCR6$^-$ (Annexe 4). Les cellules T CCR6$^+$MDR1$^+$ et MDR1$^-$ sont hautement permissives à l'infection au VIH *in vitro* (Annexe 4). Déterminer si l'expression de MDR1 à la surface des cellules Th17 pathogéniques est associée à une concentration sous optimal d'ARVs, et ainsi à la persistance préférentielle de VIH dans ces cellules est d'une importance particulière.

De plus, une étude a identifié MDR1 hautement exprimé par les cellules T CD4$^+$ exprimant l'intégrine $\alpha4\beta7$ et donc prône à migrer vers l'intestin ainsi que par les cellules T CD4$^+$ du colon [373]. De ce fait, il est possible que la concentration sous-optimale d'ARV dans les tissus soit accompagnée d'une faible concentration d'ARV dans les cellules infiltrant l'intestin causé par leur importante expression de MDR1.

6.5. DÉCONNEXION DU PROFIL TRANSCRIPTIONNEL DES TH17 ET DE LA RÉPLICATION VIRALE (Manuscrit #3)

Toujours dans le but d'identifier des facteurs de permissivité/résistance pouvant représenter de nouvelles stratégies thérapeutiques ciblées, nous avons pris pour avantage la susceptibilité non homogène des sous-populations de cellules T CD4$^+$ à l'infection par le VIH. Comme discuté plus tôt, le VIH infecte préférentiellement les cellules CCR6$^+$Th17. Ces cellules présentent donc un programme cellulaire optimal pour soutenir la réplication et la persistance du VIH. Cela signifie que les facteurs de l'hôte impliqués dans l'infection par le VIH sont exprimés préférentiellement dans les cellules Th17 et non dans la population Th1 qui est relativement résistante au VIH. Dans une étude précédente au laboratoire, nous avons identifié PPARγ comme étant fortement exprimé dans les cellules Th17 comparativement aux lymphocytes

Th1 [322]. PPARγ est pourtant décrit dans la littérature comme étant un répresseur transcriptionnel de RORC qui est le majeur régulateur transcriptionnel des cellules Th17 [324]. PPARγ contrôle donc les fonctions effectrices de la population Th17. Dans une autre étude au laboratoire, l'interférence à ARN de PPARγ dans les cellules T $CD4^+$ a induit une augmentation de la réplication virale *in vitro* [237], suggérant ainsi que PPARγ est un régulateur négatif du VIH exprimé préférentiellement dans les cellules Th17.

Rappelons que l'appauvrissement persistant de la fonction et de la fréquence des cellules Th17 intestinales chez les sujets vivant avec le VIH sous TAR est considéré comme une cause majeure de progression de la maladie et de survenue de comorbidités non-SIDA [374] [375] [376] [377] [378] [379]. La restauration des cellules Th17 intestinales est associée à une amélioration de l'homéostasie de l'immunité mucosale [320] [380]. Considérant également la contribution importante des cellules Th17 à la persistance du VIH chez les sujets sous TAR, nous avons exploré la possibilité de cibler les cellules Th17 via PPARγ dans le but d'une part de restaurer l'immunité mucosale via le rétablissement des fonctions effectrices des Th17 et d'autre part d'éradiquer le réservoir viral par sa réactivation. L'hypothèse de ce projet était que l'inhibition de PPARγ pourrait être pertinente dans la stratégie de « shock and kill », consistant à réactiver le virus latent afin que le système immunitaire puisse détecter et tuer les cellules infectées en présence d'antirétroviraux pour éviter l'infection de nouvelles cellules.

Comme attendu, la stimulation des cellules T $CD4^+$ de sujets VIH^+ sous TAR en présence de T0070907, un antagoniste de PPARγ, a entraîné une augmentation de la production d'IL-17A et de la transcription du VIH (Manuscrit #3 ; Figure 1). Contre toute attente, T0070907 a également inhibé la libération des virions et la réplication du VIH *in vitro* (Manuscrit #3 ; Figure 1-2). Les études du profil transcriptionnel au niveau du génome entier ont révélé de multiples voies de signalisation/gènes modulées par T0070907 dans les cellules Th17. En outre, une diminution des facteurs impliqués dans la permissivité au VIH (e.g. CCR5, furine) ainsi qu'une augmentation de ceux relatifs à la restriction par le VIH (e.g. TRIM22, BST2, miR29) et aux fonctions effectrices Th17 (e.g. RORγt, STAT3, BCL6, IL-17A/F, IL-21, IL-22, IL-26) ont été notées

(Manuscrit #3 ; Figure 5). D'une importance majeure, T0070907 augmente considérablement la production d'IL-21 (Manuscrit #3 ; Figure 5E), une cytokine présentant des propriétés antivirales qui sont d'une part immuno-régulatrices et d'autres parts médiées par miR-29 [381] [382] [383]. L'inhibition de miR-29 ne permet cependant pas de rétablir la réplication du VIH *in vitro* dans les cellules T cultivées en présence de T0070907 (Manuscrit #3 ; Figure 5E), ce qui suggère un programme antiviral pléiotropique déclenché par l'antagonisme de PPARγ dans les cellules Th17 (Manuscrit #3 ; Figure 8). Malgré tout, les rôles majeurs joués par IL-21 dans la survie des cellules Th17 intestinales, la diminution de l'inflammation et la restauration de l'immunité mucosale soulèvent notre plus grand intérêt. Plusieurs travaux ont d'ailleurs démontré le rôle bénéfique de l'administration de l'IL-21 dans le modèle macaque d'infection à SIV incluant la restauration des cellules Th17 intestinale, une réduction de la translocation microbienne ainsi qu'une réduction de la taille des réservoirs viraux [384] [320] [380].

En conclusion, l'antagoniste de PPARγ, T0070907, combine trois caractéristiques uniques: une capacité ***i)*** d'activer la transcription du virus latent; ***ii)*** d'inhiber la réplication virale et ***iii)*** de stimuler les fonctions effectrices des lymphocytes Th17, en partie par la régulation positive de l'IL-21, une cytokine essentielle à la survie des cellules T et à la défense antivirale (Manuscrit #3).

6.5.1. Perspectives

Malgré nos précieux avancements relatifs au rôle de PPARγ dans l'infection des lymphocytes Th17 par le VIH et aux bénéfices de son inhibition, des investigations supplémentaires sont nécessaires afin d'évaluer les effets de l'antagonisme du PPARγ sur la réplication virale dans des modèles animaux. L'induction des marqueurs T_{FH} par les cellules T $CCR6^+$ en présence de T0070907 pourrait être associée à une augmentation de la fréquence des acteurs T_{FH}Th17 circulants. Il sera donc important de documenter les effets de l'antagoniste de PPARγ sur la restauration des fonctions effectrices des Th17 et des T_{FH} *in vivo* dans des modèles animaux d'infection par le VIH. Les T_{FH} ont la propriété d'induire la production d'immunoglobuline par les cellules B naïves en particulier des IgA [216]. L'induction de la population T_{FH}Th17 pourrait

être souhaitable pour générer une réponse efficace à la vaccination et pour la conception de vaccins pour l'immunité protectrice des muqueuses.

Il serait également pertinent de comparer l'expression et l'activité de PPARγ chez les sujets non infectés avec celles d'individus infectés par le VIH et traités ou non avec des antirétroviraux. Il est possible que l'expression de PPARγ soit dérégulée chez sujets vivant avec le VIH et que cela joue un rôle dans la latence du VIH et dans le dysfonctionnement des fonctions effectrices des cellules Th17 et T_{FH} circulantes.

D'un point de vue fonctionnel, il faudrait étudier les modes d'action de l'antagoniste de PPARγ. Lors de la précédente étude au laboratoire, nous avons observé que la stimulation des cellules T $CD4^+$ en présence de Rosignitazone, un agoniste de PPARγ, entraînait la translocation nucléaire de PPARγ lui permettant d'agir comme régulateur transcriptionnel. Le T0070907, quant à lui, provoque la rétention de PPARγ dans le cytoplasme. Sachant que PPARγ et RXRα forment un complexe, il est probable que RXRα soit également retenu dans le cytoplasme. RXRα régule les gènes présentant des RAREs sur leur promoteur. Comme discuté dans le Manuscrit #1 de cette thèse, l'ATRA interagit avec RXRα afin de réguler l'expression des gènes présentant des RARE ce qui augmente la réplication virale. Il serait intéressant de déterminer si l'antagoniste de PPARγ, dans un contexte où l'on exclut l'hétérodimère PPARγ:RXRα du noyau, empêche conjointement la transcription des gènes ayant des PPRE et des RAREs sur leur promoteur.

6.6. LA PISTE DU MÉTABOLISME POUR LUTTER CONTRE LE VIH

Le métabolisme intracellulaire des cellules immunitaires joue un rôle important dans leur fonction. La glycolyse, la phosphorylation oxydative (OXPHOS), la glutaminolyse et l'oxydation des acides gras (FAO) sont des voies métaboliques qui génèrent l'énergie nécessaire pour satisfaire les fonctions cellulaires de base [385]. Les cellules T peuvent adapter leur

métabolisme, d'une voie à l'autre, afin de soutenir les processus de croissance et de fonction effectrice exigeants sur le plan bioénergétique lors d'une réponse immunitaire [385]. Les cellules T naïves utilisent la phosphorylation oxydative (OXPHOS) pour générer de l'énergie. Lors de l'activation via le TCR, les cellules T subissent une reprogrammation métabolique vers la glycolyse. Les cellules activées présentent donc un métabolisme actif permettant de fournir de l'énergie sous forme d'ATP nécessaire à la production de précurseurs biosynthétiques permettant la prolifération et la croissance cellulaire [385, 386].

Le VIH est dépendant des ressources de la cellule et il est capable de moduler le métabolisme cellulaire à son avantage afin de créer un environnement propice à la production virale et à la survie des cellules infectées. Il a été démontré que le VIH infecte préférentiellement les cellules T différenciées et métaboliquement actives. Ainsi la différence dans la susceptibilité des cellules T CD4+ à l'infection par le VIH des sous-populations est associée à l'activité métabolique des cellules [387].

mTOR est un senseur de l'état nutritionnel de la cellule qui joue un rôle important dans la croissance et la survie cellulaire de part le contrôle de la synthèse protéique, et d'autre part dans le contrôle du métabolisme. Dans les cellules T, l'activation de mTOR promeut la glycolyse aérobie. Ainsi l'inhibition de mTOR est associée à une diminution de l'expression des transports au glucose (GLUT1 et GLUT4) ainsi qu'une diminution de la glycolyse en aérobie [388].

Nous avons identifié dans le manuscrit #1, une importante expression de mTOR phosphorylé dans les cellules T CCR6+ du colon sigmoides. Ceci suggèrent que ces cellules sont métaboliquement actives ce qui fournit une explication supplémentaire à la haute permissivité à l'infection des cellules T CD4+ CCR6+ intestinales. Ainsi, l'inhibition de mTOR via la metformine (Manuscrit #2) devrait être associée à une diminution du métabolisme cellulaire et être associée à une inhibition de la réplication virale dans ces cellules. Des études transcriptionnelles sont en cours, afin de révéler les voies de signalisation impliquées dans le métabolisme et modulées par le traitement à la metformine dans les cellules totale isolées des biopsies du colon sigmoïde chez les sujets VIH+ non-diabétiques (Manuscrit #2)

PPARγ est un senseur lipidique qui joue un rôle important dans le métabolisme des lipides et du cholestérol [161, 389]. Les travaux du manuscrit #3 indiquent que l'inhibition de PPARγ est associée à une augmentation de l'expression de mTOR ainsi que des transcrits impliqués dans le métabolisme du glucose et des lipides. L'effet de l'inhibiteur de PPARγ T0070907 sur l'activité métabolique reste à être déterminé. Cependant, ces résultats suggèrent qu'il est possible de déconnecter l'activité métabolique des cellules T de la réplication du VIH.

6.7. CONCLUSIONS GÉNÉRALES

Nos études ont permis de faire l'emphase sur la place des cellules CCR6$^+$ à profil de polarisation Th17, spécifiquement celle à tropisme intestinale, dans la constitution des réservoirs du VIH à longue durée de vie. Nous avons montré que le profil transcriptionnel des cellules T CCR6$^+$ est favorable à la réplication virale. L'interférence avec un acteur clef de la polarisation Th17 et du métabolisme cellules, mTOR, permet de diminuer la susceptibilité à l'infection. Chez les sujets déjà infectés, l'inhibition de mTOR permet de diminuer la transcription du VIH, ce que ne permet aucuns ARVs. La transcription résiduelle du VIH étant une des causes de l'activation immunitaire chronique, il est probable que la metformine permette, à plus long terme, de diminuer l'inflammation et restaurer ainsi l'homéostasie de la muqueuse intestinale. Cependant, considérant l'importance des cellules Th17 dans l'immunité mucosale, il est possible qu'altérer le profil de polarisation des Th17 via mTOR ne soit pas totalement bénéfique. C'est une des raisons pour lesquelles nous avons exploré en parallèle la possibilité de restaurer les fonctions effectrices des Th17 *via* l'inhibition de PPARγ. Nos résultats innovants montrent pour la première fois qu'il est possible de dissocier la réplication virale du profil transcriptionnel des cellules Th17 dépendant de RORγt.

6.8. PERSPECTIVES GÉNÉRALES

Nos travaux ouvrent la porte vers de nouvelles investigations sur l'utilisation de mTOR et PPARγ comme cibles thérapeutiques dans le contexte de l'infection au VIH. Plusieurs

inhibiteurs de mTOR et immuno-modulateurs existent déjà en clinique (metformin, sirolimus, everolimus, temsirolimus) et d'autres sont en phase clinique de développement [390]. L'agoniste de PPARγ, la rosiglitazone (RZG), est utilisé en clinique chez les sujets vivant avec le VIH, pour le traitement de symptômes inflammatoires relatifs au VIH, mais les avantages de RGZ ne sont pas clairs [391]. Des études chez le singe montrent que les altérations hématopoïétiques causées par Nef sont dépendantes de l'activation de PPARγ, suggérant ainsi que l'utilisation d'antagonistes de PPARγ pour la rémission du VIH serait davantage appropriée [392]. Des explorations supplémentaires accompagnées d'un criblage des inhibiteurs de mTOR et des antagonistes de PPARγ sont nécessaires. Les mécanismes d'action de ces différentes drogues divergent et il est nécessaire de les tester *in vitro* sur différentes cellules immunitaire (monocytes, Mϕ, cellules épithélial).

Finalement, des études pré-clinique dans le contexte de l'infection au VIH sont nécessaires avec de mettre en place de nouveaux essais cliniques. Une attention particulière doit être portée sur la restauration de l'immunité mucosale.

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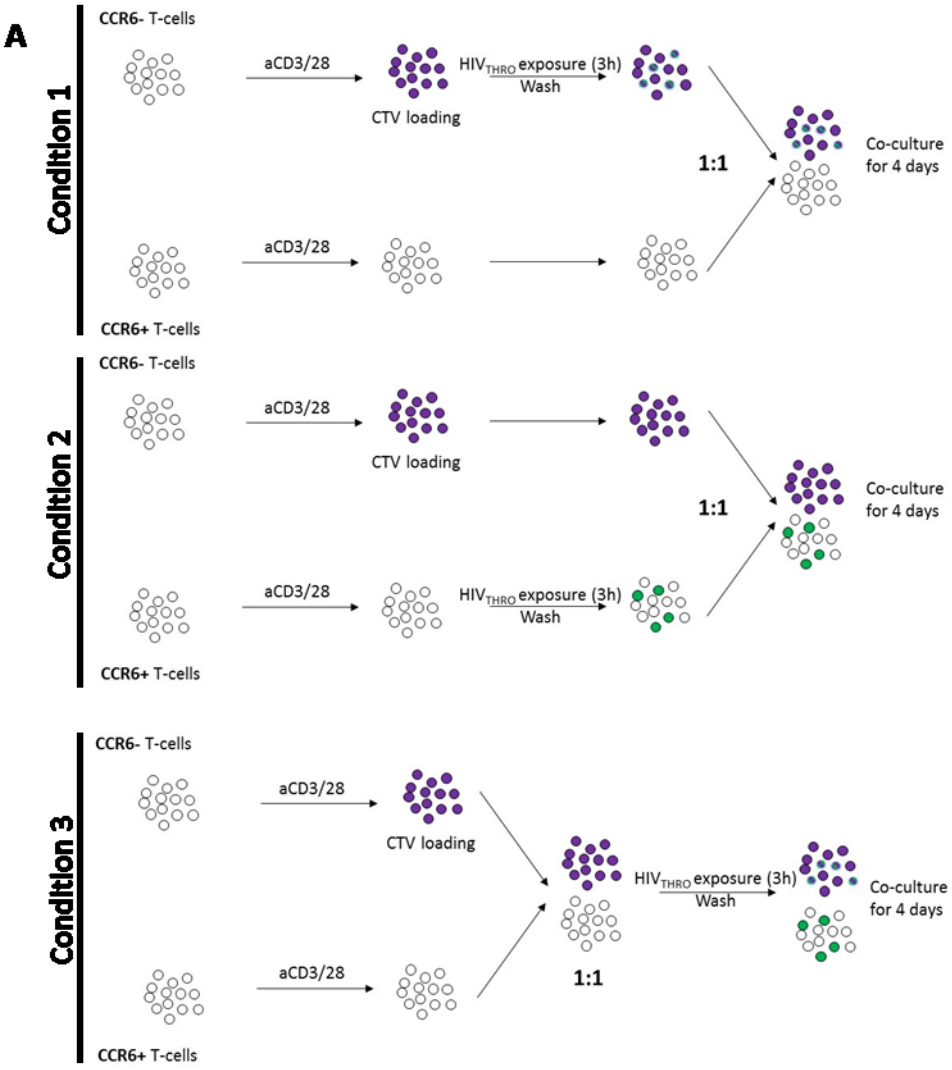
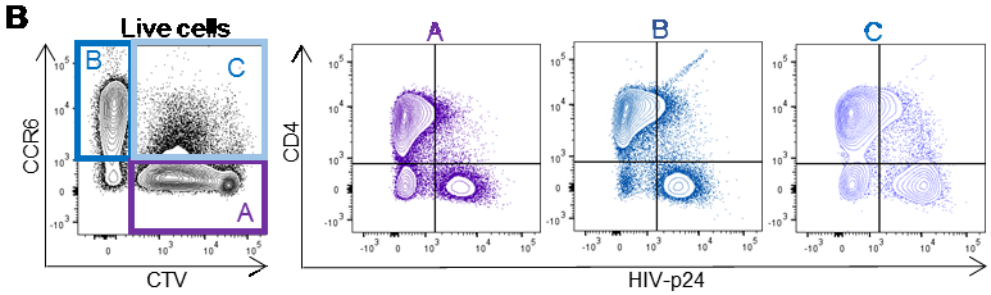
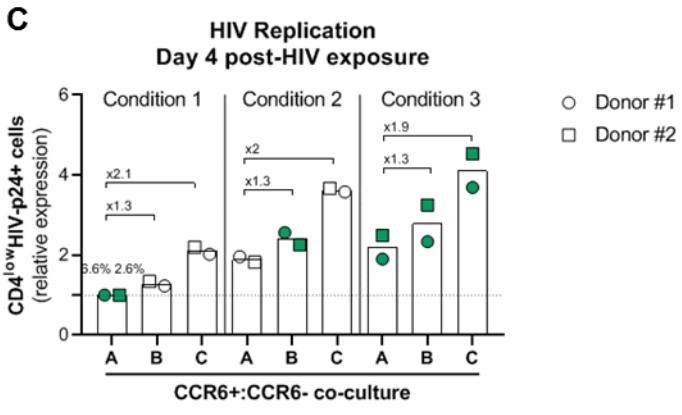
ANNEXES


A
Condition 1
CCR6- T-cells
aCD3/28
CTV loading
HIV$_{THRO}$ exposure (3h)
Wash
1:1
Co-culture
for 4 days
aCD3/28
CCR6+ T-cells
Condition 2
CCR6- T-cells
aCD3/28
CTV loading
1:1
Co-culture
for 4 days
aCD3/28
HIV$_{THRO}$ exposure (3h)
Wash
CCR6+ T-cells
Condition 3
CCR6- T-cells
aCD3/28
CTV loading
HIV$_{THRO}$ exposure (3h)
Wash
Co-culture
for 4 days
aCD3/28
1:1
CCR6+ T-cells


B
Live cells
B
C
A
CCR6
CTV
A
B
C
CD4
HIV-p24


C
HIV Replication
Day 4 post-HIV exposure
Condition 1
Condition 2
Condition 3
x2.1
x1.3
x2
x1.3
x1.9
x1.3
6.6%
2.6%
CD4^{low}HIV-p24+ cells
(relative expression)
A B C A B C A B C
CCR6+:CCR6- co-culture
Donor #1
Donor #2

Annexe 1 : Les cellules T CCR6+ initient la réplication du VIH in vitro

(A) Les cellules T CD4+CCR6+ et CCR6- ont été triées par MACS et FACS à partir des PBMCs de deux sujets non infectés par le VIH et ont été stimulées pendant 3 jours via leur CD3/CD28. Les cellules T CCR6- stimulées ont été incubées avec un traceur violet (CTV).

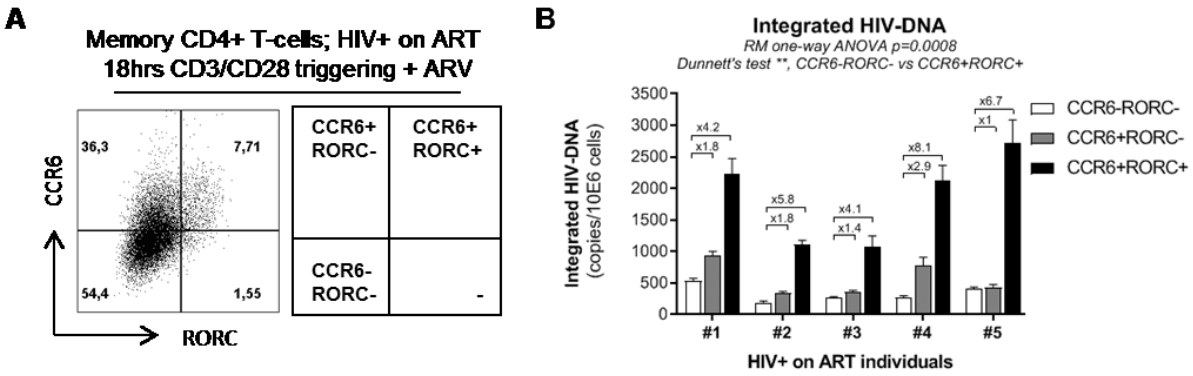
Les cellules T CCR6-CTV+ ont été exposées au VIH Transmitted/Founder (T/F) THRO (25ng/10^6 cellules) puis co-cultivées avec les cellules T CCR6+ non-infectées avec un ratio 1:1 (condition 1) pendant 4 jours.

Les cellules T CCR6+ ont été exposées au VIH T/F THRO (25ng/10^6 cellules) puis co-cultivées avec les cellules T CCR6-CTV+ non-infectées avec un ratio 1:1 pendant 4 jours (condition 2).

Les cellules T CCR6+ et CCR6-CTV+ ont été mixées à un ratio 1:1 puis exposées au VIH T/F THRO (25ng/10^6 cellules) et co-cultivées pendant 4 jours (condition 3).

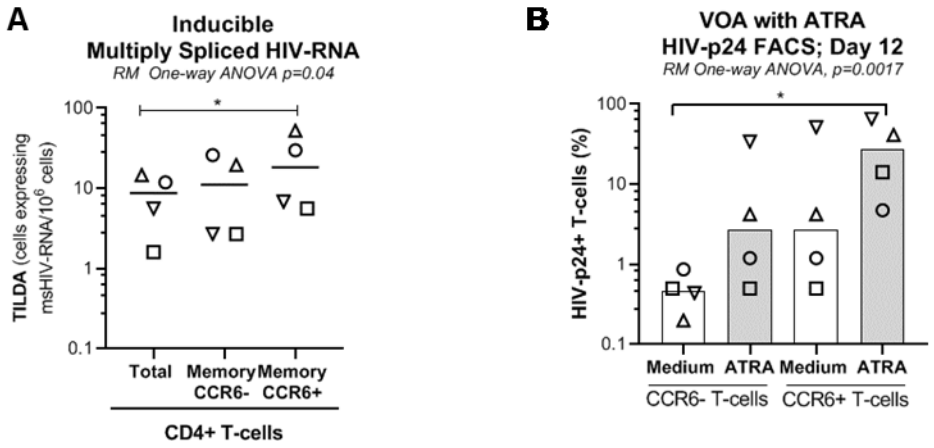
(B) Dot plot représentatif de l'expression de CTV (encadré A), de CCR6 (encadré B) et de la co-expression de CTV et CCR6 (encadré C). Les 3 dot plots colorés représentent l'expression de CD4 et VIH-p24 dans les populations CTV+ (A), CCR6+ (B) et CTV+CCR6+ (C).

(C) Analyse statistique de la fréquence de cellules T CD4low VIH-p24+ dans les populations A, B et C pour chaque condition expérimentale. Les symboles en vert indiquent la population initialement exposée au VIH pour chaque condition.



<u>Annexe 2 : Les cellules T CCR6+CD4+ mémoires exprimant RORC sont enrichies en ADN proviral chez les sujets VIH sous TAR</u>

(A-B) Les cellules T CD4+ mémoires ont été triées par sélection négative par MACS à partir de PBMCs de sujets VIH+ sous TAR. Les cellules ont été stimulées via le CD3/CD28 pendant 18h en présence d'antirétroviraux (AZT 180 nM, EFV 100nM, Ralt 200nM) pour empêcher la réplication viral *in vitro*. Les cellules ont ensuite été marquées et les cellules CCR6+RORC-, CCR6+RORC+ et CCR6-RORC- ont été triées par FACS. **(A)** L'expression de CCR6 et RORC est illustrée ici pour un donneur représentatif. **(B)** Le nombre de copies d'ADN proviral par millions de cellules a été quantifié par PCR nichée en temps réel dans chaque population triées. Le fold change pour chaque donneurs est indiqué sur le graphique. Les résultats du test statistique RM One-Way ANOVA (Friedman) sont indiqués sur le graphique.

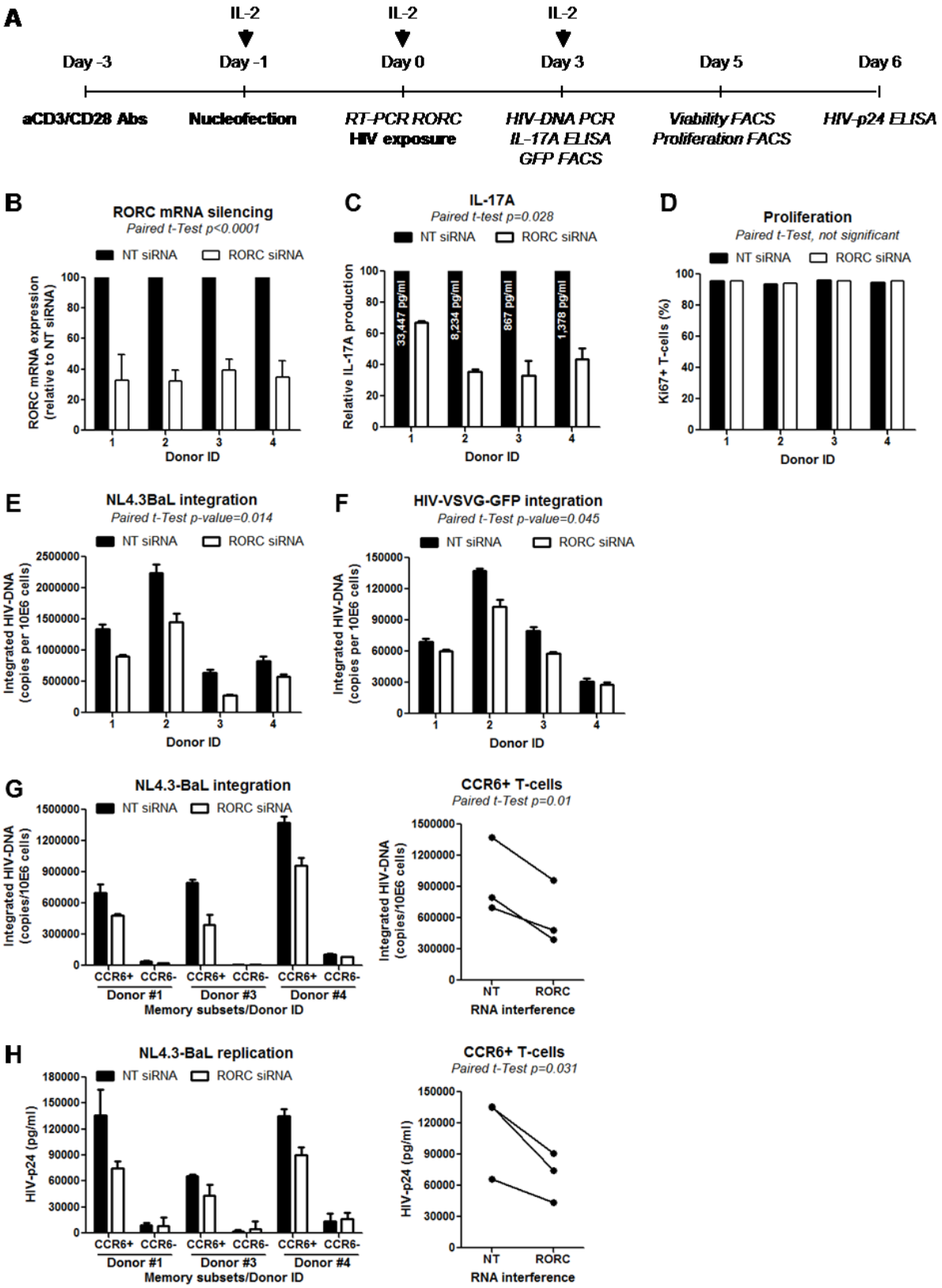


- ○ ART #1; IUPM CCR6- : 26.1 / CCR6+: 30
- □ ART #2; IUPM CCR6- : 2.7 / CCR6+: 5.6
- △ ART #3; IUPM CCR6- : 19.6 / CCR6+: 52.2
- ▽ ART #4; IUPM CCR6- : 2.7 / CCR6+: 6.2

Annexe 3 : Les cellules T CD4+CCR6+ mémoires sont enrichies en ARN multi-épissé inductible et ont une capacité augmentée à produire du virus compétent pour la réplication.

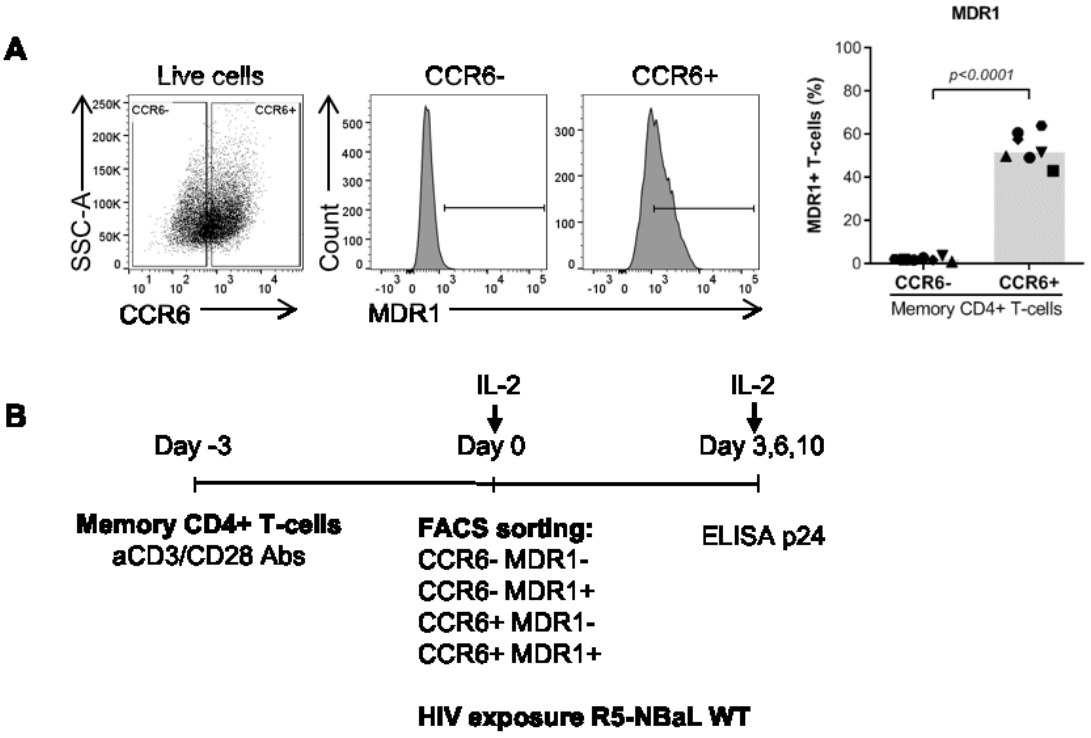
Les cellules T CD4+ ont été triées par sélection négative par MACS à partir de PBMCs de sujets infectés par le VIH et sous TAR. Les sous-populations mémoires CCR6+ et CCR6- ont été triées par FACS. **(A)** La fréquence des cellules portant de l'ARN viral (Tat/Rev) multi-épissé a été quantifiée par TILDA (Tat/Rev induced limiting dilution assay). La quantification par TILDA a été réalisée par Dr Remi Fromentin et Amélie Pagliuzza (Laboratoire Dr Chomont). Brièvement, après 3-5h de repos en présence d'ARVs (200 nM raltegravir, 100 nM efavirenz and 180 nM AZT), les cellules ont été stimulées avec 100 ng/ml de PMA et 1 µg/ml de ionomycin pendant 12 heures. Après la stimulation, les cellules ont été lavées et comptées et diluées en série à 18×10^6 cellules/ml, 9×10^6 cellules/ml, 3×106 cellules/ml et 1×10^6 cellules/ml de milieu de culture. Les transcrits Tat et Rev ont été quantifiés par RT-PCR nichée en temps réel.

(B) La réactivation du réservoir de VIH a été réalisé par un essai de réactivation viral mis au point au laboratoire. Brièvement, les cellules T CD4+ mémoires CCR6+ et CCR6- ont été cultivées à 10^6 cellules/ml et stimulées via le CD3/CD28 pendant 3 jours en présence ou absence d'ATRA (100nM). Ensuite, les cellules ont été lavées et divisées dans deux nouveaux puits et cultivées en présence d'IL-2. Les cellules ont été divisées et le milieu de culture a été rafraîchi tous les 3 jours jusqu'au jour 12. Après 12 jours de culture, l'expression intracellulaire de la protéine p24 du VIH a été quantifiée par FACS**.** Les valeurs appariées du test t sont indiquées sur les graphiques.



Annexe 4 : RORC est un régulateur positif de la production d'IL-17A et de la réplication virale.

(A) L'organigramme de l'expérience d'interférence à ARN de RORγt est illustré.
Brièvement, les cellules T CD4+ **(B-F)** ont été triées par sélection négative par MACS à partir de PBMCs de sujets non infectés et les sous-population mémoires CCR6+ et CCR6- ont été triés par FACS **(G-H)**. Les cellules ont ensuite été stimulées via le CD3/CD28 pendant 2 jours puis nucléofectées en utilisant la technologie Amaxa avec un pool de siRNA spécifiques à RORγt (Dharmacon On target smart siRNA pools specific for RORγt) ou un siRNA sans target (NT; non-targeting siRNA). **(B-C)** L'efficacité de l'interférence à ARN de RORC a été évaluée par quantification du niveau d'expression de **(B)** RORC (PCR en temps réel) et de la production **(C)** d'IL-17A (ELISA)**.** Les concentrations absolues en IL-17A sont indiquées dans les barres. **(D)** Cinq jours après la nucléofection, les cellules ont été analysées par FACS pour l'expression intracellulaire de Ki-67. En parallèle, une fraction des cellules ont été exposée à un VIH NL4.3BaL (50ng par 10^6 de cellules) compétent pour la réplication **(E)** ou à un virus VIH pseudo-typé VSVG (50ng par 10^6 de cellules) non-réplicatif **(F)**. **(E-F)** L'ADN proviral a été quantifié par PCR nichée en temps réel 3 jours après l'exposition au VIH . **(G-H)** L'effet de l'interférence à ARN de RORC après exposition au VIH NL4.3BaL (50ng par 10^6 de cellules) compétent pour la réplication dans les cellules T CCR6+ et CCR6- a été évaluée. **(G)** L'ADN proviral 3 jours après l'exposition au VIH à été quantifié par PCR nichée en temps réel. **(H)** La réplication virale a été évaluée par quantification de la protéine VIH-p24 par ELISA 6 jours post-exposition au VIH. **(G-H, panels de droite)** Analyse statistique de l'effet de l'interférence à ARN de RORC dans les cellules T CCR6+ est illustrée. **(B-H)** Les valeurs appariées du test t sont indiquées sur les graphiques.



<u>Annexe 4 : MDR1 est un marqueur des cellules T CCR6+ hautement permissives à l'infection par le VIH-1 *in vitro*.</u>

Les lymphocytes T CD4+ mémoire ont été triés par MACS et stimulés via CD3/CD28 pendant 3 jours. **(A)** Fréquence des cellules CCR6+ et CCR6- exprimant MDR1 (median; n=8). Les valeurs appariées du test t sont indiquées sur les graphiques. Pour n=2 donneurs, les cellules T CCR6+MDR1+, CCR6+MDR1-, CCR6-MDR1+ et CCR6-MDR1- ont été triées par FACS (BD Aria II) et exposées à la souche VIH NL4.3 BaL-WT (50 ng HIV-p24 / ml). Les cellules infectées ont été cultivées en présence d'IL-2 (5ng/ml) pendant 10 jours. **(B)** L'organigramme de l'expérience est illustré. **(C)** La réplication virale a été évaluée par quantification de la protéine VIH-p24 par ELISA à 3, 6 et 10 jours post-exposition au VIH.

Annexe 5 : Présentation orale et par affiche

Présentations orales :

1. **Journées Québécoises du VIH 2019, Montréal, QC, Canada.**
   D. Planas*, Y. Zhang*, J-P. Goulet, MJ. Ruiz, L. Raymond Marchand, A. Fert, H. Chen, TR. Wiche Salinas, A. Gosselin, WW. Bazie, C. Gilbert, EA. Cohen, JP. Routy, N. Chomont, P. Ancuta. *L'inhibition pharmacologique de PPARγ réactive le VIH latent, mais prévient la production/infectiosité virale tout en renforçant les fonctions Th17*

2. CAHR 2017, 26th Annual Canadian Conference on HIV/AIDS Research, Montréal, QC, Canada.
   D. Planas, A. Gosselin, R. Ponte, J-P. Routy and P. Ancuta. *Expression of HIV Dependency Factors in CCR6+CD4+ T-Cells Infiltrating the Colon of Individuals Receiving Antiretroviral Therapy -*

3. IAS 2015, 8th International AIDS society Conference on HIV, Vancouver, BC, Canada.
   Distinction: International Scholarship

   D. Planas, Y. Zhang, P. Monteiro, J-P. Goulet, A. Gosselin, J-P. Routy and P. Ancuta. *Molecular Determinants of HIV-1 Permissiveness and Persistence in Gut-Homing CD4+ T-Cells Expressing the Th17 Marker CCR6*

4. IAS 2015, Towards an HIV Cure Symposium, Vancouver, BC, Canada.
   Distinction: International Scholarship
   D. Planas, Y. Zhang, P. Monteiro, J-P. Goulet, A. Gosselin, J-P. Routy and P. Ancuta. *Molecular Determinants of HIV-1 Permissiveness and Persistence in Gut-Homing CD4+ T-Cells Expressing the Th17 Marker CCR6*

Présentations par affiche:

1. CROI 2019, Conference on Retroviruses and Opportunistic Infections, Seattle, WA, USA.
   Distinction: Young Investigator Scholarship.
   D. Planas, A. Pagliuzza, R. Ponte, A. Fert, L. Marchand Raymond, F. Dupuy, V. Mehraj, MP. Ghali, J. Angel, EA. Cohen, N. Chomont, P. Ancuta*, and JP. Routy*; *, equal contribution. *Metformin Treatment in Non-Diabetic HIV-Infected Individuals on ART*

2. 2018, Keystone Symposium on HIV and Co-Infections, Whistler, BC, Canada.
   Y. Zhang*, **D. Planas*,** H. Chen, A. J-P Goulet, T. Wiche-Salinas, A. Gosselin, M-J. Ruiz, N. Chomont, J-P. Routy, P. Ancuta. *The PPARγ antagonism boosts RORγt-mediated effector functions and locks HIV reactivation in Th17 cells.*

3. IAS 2017, 9th International AIDS society Conference on HIV, Paris, France.
   Distinction: International Scholarship
   D. Planas, Y. Zhang, P. Monteiro, J-P. Goulet, A. Gosselin, N. Grandvaux, TJ. Hope, A. Fassati, J-P. Routy and P. Ancuta. *The mammalian target of rapamycin is a druggable key regulator of HIV-1 permissiveness in gut-homing CCR6+ Th17 cells.*

4. CAHR 2016, 25th Annual Canadian Conference on HIV/AIDS Research, Winnipeg, MA, Canada.
   D. Planas, Y. Zhang, P. Monteiro, J-P. Goulet, A. Gosselin, N. Grandvaux, TJ. Hope, A. Fassati, J-P. Routy and P. Ancuta. *Retinoic acid-mediated imprinting for gut homing render Th17 cells highly permissive to HIV-1 infection by modulating the mTORC1/C2 pathways.*

5. IUMS 2014, XVIth International Congress of Virology, Montréal, QC, Canada.
   D. Planas, P. Monteiro, J-P. Goulet, A. Gosselin, J-P. Routy and P. Ancuta. *Imprinting for Gut Homing and HIV-1 Permissiveness mechanisms are specifically regulated by retinoic acid in CCR6+ T-cells.*

6. CROI 2014, 21st Conference on Retroviruses and Opportunistic Infections, Boston, MA, USA.
   Distinction: Young Investigator Scholarship.
   D. Planas, P. Monteiro, J-P. Goulet, A. Gosselin, J-P. Routy and P. *Imprinting for gut homing and HIV-1 permissiveness mechanisms are specifically regulated by retinoic acid in CCR6+ T-cells.*

Annexe 6: Publications en tant que co-auteur

1. Routy JP, Isnard S, Mehraj V, Ostrowski M, Chomont N, Ancuta P, Ponte R, **Planas D**, Dupuy FP, Angel JB; Lilac Study Group. *Effect of metformin on the size of the HIV reservoir in non-diabetic ART-treated individuals: single-arm non-randomised Lilac pilot study protocol.* **BMJ Open.** Avril 2019, 20;9(4):e028444.
2. Cattin A, Wiche Salinas TR, Gosselin A, **Planas D**, Shacklett B, Cohen EA, Ghali MP, Routy JP, Ancuta P. *HIV-1 is rarely detected in blood and colon myeloid cells during viral-suppressive antiretroviral therapy*. **AIDS**. Juillet 2019, 33(8):1293-1306.
3. Zhyvoloup A, Melamed A, Anderson I, **Planas D**, Lee CH, Kriston-Vizi J, Ketteler R, Merritt A, Routy JP, Ancuta P, Bangham CRM, Fassati A. *Digoxin reveals a functional connection between HIV-1 integration preference and T-cell activation.* **Plos Pathogens.** Juillet 2017, 13(7):e1006460
4. Gosselin A, Wiche Salinas TR, **Planas D**, Wacleche VS, Zhang Y, Fromentin R, Chomont N, Cohen ÉA, Shacklett B, Mehraj V, Ghali MP, Routy JP, Ancuta P. *Colon and blood CCR6+ T-cells contribute to HIV reservoir persistence during antiretroviral therapy*. **AIDS.** Janvier 2017, 31(1):35-48.
5. Cleret-Buhot A, Zhang Y, **Planas D**, Goulet JP, Monteiro P, Gosselin A, Wacleche VS, Tremblay CL, Jenabian MA, Routy JP, El-Far M, Chomont N, Haddad EK, Sekaly RP, Ancuta P. *Identification of novel HIV-1 dependency factors in primary CCR4+CCR6+Th17 cells via a genome-wide transcriptional approach.* **Retrovirology.** Décembre 2015, 10;12:102.