Universitat Autònoma de Barcelona

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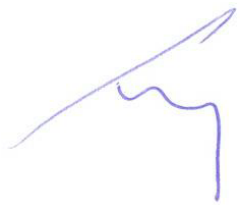

Dr. Xavier Daura Ribera

Als meus amics del poble

A la meua família

A la meua terra

Als tiets

“You do not really understand something unless you can explain it to your grandmother”

Albert Einstein

“Tu tranquil”

La Mama

ABBREVIATIONS

A	Alexa
ABC	Abacavir
ADCC	Antibody-dependent Cellular Cytotoxicity
ADCVI	Antibody-dependent Cellular Viral Inhibition
ADP	Adenosine Diphosphate
AIDS	Acquired Immune Deficiency Syndrome
ANOVA	One-way Ordinary Analysis of Variants
APC	Antigen-presenting Cell
APC-Cy7	Allophycocyanin-cyanine7 conjugate
APOBEC3	Polyprotein B mRNA Editing Enzyme Catalytic Polypeptide-like 3
ATP	Adenosine Triphosphate
AZT	Azidothymidine / Zidovudine
BAT3	HLA-B Associated Transcript 3
BCL-6	B-cell Lymphoma 6 transcription factor
BCR	B-cell antigen Receptor
Blimp-1	B Lymphocyte-Induced Maturation Protein-1
bNAb	Broadly Neutralizing Antibody
Bryo	Bryostatin A
BV	Brilliant Violet
CA	Capsid
Ca^{2+}	$Calcium^{2+}$
caHIV-1 RNA	Cell-associated HIV-1 RNA
CAR	Chimeric Antigen Receptor
cART	Combined Antiretroviral Therapy
Cas9	CRISPR associated protein nuclease 9
CAU	Caucasian
CEACAM-1	Carcinoembryonic Antigen Cell Adhesion Molecule-1
CCR	C-C Chemokine Receptor
cDNA	Complementary Deoxyribonucleic Acid
CDR	Cytosolic DNA Receptor
CD	Cluster of Differentiation
CD62L	L-selectin

CLR	C-type Lectin Receptor
CM	Central Memory T cell
CO_2	Carbon Dioxide
COB	Cobicistat
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeat
CRF	Circulating Recombinant Form
CT	Contact
CTL	CD8+ T-cell Line
CTLA-4	Cytotoxic T Lymphocyte-associated Antigen-4
CXCR	C-X-C Chemokine Receptor
DAMP	Damage-associated Molecular Pattern
DC	Dendritic Cell
dCA	Didehydro-cortistantin A
ddC	Zalcitabine
ddPCR	Droplet Digital PCR
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNMTis	DNA Methyltransferase Inhibitor
dNTP	Deoxynucleoside Triphosphate
dsDNA	Double-stranded Deoxyribonucleic Acid
DTG	Dolutegravir
eATP	Extracellular Adenosine Triphosphate
eADP	Extracellular Adenosine Diphosphate
EC	Elite Controller
Eff	Effector T cell
Ei	Early HIV-1-infected patient
ELV	Elvitegravir
EM	Effector Memory T cell
EMA	European Medicines Agency
ENTPD1	Ectonucleoside Triphosphate Diphosphohy-drolase 1
Eomes	Eomesodermin
FCS	Fetal Calf Serum

FDA	U.S. Food and Drug Administration for treating AIDS
FGL-1	Fibrinogen-related Protein-1
FITC	Fluorescein Isothiocyanate
FMO	Fluorescence Minus One
FTC	Emtricitabine
Gal	Galectin
GALT	Gut-associated Lymphoid Tissue
GFP	Green Fluorescent Protein
GRB2	Growth factor Receptor-bound Protein 2
HAVCR2	Hepatitis A Virus Cellular Receptor 2
HC	Healthy Control
HCV	Hepatitis C Virus
HDACi	Histone Deacetylase Inhibitor
HLA	Human Leukocyte Antigen
HIV	Human Immunodeficiency Virus
HMGB1	High Mobility Group Protein B1
HMTi	Histone Methyltransferase Inhibitor
ICI	Immune Checkpoint Inhibitor
IFN-I	Interferon Type-I
IFNy	Interferon y
Ig	Immunoglobulin
IL	Interleukin
IL-R	Interleukin Receptor
IN	Integrase
INSTI	Integrase Strand Transfer Inhibitor
IO	Ionomycin
IQR	Interquartile Range
IR	Inhibitory Receptor
irAE	Immune-related Adverse Event
IS	Immunological Synapse
ITAM	Immunoreceptor Tyrosine-based Activation Motif
ITIM	Immunoreceptor Tyrosine-based Inhibitory Motif

ITSM	Immunoreceptor Tyrosine-based Switch Motif
ITTM	Immunoglobulin Tyrosine Tail-like Motif
LAMP	Lysosome-associated Membrane Glycoprotein
LAT	Linker for Activation of T Cell
LAV	Lymphadenopathy-associated Virus
Lck	Lymphocyte Protein Tyrosine Kinase
LCMV C13	Lymphocytic Choriomeningitis Virus Clone 13
LPA	Latency-promoting Agent
LRA	Latency-reversing Agent
LSECtin	Liver and Lymph Node Sinusoidal Endothelial Cell C-type Lectin
LTR	Long Terminal Repeat
M	Major or Main
MA	Matrix
MAPK	Mitogen-activated Protein Kinase
MHC	Major Histocompatibility Complex
MIP-1β	Macrophage Inflammatory Protein-1β
Mg^{2+}	$Magnesium^{2+}$
MM	HLA-mismatched
MOI	Multiplicity Of Infection
MPER	Membrane-proximal External Region
MRI	Magnetic Resonance Image
mRNA	Messenger RNA
N	Non-M or Non-O
NC	Nucleocapsid
Nef	Negative Regulating Factor
NK	Natural Killer
NF-κB	Nuclear Factor-κB
NFAT	Nuclear Factor of Activated T cell
NNRTI	Non-nucleoside Reverse Transcriptase Inhibitor
NRTI	Nucleoside Reverse Transcriptase Inhibitor
NSCLC	Non-small-cell Lung Carcinoma

O	Outlier
ORF	Open Reading Frame
P	Putative
PAMP	Pathogen-associated Molecular Pattern
PB	Pacific Blue
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PE	Phycoerythrin
PerCP-Cy5.5	Peridinin Chlorophyll Protein-cyanine 5.5
PET-CT	Positron Emission Tomography-Computed Tomography
PD-1	Programed Cell Death 1
PD-L	Programed Death Ligand
PI	Protease Inhibitor
PIC	Pre-Integration Complex
PI3K/Akt	Phosphatidylinositol 3-kinase/Protein Kinase B
PKC	Protein Kinase C
PLCγ1	Phospholipase C γ 1
PMA	Phorbol 12-myristate 13-acetate
PNBN	Panobinostat
PR	Protease
PRR	Pattern Recognition Receptor
PT	Patient
PtdSer	Phosphatidyl Serine
RAL	Raltegravir
RCC	Renal Cell Carcinoma
RELI	Resting-like Cell
Rev	RNA Splicing Regulator
RI	Receptor de Inhibición
RMD	Romidepsin
RNA	Ribonucleic Acid
RT	Room Temperature
RT	Reverse Transcriptase
RTV	Ritonavir

SAHA	Suberoyl Aniline Hydroxamic Acid
SAMHD1	SAM Domain and HD Domain 1
SD	Standard Deviation
SEB	Staphylococcus Aureus Enterotoxin B
SEM	Standard Error of the Mean
SIV	Simian Immunodeficiency Virus
SIVcpz	Simian Immunodeficiency Virus of Central African Chimpanzee
SIVsm	Simian Immunodeficiency Virus of Sooty Mangabey
ssRNA	Single Stranded Ribonucleic Acid
STAT	Signal Transducer and Activator of Transcription
TALEN	Transcription Activator-like Effector Nuclease
TARc	Tratamiento Antirretroviral Combinado
Tat	Transactivator Protein
T-bet	Transcription Factor T-box Expressed in T Cell
TBP	TATA-binding protein
$TCID_{50}$	50% Tissue Culture Infective Dose
TCR	T-cell Receptor
TDF	Tenofovir
TDF/FTC	Truvada
Tex	T-cell exhaustion
TIGIT	T-cell Immunoglobulin and ITIM domain
TIL	Tumor-infiltrating Lymphocyte
TIM-3	T-cell Immunoglobulin-3
TLR	Toll-like-receptor
TM	Transitional Memory T cell
TNF	Tumor Necrosis Factor
Tricho	Trichostatin A
TRIM5α	Tripartite Motif 5α
t-SNE	t-Distributed Stochastic Neighbor Embedding
UNAIDS	United Nations Program on HIV/AIDS
usVL	Ultrasensitive Viral Load
UT	Untreated

V	Violet
Vif	Viral Infectivity Factor
VL	Viral Load
Vpr	Virus Protein R
Vpu	Virus Protein Unique
W - Wk	Week
WHO	World Health Organization
Zap-70	Zeta-chain Associated Protein Kinase 70
ZFN	Zinc-finger Nuclease
3TC	Lamivudine
αIR	Anti-inhibitory Receptor

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SUMMARY, RESUMEN & RESUM

Human Immunodeficiency Virus type 1 (HIV-1) infection is currently one of the most important pandemics worldwide. Nearly 38 million individuals are currently living with HIV-1 and more than 33 million deaths have been reported since the beginning of the pandemic. Although the development of combined antiretroviral treatment (cART) is one of the most important advances to treat HIV-1 infection, cART is unable to cure the infection completely as a consequence of viral perpetuation in the form of the reservoir. For this reason, the development of effective cure strategies is currently the major goal for the HIV-1 scientific community in its search for a cure for HIV-1.

HIV-1-specific CD8+ T cells play a major role in controlling viral replication through the recognition and killing of infected cells. However, viral persistency and sustained immune activation cause exhaustion of CD8+ T cells, which progressively lose their function with an increase in the expression of inhibitory receptors (IRs). Although cART effectively controls viral replication and partially contributes to immune recovery, HIV-1-specific CD8+ T cells remain dysfunctional. Therefore, the contribution of IR expression in the regulation of CD8+ T cells needs to be overcome in HIV-1 infection and also cART. To address this, the aim of my thesis is to provide a better understanding of the immunological bases of CD8+ T-cell exhaustion mediated by IRs behind HIV-1 infection and how IRs may impact current immunotherapeutic strategies for HIV-1 cure. Indeed, reversion of the exhaustion state in CD8+ T cells may be key for achieving a cure for HIV-1.

Based on the fundamental role of HIV-1-specific CD8+ T cells for the success of cure strategies, the first chapter of this thesis is focused on a first study aimed at identifying the immunological barriers that prevent shock and kill strategies from successfully clearing the viral reservoir. Our results demonstrated that the potency of LRAs is crucial to modulate the speed of HIV-1-antigen recognition and also the magnitude of killing by HIV-1-specific CD8+ T cells. In addition, we observed an interdependency between CD8+ T-cell function, the expression patterns of IRs, and the killing of reactivated cells, indicating the need to understand the role of IRs in HIV-1-specific CD8+ T-cell responses as functional limitations on shock and kill strategies and thus on success in achieving a cure for HIV-1.

As result of these findings, in the second study described in chapter II, we performed a high-resolution mapping of IR expression across three levels in the landscape of CD8+ T-cell populations in HIV-1 infection and long-term cART. TIGIT+ and TIGIT+TIM-3+ expression persisted longitudinally elevated in CD8+ T-cell subsets after long-term cART and TIGIT+ correlated inversely with CD4+ T-cell counts. Our single-cell data revealed wide IR heterogeneity with homeostatic changes including contraction and expansion of CD8+ T-cell clusters in the course of HIV-1 infection and long-term cART. Moreover, we characterized a selective depletion of a differential cluster with $CD107a^{hi}$ $TIGIT^{hi}$ memory HIV-1-specific CD8+ T cells after long-term cART. TIGIT blockade effectively restored degranulation profiles of HIV-1-specific CD8+ T cells. These results led us to propose TIGIT as a new immunotherapeutic target to reverse the exhausted state of CD8+ T cells in HIV-1 infection.

In order to evaluate the potential therapeutic effect of immune checkpoint inhibitors (ICIs) in humans as a curative strategy, we addressed the impact of ICIs in a clinical case report, detailed in the third chapter. The study focused on an HIV-1-infected individual on cART that received αPD-1 as therapy for metastatic melanoma. In terms of HIV-1, we showed that despite a transient increase in activation markers and TNF production in HIV-1-specific CD8+ T-cell responses, the effect of the αPD-1 treatment was not sufficient to consistently reduce the size of the viral reservoir, thus calling into question the stand-alone effect of ICIs in terms of shock and kill as HIV-1 curative strategies.

Altogether, this thesis provides an overview of current curative strategies that are being used to attain a cure for HIV-1, starting from identifying the limitations of using HIV-1-specific CD8+ T cells to kill reactivated cells, going on to offer a thorough exploration of the role of IRs in HIV-1 infection and cART for the identification of novel molecular targets, and finally analyzing the impact of current ICIs therapies as a cure strategy for HIV-1. We believe that the key to an effective cure of HIV-1 hinges on finding a way to reactivate all the provirus from latently infected cells and enhancing HIV-1-specific CD8+ T cells through vaccines or ICIs to clear the infected cells. We hope that all the scientific work described in this thesis will contribute to a better understanding of the role of IRs in the development of new and effective HIV-1 cure strategies.

La infección por el virus de la inmunodeficiencia humana tipo 1 (VIH-1) es una de las pandemias más importantes del mundo ya que actualmente consta con casi 38 millones de personas infectadas y con más de 33 millones de muertes desde su inicio. Aunque el tratamiento antirretroviral combinado (TARc) es uno de los avances más importantes para controlar dicha infección, es incapaz de curarla ya que el virus permanece de forma persistente como reservorio viral. Por consiguiente, el desarrollo de estrategias de curación es uno de los principales objetivos de la comunidad científica para lograr el fin de la pandemia. Las células T-CD8+ específicas para el VIH-1 ejercen un papel esencial en el control de la infección, ya que reconocen las células infectadas por el virus y las destruyen. Sin embargo, debido a la persistencia viral y la activación sostenida inmune, estas se agotan y pierden su funcionalidad a través del aumento progresivo de la expresión de los receptores de inhibición (RIs). Aunque el TARc controla la replicación viral y contribuye a la recuperación inmunitaria, las células T-CD8+ siguen siendo disfuncionales. Por lo tanto, es fundamental entender el papel de los RIs en la infección por el VIH-1 y así, poder desarrollar dianas inmoterapéuticas para recuperar la respuesta antiviral. El objetivo de mi tesis se centra en proporcionar una mejor comprensión de las bases inmunológicas de los RIs durante el agotamiento de T-CD8+ en la infección por el VIH-1 y TARc, y además en estudiar cómo los RIs afectan a las estrategias inmunoterapéuticas más usadas actualmente en la cura para el VIH-1.

Debido al papel fundamental de las células T-CD8+ específicas para el VIH-1 en las estrategias de curación, el primer capítulo de esta tesis se centra en la identificación de las barreras viro-inmunológicas que evitan la eliminación del reservorio viral durante la estrategia de “shock and kill”. Nuestros resultados muestran que la potencia de los agentes reactivadores de la latencia (LRAs) es crucial para la modulación de la velocidad de reconocimiento del VIH-1 y la magnitud con la que las células T-CD8+ específicas para VIH-1 actúan. Además, observamos una relación entre la funcionalidad de las células T-CD8+, los patrones de expresión de los RIs y la destrucción de las células reactivadas, indicando la necesidad de una mejor comprensión de los RIs en los linfocitos T-CD8+ específicos para el VIH-1 como limitadores funcionales del “shock and kill” y, por lo tanto, en conseguir una cura para el VIH-1.

Tras estos resultados, en el segundo capítulo realizamos un mapeo en tres niveles de la expresión de los RIs en T-CD8+ durante la infección por VIH-1 y tras largos periodos de TARc. Lo que identificamos fue que la expresión de TIGIT+ y TIGIT+TIM-3+

persistieron longitudinalmente elevados en T-CD8+ tras largos periodos de TARc y que la expresión de TIGIT+ correlacionó inversamente con T-CD4+. Nuestros datos de single-cell revelaron una amplia heterogeneidad de IRs con cambios homeostáticos durante la infección y tras largos periodos de TARc incluyendo contracciones y expansiones de clústeres de T-CD8+. También, caracterizamos una depleción selectiva tras largos periodos de TARc de un cluster diferencial con T-CD8+ específicos para el VIH-1 con fenotipo de memoria y $CD107a^{hi}$ $TIGIT^{hi}$. El bloqueo de TIGIT recuperó eficazmente los perfiles de degranulación de T-CD8+ específicos para el VIH-1. Por lo tanto, proponemos TIGIT como nueva diana inmunoterapéutica para revertir el estado de agotamiento en las células T-CD8+ durante la infección por VIH-1.

Con el fin de evaluar el potencial efecto inmunoterapéutico en humanos de los inhibidores de puntos de control inmunitario (ICIs) como estrategia curativa, en el tercer capítulo abordamos el impacto de esta estrategia en un caso clínico. El estudio se centró en un individuo infectado por VIH-1 bajo tratamiento antirretroviral que recibió αPD-1 como terapia para tratar el melanoma metastásico. En términos de VIH-1, encontramos que a pesar del aumento transitorio de los marcadores de activación y la producción de TNF en las células T-CD8+ específicas para el VIH-1, el efecto del αPD-1 no fue suficiente para reducir considerablemente el tamaño del reservorio viral, cuestionando así, el efecto independiente de los ICIs en términos de “shock and kill” como estrategias curativas del VIH-1.

En conjunto, esta tesis proporciona una descripción general de las estrategias inmunoterapéuticas actuales que se están utilizando para lograr la curación del VIH-1, empezando por la identificación de las limitaciones de las células T-CD8+ agotadas para eliminar las células reactivas, seguido por una mejor comprensión de los RIs en la infección por VIH-1 y TARc y, acabando con el efecto de los ICIs *in vivo* como estrategias de curación para el VIH-1. Creemos que la clave para lograr dicha curación se centraría en reactivar de alguna manera todos los provirus de las células infectadas de forma latente y en mejorar funcionalmente, mediante vacunas o ICIs, las células T-CD8+ agotadas, para así poder eliminar todas las células infectadas. Esperamos que todo el trabajo que se ha realizado en esta tesis pueda contribuir a comprender mucho mejor el papel de los RIs, y así poder utilizar esta comprensión para el desarrollo de nuevas y efectivas estrategias de curación del VIH-1.

La infecció pel virus de la immunodeficiència humana tipus 1 (VIH-1) és una de les pandèmies més importants del món ja que actualment consta de quasi 38 milions de persones infectades i amb més de 33 milions de morts des del seu inici. Encara que el tractament antiretroviral combinat (TARc) és un dels avanços més importants per a controlar aquesta infecció, és incapaç de curar-la ja que el virus roman de manera persistent com a reservori viral. Per tant, el desenvolupament de noves estratègies de curació és un dels principals objectius de la comunitat científica per assolir la fi de la pandèmia. Les cèl·lules T-CD8+ específiques per al VIH-1 tenen un paper essencial en el control de la infecció ja que reconeixen les cèl·lules infectades i les destrueixen. No obstant això, la persistència viral i l'activació immune sostinguda provoquen un esgotament d'aquestes i fa que la seua funció es perdi mitjançant un augment progressiu de l'expressió dels receptors d'inhibició (RIs). Tot i que el TARc controla eficaçment la replicació viral i contribueix a la recuperació immunitària, les cèl·lules T-CD8+ segueixen sent disfuncionals. Per tant, és fonamental entendre el paper dels RIs en la infecció pel VIH-1 i així poder desenvolupar dianes immoterapèutiques per recuperar la resposta antiviral perduda. L'objectiu de la meva tesi se centra a proporcionar una millor comprensió de les bases immunològiques dels RI durant l'esgotament de les cèl·lules T-CD8+ en la infecció pel VIH-1 i sota TARc, ia més a estudiar com els RIs afecten a les estratègies immunoterapèutiques més usades actualment per la cura del VIH-1.

A causa del paper fonamental que exerceixen les cèl·lules T-CD8+ específiques per al VIH-1 en les estratègies de curació, el primer capítol d'aquesta tesi es centra en la identificació de les barreres viro-immunològiques que eviten l'eliminació del reservori viral durant l'estratègia de "shock and kill". Els nostres resultats van mostrar que la potència dels agents reactivadors de la latència (LRAs) és crucial per a la modulació de la velocitat de reconeixement del VIH-1 i la magnitud què les cèl·lules T-CD8+ específiques per al VIH-1 actuen. A més a més, vam observar una relació entre la funcionalitat de les cèl·lules T-CD8+, els patrons d'expressió dels RIs i la destrucció de les cèl·lules reactivades, la qual cosa indica la necessitat d'una millor compressió dels RIs en els limfòcits T-CD8+ específics per al VIH-1 com limitadors funcionals d'estratègies de “shock and kill” i, per tant, en aconseguir una cura per al VIH-1.

Després d'aquests resultats, al segon estudi del capítol II, vam realitzar un mapatge en tres nivells d'alta resolució de l'expressió dels RIs a les poblacions de T-CD8+ durant

la infecció per VIH-1 i TARc a llarg termini. El que vam identificar va ser que l'expressió de TIGIT+ i TIGIT+TIM-3+ va persistir longitudinalment elevada a les subpoblacions de T-CD8+ després de llargs períodes de TARc. A més a més que l'expressió de TIGIT+ va correlacionar inversament amb T-CD4+. Les nostres dades de single-cell van revelar una àmplia heterogeneïtat d'IRs amb canvis homeostàtics durant el curs de la infecció pel VIH-1 i després de llargs terminis de TARc incloent contraccions i expansions de clústers cel·lulars de T-CD8+. També vam caracteritzar una depleció selectiva després de llargs períodes de TARc d'un clúster diferencial de cèl·lules T-CD8+ específiques per al VIH-1 amb fenotip de memòria i CD107a^{hi} TIGIT^{hi}. El bloqueig de TIGIT va recuperar eficaçment els perfils de degranulació de les cèl·lules T-CD8+ específiques per al VIH-1. Aquests resultats ens van portar a proposar TIGIT com a nova diana inmunoterapèutica per a revertir l'estat d'esgotament de les cèl·lules T-CD8+ en la infecció per VIH-1.

Amb la finalitat d'avaluar el potencial efecte inmunoterapèutic en humans dels inhibidors de punts de control immunitari (ICIs) com a estratègia curativa, en el tercer capítol vam abordar l'impacte d'aquesta estratègia en un cas clínic. L'estudi es va centrar en un individu infectat per VIH-1 sota TARc que va rebre αPD-1 com a teràpia per a tractar el melanoma metastàtic. En termes de VIH-1, vam trobar que malgrat l'augment transitori dels marcadors d'activació i la producció de TNF a les cèl·lules T-CD8+ específiques per al VIH-1, l'efecte del αPD-1 no va ser suficient per a reduir considerablement la grandària del reservori viral, qüestionant així, l'efecte independent dels ICIs en termes de “shock and kill” com a estratègia curativa del VIH-1.

En conjunt, aquesta tesi proporciona una descripció general de les estratègies inmunoterapèutiques més actuals que s'estan utilitzant per a aconseguir la curació del VIH-1, començant per la identificació de les limitacions de les cèl·lules T-CD8+ esgotades per a eliminar les cèl·lules reactives, seguit per una millor comprensió dels RIs en la infecció per VIH-1 i TARc i, acabant amb l'efecte dels ICIs *in vivo* com a estratègia de curació del VIH-1. Creiem que la clau per a aconseguir una estratègia de curació se centraria en reactivar d'alguna manera tots els provirus de les cèl·lules infectades de forma latent i en millorar funcionalment, mitjançant vacunes o ICIs, les cèl·lules T-CD8+ esgotades, per així poder eliminar totes les cèl·lules infectades. Esperem que tota la feina que s'ha realitzat en aquesta tesi pugui contribuir a comprendre molt millor el paper dels RIs, i així poder utilitzar aquesta comprensió per al desenvolupament de noves i efectives estratègies de curació del VIH-1.

INTRODUCTION

1. Human Immunodeficiency Virus

1.1. The HIV pandemic

Phylogenetic analyses suggest that the Human Immunodeficiency Virus (HIV) was exported from Africa to Haiti in 1966 and then introduced to North America in the early 1980s [1], [2]. Its presence was first formally noted on 5 June 1981, when the United States health protection agency reported an unusual type of pneumonia in five gay individuals in Los Angeles. This represented the first description of a new Acquired Immune Deficiency Syndrome or AIDS [3].

Two years later, a French research group headed by Dr. Françoise Barré-Sinoussi and Dr. Luc Montagnier who were subsequently awarded the Nobel Prize in Physiology or Medicine in 2008 [4], [5], isolated for the first time a new human retrovirus from samples of a subject presenting lymphadenopathy [6]. This novel virus was designated a lymphadenopathy-associated virus (LAV) and renamed HIV by the International Committee on the Taxonomy of Viruses in 1986 [7]–[9].

Since the discovery of HIV as the causative agent of AIDS, the United Nations Program on HIV/AIDS (UNAIDS) has reported more than 75 million people infected with HIV and more than 33 million AIDS-related deaths since the beginning of the pandemic [10]. In addition, the percentage of people worldwide living with HIV has risen to 37.9 million in recent decades, with a global prevalence of 0.7% in 2019 (**Figure 1**). In Spain, about 150,000 people have been estimated by UNAIDS to be living with HIV [10].

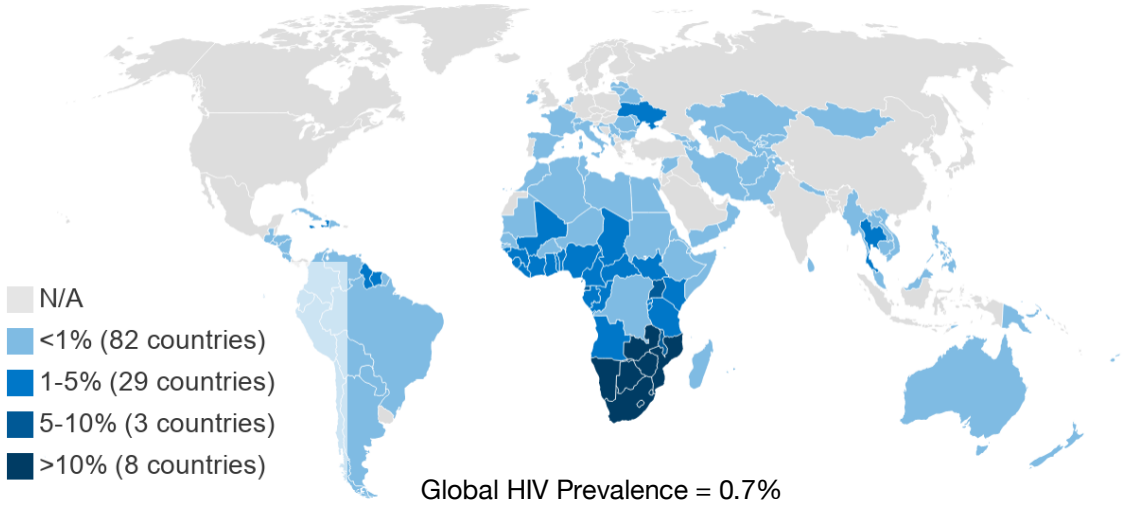

Figure 1. Global prevalence of HIV in adults aged 15 to 49 in the different regions of the world in 2019. Data are estimates. Each country is color coded according to the percentage of prevalence. Image adapted from Kaiser Family Foundation and UNAIDS, accessed 2021, using Adobe Illustrator CC 2018 [11].

In 2013, UNAIDS proposed an ambitious 90-90-90 target for HIV treatment: 90% of HIV-infected people should know their HIV status, of whom 90% should be receiving combined antiretroviral therapy (cART), and 90% of those receiving therapy should be virally suppressed [12]. UNAIDS projected that achieving these targets by 2020 would make it possible to bring the HIV pandemic to an end by 2030. Although the current aware-treated-suppressed ratio is 79-62-53 worldwide, the original target is almost achieved in Spain being 86.2-93.4-90.4 [10]. Despite the failure to achieve the goal on a global scale, the numbers of AIDS-related deaths have continued to decline gradually from a peak of 1.7 million in 2004 to 770,000 in 2018 [10]. Also, the global incidence-prevalence ratio has dropped from 11.2% in 2000 to 4.6% in 2018. Nonetheless, given these numbers, the HIV pandemic is still a huge challenge for global health and the world is still far from eradicating AIDS by 2030.

1.2. The virology behind HIV

Classification and diversity

HIV is classified as a member of the *Lentivirus* genus, *Retroviridae* family and, *Orthoretrovirinae* subfamily [13]. On basis of the organization of the HIV genome, HIV is classified into type 1 (HIV-1) and type 2 (HIV-2) [14]. While HIV-1 is responsible for the worldwide spread of the infection, HIV-2 is restricted to Western and Central Africa [14][15]. Phylogenetic data support the hypothesis that zoonotic transmission occurred at the beginning of the 20th century, with non-human primate immunodeficiency viruses transmitted from Central African chimpanzees (SIVcpz) and West African sooty mangabeys (SIVsm) to humans being the origins of HIV-1 and HIV-2, respectively [14], [16]–[18].

Focusing on HIV-1 as the main cause of the pandemic, HIV-1 is classified into several groups according to the degree of genetic diversity: Major or Main (M), Outlier (O), Non-M or Non-O (N), and Putative (P) [19]. Each group has originated from cross-species transmissions between non-human primates and humans [14], [19]–[21]. Moreover, the M group is subdivided into subtypes A-D, F-H, J, and K and also into 103 circulating recombinant forms (CRFs), including subtypes E, G, and I. Globally, subtype C is the most prevalent and responsible for 46% of the infections worldwide. Subtype C tends to be predominant in the low-income countries of southern Africa as well as in India and Ethiopia. Despite the prevalence of subtype C, the majority of HIV-1 research is focused on subtype B, which constitutes 12% of infections worldwide and is predominant in Europe, North and South America and, Oceania [14], [19]–[21].

Genomic organization and particle structure

The HIV-1 genome is 9.7 kb in length and consists of two polarized positive single-stranded (ss) RNA molecules with three overlapping open reading frames (ORF) that encode for 15 proteins and are flanked by long terminal repeats (LTRs) (**Figure 2A**). The viral genome encodes for nine functional genes including three structural (Gag, Pol, Env), two regulatory (Tat, Rev), and four accessory genes (Vif, Vpu, Vpr, Nef) [22]–[27]. The sequence of the Gag gene encodes for Gag-polyprotein precursors that are sequentially cleaved by the viral protease to generate the capsid (CA, p24), the matrix (MA, p17), the nucleocapsid (NC, p7), and the p6 protein (**Figure 2A**). In addition, the protease (PR, p12) is also encoded by the Gag-Pol reading frame. Adjacent to this, viral reverse transcriptase (RT, p51) and integrase (IN, p32) are also synthesized by the Pol gene. Contiguously, the Env gene encodes for the gp160 precursor, which undergoes proteolytic cleavage into gp41 and gp120.

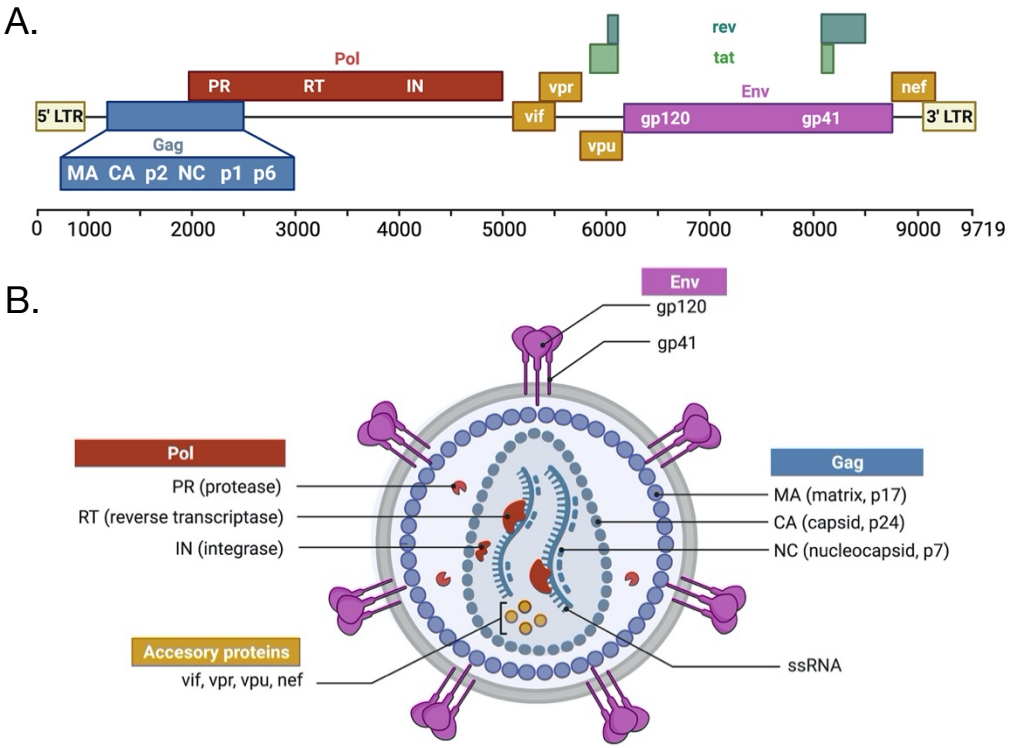

Figure 2. HIV-1 genome and viral particle structure. A. Sequence of the HIV-1 genome. The viral genome is made up of structural (Gag in blue, Pol in red and Env in yellow), accessory (orange) and regulatory genes (green). The different processed structural proteins are represented in the compartment of each structural gene. **B.** Schematic structure of HIV-1 virion. Image adapted from Cervera *et al*., 2019, using Biorender and Adobe Illustrator CC 2018 [25].

The HIV-1 virion is a spherical particle 119-207 nm in diameter. The viral particle is wrapped in an envelope composed of a lipidic bilayer from the host cells and by gp41-gp120 viral spikes (**Figure 2B**). These viral glycoproteins protrude from the lipidic bilayer

as a trimeric structure and are involved in the process of viral entry. On the inner surface of the membrane, MA forms an icosahedral symmetric assembly that surrounds the condensed cone-shaped capsid [28]. The p24 viral shell houses the viral RT, IN, and PR, as well as the p6 protein and NC, which stabilizes the two identical strands of RNA [25], [28], [29]. In addition, other accessory proteins required for viral replication, budding, and pathogenesis are also located inside the viral capsid (**Figure 2B**). These accessory proteins include Tat (transactivator protein, p14), which elongates viral transcripts, Rev (RNA splicing-regulator, p19), which promotes the nuclear export of spliced RNA strands, Nef (negative regulating factor, p27), which enhances the infectivity and downregulates CD4+ expression and Vif (viral infectivity factor, p23), Vpr (virus protein r, p15) and Vpu (virus protein unique, p16), which produce infectious virus and regulate the transcriptional outputs [22]–[27].

Replication cycle

Once the virion enters the body, HIV-1 comes into contact with CD4+ T cells, dendritic cells (DCs), monocytes or macrophages, and uses their machinery to multiply and spread throughout the body [30], [31]. This process is carried out in four steps (**Figure 3**): A) binding, fusion, and uncoating, B) reverse transcription and integration, C) transcription, splicing and translation, and D) assembly, release, and maturation.

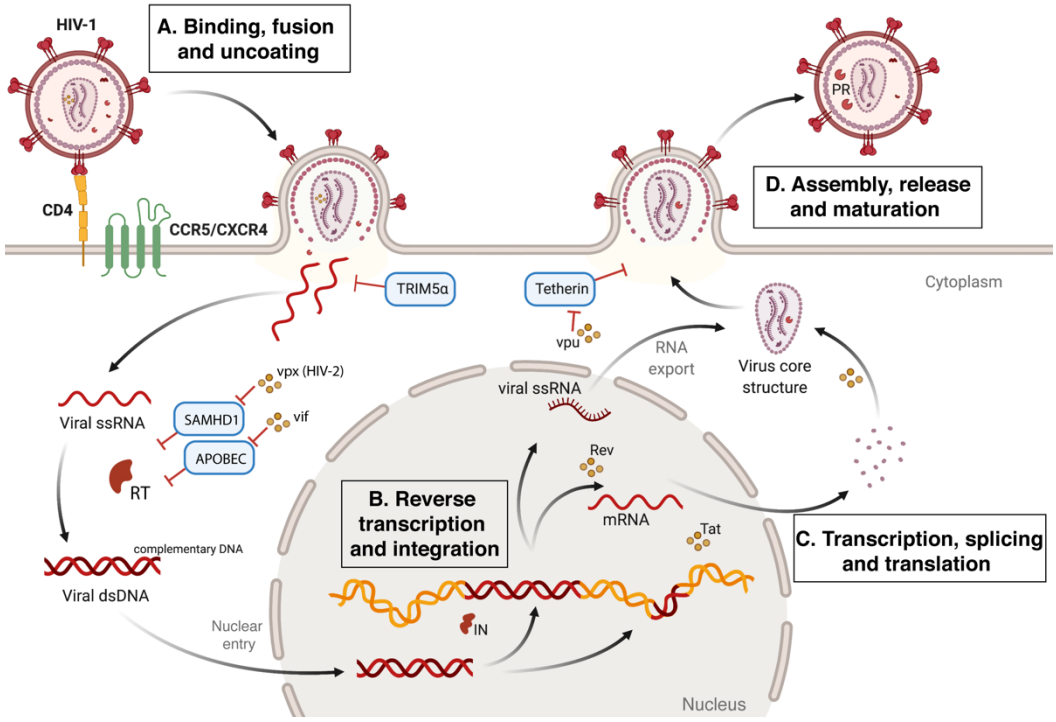

Figure 3. HIV-1 cycle of replication. A. Binding, fusion, and uncoating. **B.** Reverse transcription and integration. **C.** Transcription, splicing, and translation. **D.** Assembly, release, and maturation. Blue squares represent the host restriction factors, black arrows indicate continuity, and red lines inhibition. Image recreated from viralzone.expasy using Biorender and Adobe Illustrator CC 2018 [32].

Binding, fusion, and uncoating

These are the first steps the virus takes to enter the target cells and establish viral infection (**Figure 3A**). The receptor CD4 (cluster of differentiation 4) triggers the remodeling of gp120, thus allowing the binding of the virus to the co-receptors, which are either C-C chemokine receptor 5 (CCR5) or C-X-C chemokine receptor 4 (CXCR4) depending on viral tropism [15], [27], [33]. In general, viruses using CCR5 (R5-tropic) are responsible for viral transmission, and those using CXCR4 (X4-tropic) or both (dual- or R5X4-tropic) emerge later during disease progression [34], [35]. After the conformational changes of gp120, the hydrophobic region of gp41 is exposed to the cell membrane, allowing the fusion of the virus with the target cell. Consequently, the viral capsid can enter the cytoplasm of susceptible cells [36].

Reverse transcription and integration

Once inside the cell, the virus moves towards the nucleus using microtubules and the uncoating process begins (**Figure 3B**). The viral RT uses the pools of nucleotides and the HIV-1 ssRNA is reverse-transcribed into complementary DNA (cDNA) [37]. As it goes, the RT degrades the ssRNA and completes the synthesis of double-strand DNA (dsDNA) [38]. When the dsDNA reaches the nucleus, the viral IN together with cellular proteins binds to the ends of the dsDNA and forms the pre-integration complex (PIC) [37]. Once inside the nucleus, dsDNA is ligated to the cell genome by IN and defines the provirus [37].

The integration and generation of provirus preferentially occur in activated CD4+ T cells where genes are transcriptionally active for productive HIV-1 infection. However, these activated CD4+ T cells can revert into a long-lived memory resting state, known as latently HIV-1-infected CD4+ T cells, where provirus is transcriptionally silent but retains the capacity to produce viral particles upon stimulation. Of note, some studies also support direct infection of resting memory CD4+ T cells [39].

Once CD4+ T cells are latently HIV-1-infected, cells can homeostatically proliferate and expand clonally in anatomical sites where the virus can persist in the form of a latent viral reservoir. In this context, anatomical compartments such as lymph nodes, the central nervous system, liver, or adipose tissue are considered sites for the HIV-1 latent reservoir. There is evidence indicating ongoing active viral replication at these sanctuary sites [30], [39]–[42].

Transcription, splicing, and translation

During this step, the cellular RNA polymerase II transcribes the proviral HIV-1 DNA into the earliest messenger RNA (mRNA). This mRNA becomes fully spliced and is exported into the cytoplasm to be translated into Tat, Rev, or Nef regulatory proteins (**Figure 3C**). Then, Tat and Rev return to the nucleus, where Tat promotes the elongation of mRNAs and Rev leads the transcription of spliced and unspliced mRNAs. Longer mRNAs with Gag, Pol, or Env genes are translated and processed into the structural proteins destined for packaging into new virus particles [37], [43], [44].

Assembly, release, and maturation

At the cell membrane, thousands of precursors of Gag-polyproteins and viral enzymes are assembled with the viral ssRNA and constitute a tightly packed lattice that forces the membrane to bulge out. During the budding process, the ESCRT-IIIs cellular proteins are also recruited to pull the membrane at the bud neck and undergo fission. Once the virion is released (**Figure 3D**), it undergoes a maturation process to generate fully infectious HIV-1 particles through the action of the PR, which cleaves the Gag-polyprotein precursors. Finally, virions are released to establish new infections [37], [45], [46].

2. The natural course of HIV-1 infection

HIV-1 transmission is mainly through body fluids from an HIV-1-infected individual to another individual. The main routes of viral transmission are; I) sexual contact across mucosal surfaces including vaginal, anal, or oral, II) vertical transmission from mother to child during pregnancy, delivery, or breast-feeding, and III) percutaneous inoculations through transfusions with HIV-1-infected blood, after sharing injection material or other cutting objects. The risk of transmission is dependent on various factors including the viral load, the presence of other sexually transmitted diseases, and the susceptibility of the contact partner. Of note, UNAIDs estimated in 2019 that unprotected sex is the main cause of new HIV-1 infections [47].

The natural course of HIV-1 infection is characterized by an exponential rise in the viral load, progressive decline in CD4+ T cells, and chronic immune activation. The natural course of HIV-1 infection can be divided into three phases, namely acute, chronic, and AIDS, as represented in **Figure 4**.

Acute infection

HIV-1 exists as a quasispecies or high numbers of variant genomes, termed mutant spectra. However, only one or a small number of founder viruses establish the infection [48], [49]. Following viral exposure, HIV-1 targets DCs, Langerhans cells, and CD4+ T cells through interactions at mucosal sites. This period, termed the "eclipse" phase, generally takes between 10 to 12 days and viremia and symptoms are still undetectable [50][51]. After early infection but still in the acute phase, HIV-1 starts to disseminate throughout the body leading to an increase in plasma viremia (**Figure 4A**). This defines the systemic phase of infection and is characterized by high titers of virus in circulation (>10^5 RNA copies/ml) and a massive depletion of circulating CD4+ T-cell counts due to the direct viral cytopathic effect. In addition, most of the non-specific signs and symptoms including fever, fatigue, skin rash, myalgia, pharyngitis, and cervical adenopathy are manifested during this phase [27], [50].

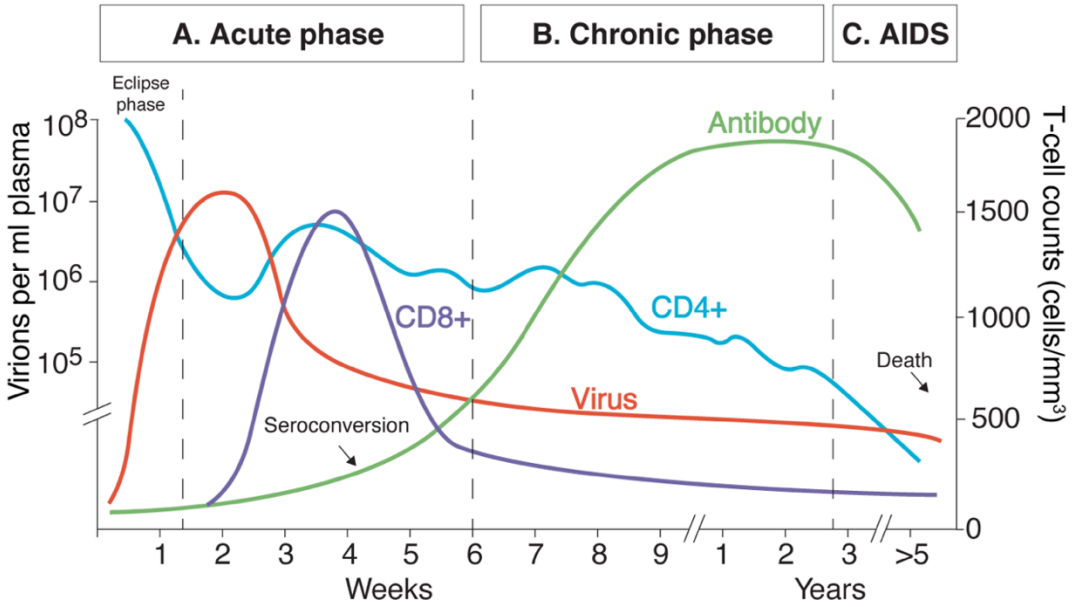

Figure 4. The natural course of HIV-1 infection. **A.** After viral exposure, viral load peaks at 2 to 3 weeks with a consequent decline in CD4+ T-cell counts. HIV-1-specific CD8+ T-cell count peaks at 4 to 5 weeks and humoral responses at 12 weeks and both reduce plasma viremia. If neutralizing antibodies appear, they do so after 3 to 6 months of infection. **B.** In the course of the chronic phase, CD4+ T-cell counts progressively decline and viral loads remain stable. **C.** Finally, after 8-10 years AIDS is manifested in the absence of cART. Image adapted from Goulder *et al*., 2004, using Adobe Illustrator CC 2018 [52].

Following the peak of the virus in circulation and after the first wave of innate immunity, adaptive immunity emerges to further control viral replication. The components of the adaptive immune responses include the production of antibodies and the expansion of virus-specific T-cell responses. As a result, plasma viremia is reduced to the viral set point and CD4+ T-cell counts return to lower levels than those present at the beginning of infection.

Chronic phase

This phase is generally characterized by clinical latency and can last from one to ten years [15], [27], [50], [53]. In this period, circulating CD4+ T-cell counts progressively decline and viral loads remain stable (**Figure 4B**). However, as HIV-1 continues to replicate at the level of viral reservoirs, the persistent antigen exposure leads to sustained immune activation and, consequently, HIV-1 leads to progressive loss of effector functions of CD8+ T cells, which causes a lack of immune control. This exhausted state is described in the following sections.

AIDS

After the chronic infection phase (**Figure 4C**), the HIV-1-infected individual develops long-term impaired immunity and severe immunodeficiency, which is concomitant with a gradual increase in viremia and a drop in CD4+ T-cell counts (50-100 cells/mm^3) [27], [50]. Subsequently, opportunistic illnesses appear, including concomitant infections and cancers and life expectancy falls to about one to three years [54].

This scenario can now be completely altered by the introduction in HIV-1-infected individuals of cART, which modifies the natural course of HIV-1 infection. cART reduces viral load to <50 copies/ml, with the result that morbidity, mortality, and viral transmission are also reduced. In addition, normal CD4+ T-cell counts can be recovered, and life expectancy becomes similar to that of uninfected individuals [55]. Nevertheless, cART still faces several challenges and drawbacks, which will be detailed in subsequent sections.

3. Immune responses in HIV-1 infection

Similar to other pathogens, HIV-1 infection induces both innate and adaptive immune responses. While innate immunity is non-specific and immediate and provides the first line of defense against pathogens, adaptive immunity is highly specific and long-lasting and provides the second line of defense. **Figure 5** provides an overview of both innate and adaptive immunity during HIV-1 infection.

3.1. Innate immunity against HIV-1 infection

Innate immunity is the best-conserved arm of the immune system and generates a rapid and non-specific inflammatory response. The cellular components of innate immunity

are mainly leukocytes, including basophils, eosinophils, Langerhans cells, mast cells, and antigen-presenting cells (APCs) such as monocytes, macrophages, neutrophils, and DCs. Other cells involved in innate responses are the so-called Natural Killer (NK) cells, which are lymphocytes without antigen specificity. For activation, innate cells use Pattern Recognition Receptors (PRR) that identify conserved molecular structures in the surface of pathogens such as pathogen- and damage-associated molecular patterns (PAMPs and DAMPs, respectively).

In HIV-1 infection, innate immunity plays an important role during the first week (**Figure 5A**). The HIV-1 particle is sensed by several PRRs, including Toll-like-receptors (TLRs), C-type lectin receptors (CLRs), and cytosolic DNA receptors (CDRs) expressed in APCs [56]–[59]. Also, host restriction factors are important to limit the HIV-1 replication, of which some of the best-studied are APOBEC3, TRIM5α, SAMHD-1, and Tetherin [58], [59].

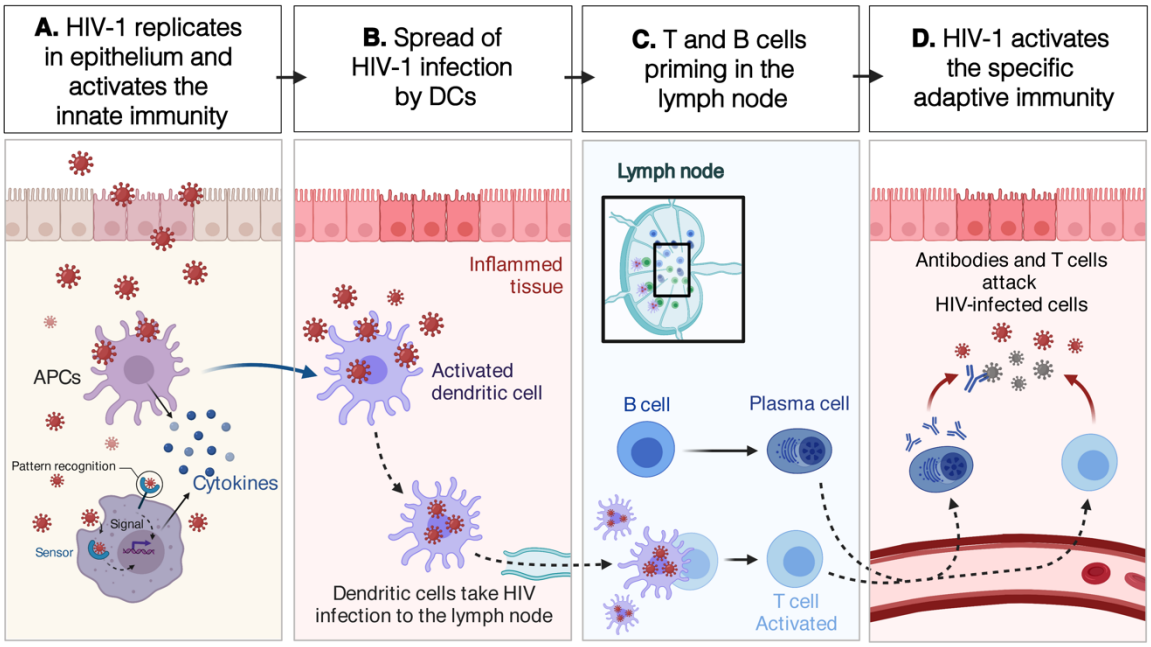

Figure 5. Immune responses to HIV-1 infection. A. Innate immunity uses pattern recognition receptors to induce immune activation and antiviral properties. **B.** DCs take the viral particles and the virus starts to disseminate. **C.** In the lymph node, T and B cells recognize the HIV-1-specific antigens and adaptive immunity against HIV-1 is activated. **D.** Antibodies and T cells attack HIV-1-infected cells. Image created with Biorender and Adobe Illustrator CC 2018.

Once the aforementioned PRRs recognize HIV-1 PAMPs or DAMPs, innate cells such as DCs capture the viral particles and the virus starts to disseminate (**Figure 5B**). In addition, DCs produce inflammatory cytokines or interact with other cells to activate the adaptive immune system. Innate and adaptive immune systems work together to control the HIV-1 replication [58], [59].

3.2. Adaptive immunity against HIV-1 infection

Once HIV-1 antigens are presented by DCs to lymphocytes at the lymph node (**Figure 5C**), activated lymphocytes remain either in the lymphoid organs to help activation or migrate to the sites of viral infection to orchestrate more cells for virus elimination. B cells produce antibodies, CD8+ T cells directly destroy HIV-1-infected cells and CD4+ T cells regulate CD8+ T-cell and B-cell functions, as explained in the following sections (**Figure 5D**).

B-cell responses

As soon as HIV-1 particles are captured by DCs and transported to B-cell areas, the process of B-cell activation is initiated. B cells recognize HIV-1 antigens by the B-cell antigen receptor (BCR) engaging T-cell-independent or -dependent humoral responses. Briefly, T-independent humoral responses are generated by the cross-link of HIV-1 PAMP or DAMP signals recognized by BCRs to initiate the activation of B cells. By contrast, T-dependent humoral responses require the interaction with HIV-1-specific CD4+ helper T cells to be activated and clonally expanded (**Figure 6A**).

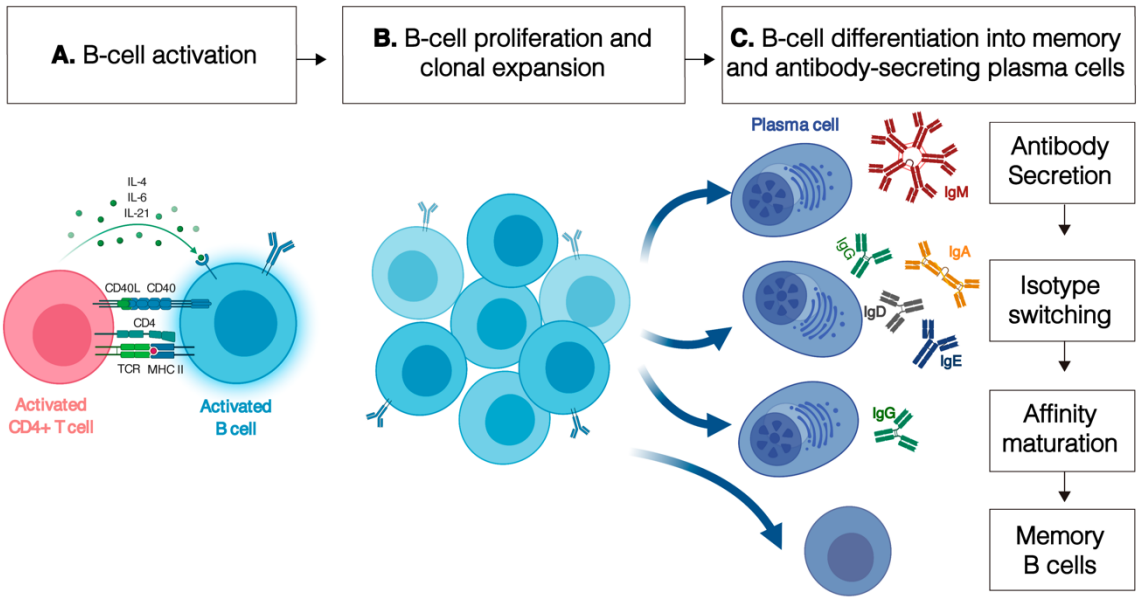

Figure 6. T-cell-dependent humoral responses. A. B-cell activation is initiated by specific recognition of HIV-1 antigens in the context of CD4+ T cells. **B.** Once B cells are activated, they proliferate and clonally expand. **C.** Progeny of the clone may differentiate into plasma cells that produce IgM, may undergo class switching and affinity maturation, or may persist as memory cells. Image adapted from Abbas, Cellular and Molecular Immunology 9th ed., 2018, using Biorender and Adobe Illustrator CC 2018 [60].

Once B cells are activated and expanded, part of the progeny differentiate into short-lived antibody-secreting plasma cells and others undergo affinity maturation (**Figure 6B**) [61], [62]. The affinity maturation process results in somatic mutations for the

immunoglobulin heavy and light chain genes to generate polyclonal antibodies with different affinities targeting HIV-1. As a result, B cells with the highest affinity for HIV-1 are selected to survive in the form of memory B cells and also differentiate into antibody-secreting plasma cells (**Figure 6C**). In addition, activated B cells can switch to different immunoglobulin isotypes which can mediate various effector functions [61], [62].

HIV-1-specific humoral responses start to be detectable within five to nine weeks after infection and mainly target the envelope region. Anti-HIV-1 antibodies against Gag and Pol products are also found. The first antibodies are IgM, IgG, and IgA isotypes and are characterized by their low neutralization capacity and consequently low contribution to viral control [63], [64]. After two to three months post-infection with multiple rounds of antibody selection and extensive somatic hypermutations, HIV-1-specific B cells produce broadly neutralizing antibodies (bNAbs) to neutralize multiple strains of HIV-1. Only about 15% to 20% of chronic HIV-1-infected individuals develop bNAbs [65]. During recent years, the identification and isolation of bNAbs have made it possible to identify vulnerable regions of HIV-1 as represented in **Figure 7**.

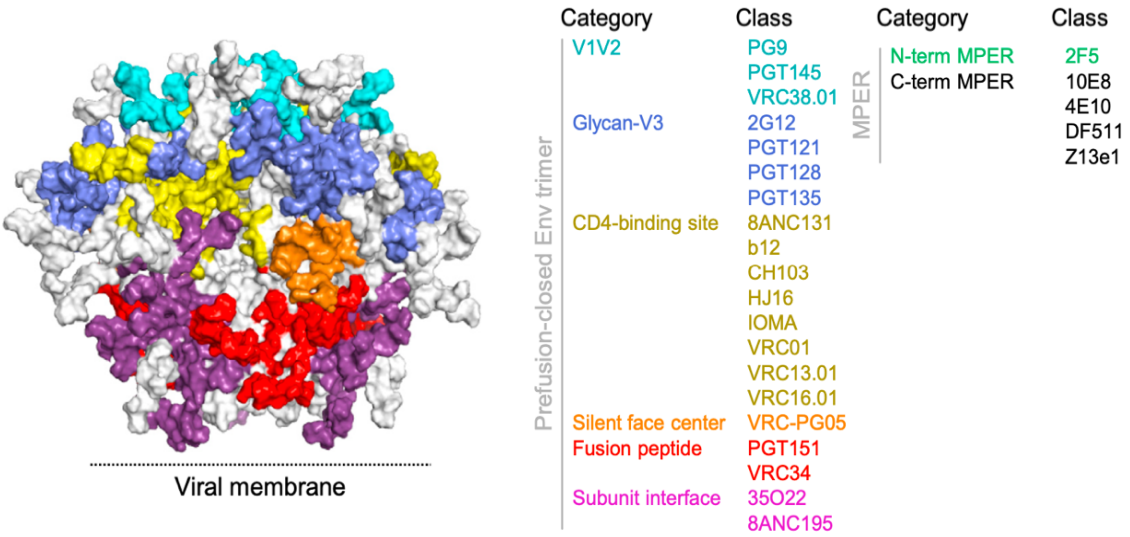

Figure 7. Categories and classes of bNAbs. The image at left shows the major sites of vulnerability in the molecular surface of gp41-gp120 trimer which are colored according to the category. The table at the right shows the categories defining the epitope and the 25 classes of neutralizing antibodies. Image adapted from Zhou *et al*., 2018, using Adobe Illustrator CC 2018 [66].

These bNAbs target specific sites including the V2 site, the N332 supersite, the CD4 binding site, the gp41-gp120 interface, the membrane-proximal external region (MPER), the silent face center, and the fusion peptide (**Figure 7**) [66], [67]. However, although a lot of work is being performed to try to stimulate this potent response, no immunogen to date has been able to mimic the generation of bNAbs *in vivo*.

HIV-1-specific T-cell responses

Aside from the humoral responses, a critical part of the adaptive immune system is the activation of T-cell responses. The adaptive immune responses are mainly mediated by CD4+ and CD8+ T cells. HIV-1-specific CD4+ T cells mediate immune response through the secretion of specific cytokines involved in the activation of innate immune cells, B cells, and CD8+ T cells, as well as nonimmune cells. By contrast, HIV-1-specific CD8+ T cells play a major role in the killing of HIV-1-infected cells.

For the purposes of this thesis, we will summarize the importance of CD8+ T cells in HIV-1 control in this paragraph and their functional regulation in the following section. Over the last decade, a continuous series of scientific publications have demonstrated the temporal correlation between the appearance of HIV-1-specific CD8+ T-cell responses and the partial control of viral replication after acute infection [68]–[72]. These observations have been further supported by studies in SIV-infected rhesus macaques where depletion of CD8+ T cells resulted in an increase in plasma viremia and repopulation with CD8+ T cells was associated with the reestablishment of SIV control. [73], [74]. Moreover, particular HIV-1-specific CD8+ T cells restricted by certain human leukocyte antigens (HLA) are associated with slow disease progression and natural control of HIV-1 replication [15], [27], [50], [75]–[77]. Further evidence centers around HIV-1 mutational escape from HIV-1-specific CD8+ T-cell responses. The fact that viral escape mutations evade the function of HIV-1-specific CD8+ T cells, even at the cost of defective replicative capacity, suggests that CD8+ T cells directly affect HIV-1 replication [75], [78], [79].

Therefore, based on this fundamental role of CD8+ T cells in controlling HIV-1 infection and its importance for this thesis, in the following sections, we will focus on CD8+ T cells. In this context, we provide a general description of CD8+ T-cell activation and differentiation as well as a summary of the regulation of HIV-1-specific CD8+ T cells through inhibitory receptors (IRs).

3.3. Activation and differentiation of HIV-1-specific CD8+ T cells

The generation of HIV-1-specific CD8+ T cells depends on a complex network of cellular signaling and molecular pathways for activation and also differentiation.

Once formed in the bone marrow, T progenitor cells migrate to the thymus to mature and become T cells. This is characterized by the acquisition of T-cell receptors (TCRs) and CD8 receptors. CD8+ T cells previously undergo a rearrangement of the

chromosomal V(D)J segments to generate unique TCRs which can recognize highly specific antigens presented by HLA (**Figure 8A**) [80]–[82].

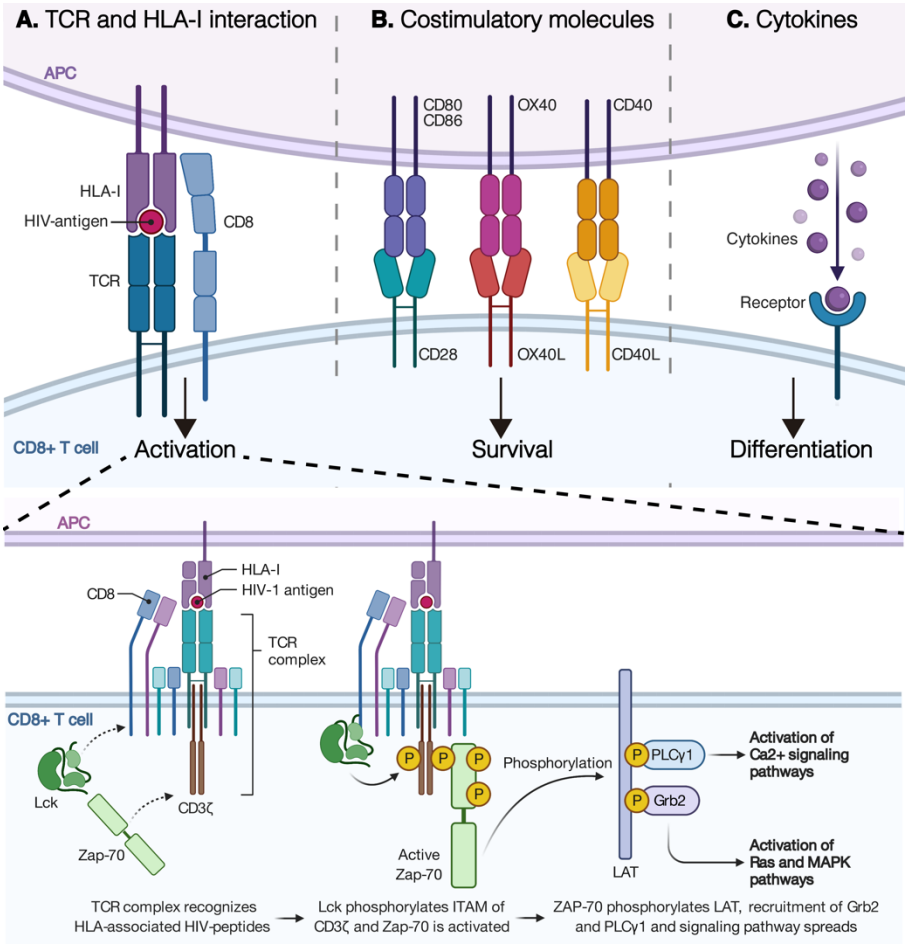

Figure 8. Mechanisms of CD8+ T-cell activation. A. TCR from CD8+ T cells recognizes HIV-1 epitopes presented by HLA-I. After viral recognition, Lck kinase phosphorylates ITAM. Then, Zap-70, which can phosphorylate LAT, is recruited and activated. After that, LAT becomes docking for PLCγ1 and Grb2, which spread the signaling by the activation of Ca^{2+}, Ras, MAPK, and NF-κB pathways [74], [77]. **B.** Costimulatory molecules together with the TCR activation prevent the anergy state. **C.** Inflammatory cytokines are fundamental to complete CD8+ T-cell activation and activate cellular differentiation. The major surface receptors of CD8+ T cells and the ligands on APCs are shown. Image adapted from Abbas, Cellular and Molecular Immunology, 9th ed., 2018, using Biorender and Adobe Illustrator CC 2018 [60].

HLAs are surface-expressed molecules on APCs and are encoded by the major histocompatibility complex (MHC) genes located in chromosome six [83]. HLAs are divided into HLA class I (-A, -B, and -C) and class II (-DP, -DM, -DOA, -DOB, -DQ, and -DR). CD8+ T cells can recognize the peptides of eight to ten amino acids of length from HLA-I presentation [75], [76], [83]. In the context of an antiviral immune response in HIV-1 infection, as soon as TCR from a CD8+ T cell binds to HLA-associated HIV-1-specific peptides, immunoreceptor tyrosine-based activation motifs (ITAM) are phosphorylated and T-cell signaling promotes the activation of CD8+ T cells as represented in **Figure 8A** [82], [84]. Together with the TCR signaling, costimulatory molecules are also

essential to complete the CD8+ T-cell activation, which is fundamental for survival and preventing cellular anergy and prolonged unresponsiveness (**Figure 8B**). CD28, one of the most thoroughly studied costimulatory molecules, binds to B7-1 (CD80) and B7-2 (CD86). CD40, OX40, and other molecules such as the integrins are also involved in CD8+ T-cell activation with the generation of the immunological synapse (IS), which constitutes a physical stabilization between CD8+ T cells and APCs [60]. Inflammatory cytokines also act directly on the responding CD8+ T cells to regulate their expansion and differentiation (**Figure 8C**). In particular, both type I interferons (IFN-I) and interleukin 12 (IL-12) have been described as critical survival signals for CD8+ T-cell expansion [85].

CD8+ T-cell differentiation is essential to provide protection for HIV-1 infection and to generate a successful antiviral memory response. Once CD8+ T cells are fully activated upon antigen recognition (**Figure 9A**), CD8+ T cells pass through subsequential stages of cellular differentiation to generate effector and memory CD8+ T cells.

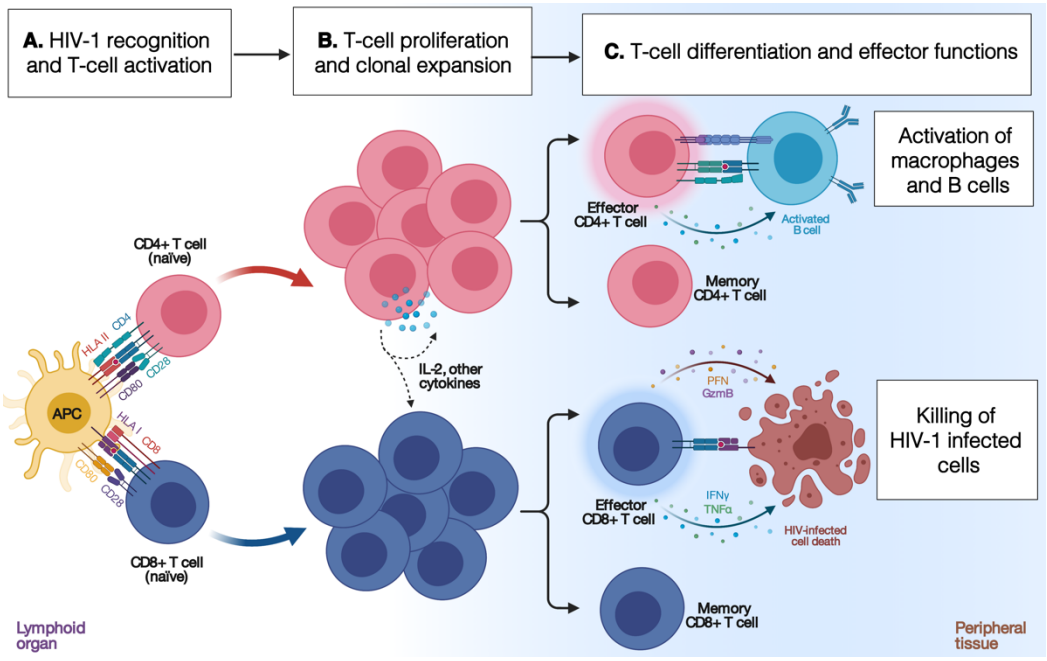

Figure 9. T-cell activation and differentiation. A. HIV-1-antigen recognition and full T-cell activation. **B.** T-cell proliferation and clonal expansion. **C.** T-cell differentiation into effector and memory T cells. CD4+ T cells are represented in pink and CD8+ T cells in blue. Image adapted from Abbas, Cellular and Molecular Immunology, 9th ed. 2018, using Biorender and Adobe Illustrator CC 2018 [60].

The expansion phase is initiated in the lymphoid tissues and naïve HIV-1-specific CD8+ T cells proliferate and clonally expand (**Figure 9B**). After clonal expansion, naïve HIV-1-specific CD8+ T cells enter a transitional stage and differentiate into effector and

memory CD8+ T cells with the consequent cellular reprogramming to complete the adaptive immune responses against HIV-1 (**Figure 9C**).

HIV-1-specific effector (Eff) CD8+ T cells have a reduced potential for homing to lymph nodes owing to their decreased expression of lymph-node-homing receptors such as CCR7 and L-selectin (CD62L) and a greater capacity to migrate to inflamed tissues owing to their increased expression of CCR5 and CCR2. Based on transcriptional programs, HIV-1-specific Eff CD8+ T cells are characterized to express T-bet, Blimp-1, and STAT4, which are associated with a reduction in proliferative capacity and longevity. Moreover, Eff CD8+ T cells are cytotoxic and secrete lytic granules to induce the killing of HIV-1-infected cells [86]. These lytic granules contain perforins and granzymes. While perforins facilitate the delivery of granzymes into the target cell, granzymes cleave the pro-caspase 3 to initiate the apoptosis of the target cell. In addition, the membrane of these granules contains lysosome-associated membrane glycoproteins (LAMP) such as LAMP-1 (CD107a), which is a marker that is widely used to detect cytolytic activity in CD8+ T cells [87].

By contrast, HIV-1-specific memory CD8+ T cells express different patterns of surface proteins involved in cell adhesion and chemotaxis such as IL-7R and CXCR3. These receptors allow the cells to extravasate into non-lymphoid tissues and mucosal sites waiting to find the same HIV-1 antigens that were activated. Based on transcription factors, HIV-1-specific memory CD8+ T cells are characterized by their expression of Eomesodermin (Eomes), BCL-6, and STAT3, which prevent the terminal differentiation into Eff CD8+ T cells. The mechanisms that determine CD8+ T-cell differentiation in memory cells are still poorly understood. Several studies suggest that the balance between the transcription factors T-bet or Eomes could influence the choice between the development of Eff or memory CD8+ T cells [88]–[94].

Conventionally, studies have defined subsets of memory CD8+ T cells according to their phenotypic markers, anatomical locations, and functions. Central memory (CM) CD8+ T cells constitute the main pool of memory cells and have limited capacity to perform effector functions upon antigen presentation. These are prone to home mainly on lymph nodes because they express the $CCR7^{high}$ homing marker and the costimulatory molecule $CD27^{high}$. Effector memory (EM) CD8+ T cells, on the other hand, are characterized by $CCR7^{low}$ $CD27^{low}$ and mainly express chemokine receptors for

migration to peripheral tissues. Under stimulation, EM CD8+ T cells rapidly become cytotoxic, proliferate and produce cytokines such as IFNγ. Another cell type is the transitional memory (TM) CD8+ T cell, which displays an intermediate phenotype between CM and EM CD8+ T cells. TM CD8+ T cells constitute a pool of highly proliferative and polyfunctional cells capable of generating high numbers of Eff CD8+ T cells [91]–[93].

Nevertheless, as a consequence of continuous HIV-1 antigen exposure and chronic immune activation, memory and Eff CD8+ T-cell populations progressively lose functions and some are even physically deleted. In the following section, we will introduce the role of inhibitory receptors (IRs) in the regulation of HIV-1-specific CD8+ T-cell function and also in CD8+ T-cell exhaustion.

3.4. Regulation of HIV-1-specific CD8+ T cells and exhaustion

In response to pathogens, CD8+ T cells are activated to clear the infection and generate immunological memory. However, this process is different between pathogens that can be eliminated (i.e., acute infection) and those that persist in the body for prolonged periods (i.e., chronic infection). For example, in acute infections such as flu, naïve CD8+ T cells are activated and differentiate into cytotoxic Eff CD8+ T cells to control and eliminate the pathogen. These Eff CD8+ T cells are characterized to express high levels of IRs driving the control of T-cell overactivation (**Figure 10, black line**). Once the antigen is cleared, most Eff CD8+ T cells undergo massive apoptosis while a minority survive and differentiate into long-lived memory CD8+ T cells with down-modulation of the expression of IRs for homeostasis (**Figure 10, black line**) [70]. By contrast, in face of persistent antigen stimulation such as HIV-1 infection, tumors, or other chronic viral infections, CD8+ T cells initially acquire effector functions. However, the duration of activation progressively impacts their function and consequently, CD8+ T cells are found to degenerate into a special hyporesponsive state known as T-cell exhaustion (Tex). HIV-1-specific CD8+ T cells become dysfunctional with high expression of IRs and consequently, fail to continuously control chronic HIV-1 infection (**Figure 10, dark blue line**). Although cART administration can normalize the levels of IRs, their expression remains high in HIV-1-specific CD8+ T cells and contributes to Tex (**Figure 10, light blue line**) [95]–[101].

Multiple extrinsic and intrinsic factors have been put forward to account for the possible mechanisms of Tex, including the expression of IRs. The phenomenon of Tex was first

described in mice with chronically lymphocytic choriomeningitis virus clone 13 (LCMV C13) infection in 1993 [102]. Tex is characterized by numerous functional defects of CD8+ T cells [103]–[106], including; I) impairment of proliferation, II) reduction of inflammatory cytokines such as IL-2, TNF, and IFNγ, III) decrease of cytotoxic activity, IV) alteration of cell adhesion molecules, thus changing the migratory properties, V) upregulation of transcription factors such as Eomes and Blimp-1 and also downregulation of T-bet and Fos and, finally, VI) a sustained expression of IRs.

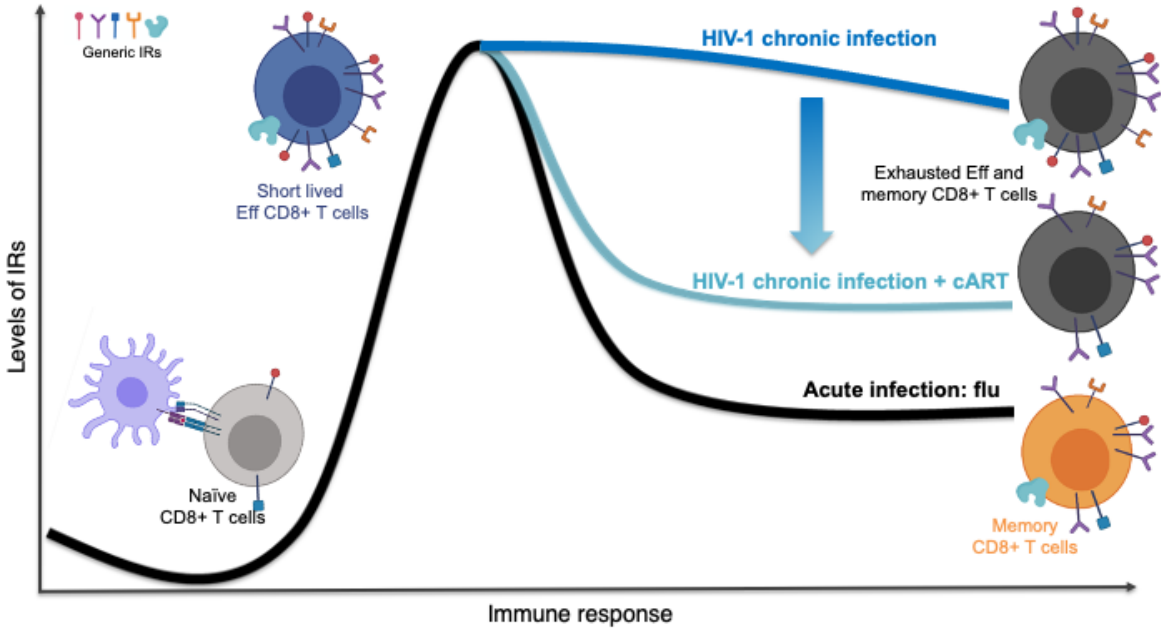

Figure 10. Regulation of IR expression and development of HIV-1-specific CD8+ T cells. Different scenarios of IR expression depend on the type of infection. In acute infection (black line), memory CD8+ T cells down-modulate the expression of IRs for homeostasis. In HIV-1 infection (blue line), levels of IRs remain high in Eff and memory CD8+ T cells and HIV-1-specific CD8+ T cells become exhausted. cART administration (light blue line) normalizes the expression of IRs but levels remain high. Image adapted from Fuertes Marroco *et al*. 2015, using Biorender and Adobe Illustrator CC 2018 [106].

IR definition and its role in HIV-1 infection and T-cell exhaustion

IRs were first described in NK cells in the early 1990s. At that time, IRs were defined by the expression of immunoreceptor tyrosine-based inhibitory motif (ITIM), which recruits phosphatases to attenuate the downstream signals of costimulatory molecules [107], [108]. Today, the definition has been refined and IRs are defined as inhibitory molecules that negatively interfere in cellular activation and function through several mechanisms; I) by competing with costimulatory molecules for the same ligands, II) by interfering with downstream signals and III) by upregulating genes involved in dysfunction [106], [109].

In HIV-1 infection, the expression of IRs can be a hallmark of Tex, showing dysfunctional HIV-1-specific CD8+ T cells. However, the role of IRs goes beyond exhaustion. In a “tide

model", the expression of co-signaling molecules including IRs and costimulatory molecules is tightly regulated and they cooperate to shape the inflammatory responses to limit damage in the surrounding areas. In this context, IRs are found in physiological immune processes and act as a mechanism to balance the activity of CD8+ T cells to ensure immune homeostasis [110]. For instance, in LCMV-specific CD8+ T cells from mice, the expression of IRs such as PD-1 in healthy humans was not significantly correlated with Tex gene signatures and did not directly affect cytokine secretion [111]. Moreover, the absence of PD-1 does not reverse Tex in tumor-infiltrating CD8+ lymphocytes (TILs) during tumorigenesis [112]. In addition, there is increasing evidence that IRs connect with the metabolic activity and are responsible for 'switching' from oxidative phosphorylation to aerobic glycolysis to enable cellular energy requirements for CD8+ T-cell activation. Indeed, PD-1 signaling modulates the glucose and glutamine catalysis generated after TCR and CD28 activation [113]. Further evidence is provided by the opposite effects of IRs depending on the molecules that participate. For example, TIM-3 expression inhibits the functionality of CD8+ T cells only when they interact with the ligand CEACAM-1 [114].
In addition to these insights, some of the strongest evidence for the connection between IR expression and cellular differentiation is the preferential expression tightly linked to the differentiation status of CD8+ T cells [106], [110]. Thus, the overlap of IRs between dysfunction and activation complicates the identification of CD8+ Tex cells. As a result, IR expression alone may not be sufficient to distinguish CD8+ T-cell activation from Tex as there is an activation-dependent exhaustion program.

In terms of structure and function, IRs are heterogeneous proteins in structure, ligand recognition, and function. However, the contribution of IR expression to the regulation of HIV-1-specific CD8+ T cells remains to be fully understood. Based on current data, we have summarized a subgroup of IRs important to understand the following chapters of this thesis. IRs including PD-1, TIGIT, LAG-3, TIM-3, and CD39 have been involved in CD8+ Tex in HIV-1 infection. **Table 1** and **Figure 11** provide an overview of the current knowledge.

PD-1 (Figure 11A)

The high expression of PD-1 is a marker of functional impairment of HIV-1-specific CD8+ T cells. The level of PD-1 expression correlates with impaired function, as well as with plasma viral load and inversely with CD4+ T-cell counts. Indeed, in untreated HIV-1-

infected subjects and SIV-infected macaques, several studies have revealed a direct correlation between PD-1 expression in HIV-1 or SIV-specific CD8+ T cells and viral load [101], [115], [116]. Moreover, co-expression of PD-1 with other IRs in CD8+ T cells has been strongly correlated with both viral load and impaired cytokine production [96], [117], [118]. Also, PD-1 expression in CD4+ T cells has been directly correlated with HIV-1 DNA levels [119].

TIGIT (Figure 11B)

High levels of TIGIT are present in blood and lymphoid tissue in Eff CD8+ T cells during SIV infection. Moreover, TIGIT has been described as a marker of Tex and dysfunction in HIV-1 and SIV-specific CD8+ T cells [100]. Co-expression of TIGIT with other IRs has been shown to impair proliferation and decrease cellular metabolism, cytokine production, and degranulation capacities in CD8+ T cells [100], [117]. Several studies have demonstrated that increased frequencies of TIGIT but also TIGIT co-expressed with PD-1 in CD8+ T cells correlate with T-cell activation, plasma viral load, and CD4+ T cell-associated HIV-1 DNA, suggesting that TIGIT is involved in HIV-1 disease progression [100]. Like PD-1, TIGIT in CD4+ T cells is directly correlated with integrated HIV-1 DNA [119].

LAG-3 (Figure 11C)

The expression of LAG-3 in HIV-1-specific CD8+ T cells has been directly correlated with high viral loads and time to viral rebound following cART interruption [120]. Moreover, some data support the role of LAG-3 expression in the generation of HIV-1 sanctuaries in CD4+ T cells and the establishment of viral latency [119]. LAG-3 inhibits NF-κB and NFAT transcription factors which both promote the HIV-1 transcription in CD4+ T cells [121].

TIM-3 (Figure 11D)

In untreated chronic HIV-1 infection, the expression of TIM-3 in CD8+ T cells has been associated with CD4+ T-cell loss [122], high viral load, and also high CD38 expression [123]. In addition, co-expression of TIM-3 with PD-1 in CD8+ T cells has been inversely correlated with CD4+ T-cell counts [122]. In progressive HIV-1 infection, HIV-1-specific CD8+ T cells expressing TIM-3 failed to produce cytokines or proliferate in response to antigen [123]. Moreover, TIM-3 expression in CD4+ T cells dampens viral replication and potentially facilitates the persistence of reservoirs [124]. However, the role of TIM-3 in T-

cell function is still controversial as its expression in the LCMV C13 model has been associated with optimal Eff CD8+ T-cell responses [125].

CD39 (Figure 11E)

The expression of CD39 in HIV-1-specific CD8+ T cells correlates with terminally exhausted phenotype showing Eomes^{high} and T-bet^{low} [126]. CD39 can be co-expressed with PD-1 and LAG-3 in HIV-1-specific CD8+ T cells, resulting in the reduction of IFNγ and TNF production. Moreover, high expression of CD39 in CD8+ T cells correlates with viral load in both hepatitis C virus (HCV) and HIV-1 infection [126]. Of note, CD39 expression seems to be involved in mediating CD8+ T-cell suppression through the activation of regulatory CD4+ T cells in HIV-1 infection. In this context, a recent study showed significantly increased HIV-1 DNA levels in regulatory CD4+ T cells expressing CD39 from patients with advanced HIV-1 infection, suggesting that CD39 may constitute a viral reservoir and contribute to HIV-1 persistence [127].

Based on the fundamental role of IRs in the regulation of HIV-1-specific CD8+ T cells and also in Tex, the manipulation of IRs through therapeutic strategies may be essential in the search for a cure for HIV-1. Thus, the need to reverse the Tex state in HIV-1-infected individuals may be key for the success of curative strategies. In the following section, we provide an overview of current challenges to curing HIV-1 as well as the most recent advances, positioning the blockade of IRs as a promising new therapeutic strategy to boost the immune system.

Table 1. PD-1, TIGIT, LAG-3, TIM-3 and CD39.

	PD-1 (Figure 11A)	**TIGIT** (Figure 11B)	**LAG-3** (Figure 11C)	**TIM-3** (Figure 11D)	**CD39** (Figure 11E)
Full name/ other names	Programmed cell death protein 1, PDCD-1 or CD279 [128].	T-cell immunoglobulin and ITIM domain or Vstm3 [129].	Lymphocyte-activation gene-3 or CD223 [129].	T-cell immunoglobulin-3, CD366 or HAVCR2 [130].	Ectonucleoside triphosphate diphosphohy-drolase 1 or ENTPD1 [126].
Year of discovery	1992 [131].	2009 [132], [133].	1990 [129].	2002 [130].	1996 [126].
Cell type expression	CD4+ and CD8+ T cells, B cells, monocytes, NKs, DCs, myeloid cells and cancer cells [134], [135].	CD4+ and CD8+ T cells, and NKs [136]–[138].	CD4+ and CD8+ T cells, B cells, DCs and NKs [137].	CD4+ and CD8+ T cells, NKs, monocytes, macrophages and DCs [137], [138].	CD4+ and CD8+ T cells, B cells, monocytes, NKs, vascular endothelial cells, fibroblasts and tumors [139].
Receptor family	CD28 Ig receptor [110].	PVR [140].	CD4 co-receptor [129].	TIM family [137].	Ecto-enzyme [126].
Structure	IgV-like, transmembrane, ITIM and ITSM [128].	IgV, transmembrane, ITIM and ITT-like motif [132], [133], [137], [138], [140].	Ig-like and transmembrane domain and KIEELE motif [120], [141]	IgV and transmembrane domains, a mucin stalk with glycosylation sites and 5 conserved Tyr residues [129], [137].	Two transmembrane domains and a hydrophobic region, Ca^{2+} and Mg^{2+} dependent manner [139], [142].
Action	Antagonize TCR [128].	Competing for the same ligands. Unknown signaling [132], [133], [137], [138], [140].	KIEELE phosphorylation but unknown signaling in CD8+ T cells [120], [141]	Not fully understood, balance of Bat3 and Fyn may a key determinant [129], [137].	Adjust ATP/adenosine and fine-tune the function of immune cells [139], [142].
Ligands (cell types expression)	• PD-L1 (CD4+, CD8+, B cells, DCs, macrophages, stem, mast cells and tumor). • PD-L2 (DCs, macrophages, mast cells and tumor) [106], [128], [134], [135], [143].	• CD155 (DCs / tumor) • CD112 (DCs / tumor) • Fusobacterium nucleatum (colorectal carcinomas) [136]–[138].	• HLA- II (APCs /tumor) • LSECtin (stromal) • Gal-3 (tumor) • FGL-1 (tumor) [137], [138], [144]–[146].	• Gal-9 (tumor) • PtdSer (apoptotic cells) • HMGB1 (tumor) • CEACAM-1 (T cells/tumor) [114], [129], [137], [138].	• eATP • eADP [139], [142].
Other information	Control T-cell tolerance [128], influenced by cytokines (IL-2, IL-21, IFN-I) [147] and Nef in vitro [148]. Expressed in TILs in melanoma [117], [128].	Important in autoimmunity and tolerance [149], high expression associated with progression in melanoma, NSCLC [117], [150].	Required in homeostasis and autoimmunity [151]. influenced by cytokines (IL-2, IL-21, IFN-I) [147] and T-bet or Eomes [152].	Important for tolerance [153], poor disease prognosis in cancer [154], essential for Eff CD8+ T cells [125].	New immune checkpoint mediator, function with CD73, expression associated with tumor progression [139], [155], [156].

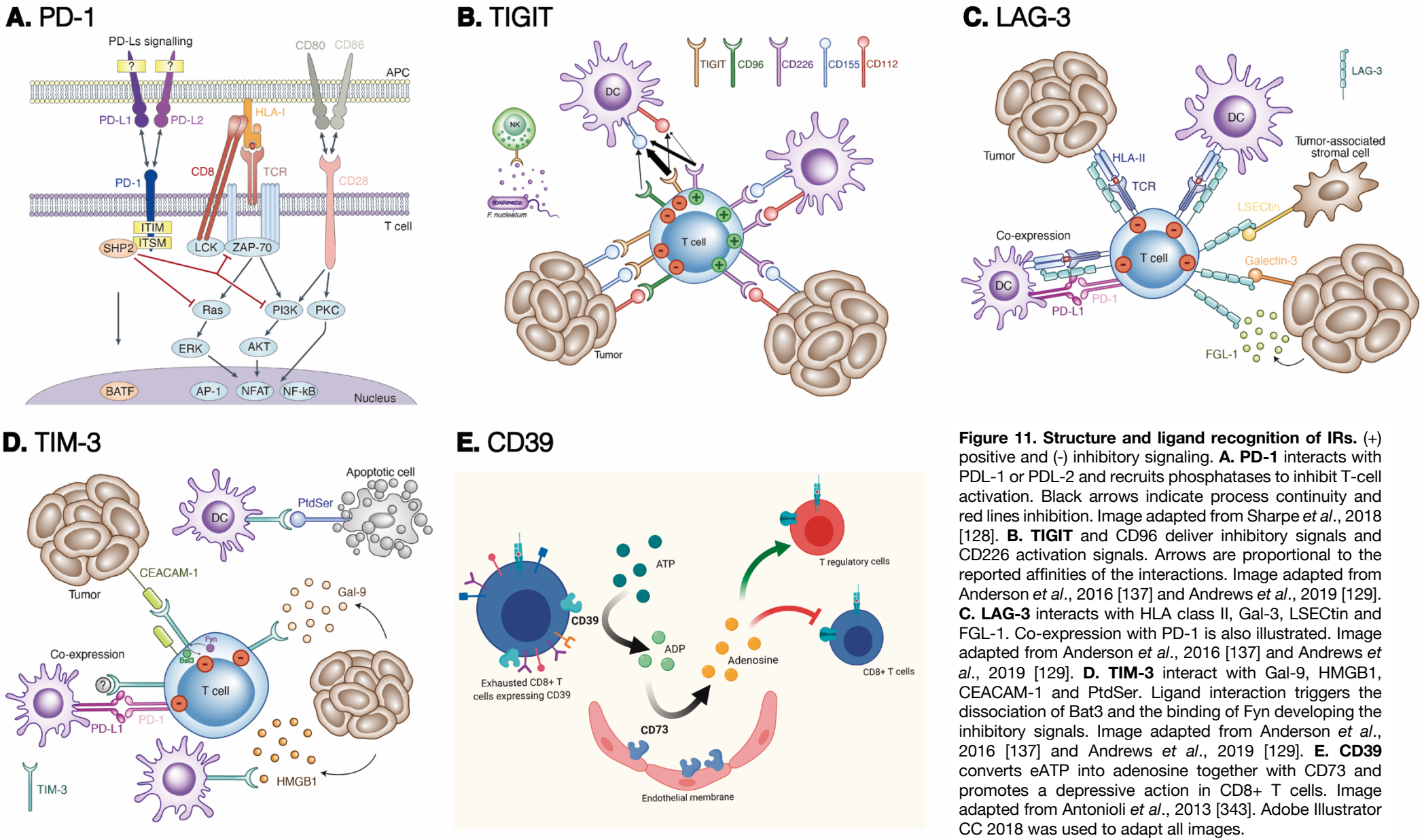

Figure 11. Structure and ligand recognition of IRs. (+) positive and (-) inhibitory signaling. **A. PD-1** interacts with PDL-1 or PDL-2 and recruits phosphatases to inhibit T-cell activation. Black arrows indicate process continuity and red lines inhibition. Image adapted from Sharpe *et al*., 2018 [128]. **B. TIGIT** and CD96 deliver inhibitory signals and CD226 activation signals. Arrows are proportional to the reported affinities of the interactions. Image adapted from Anderson *et al*., 2016 [137] and Andrews *et al*., 2019 [129]. **C. LAG-3** interacts with HLA class II, Gal-3, LSECtin and FGL-1. Co-expression with PD-1 is also illustrated. Image adapted from Anderson *et al*., 2016 [137] and Andrews *et al*., 2019 [129]. **D. TIM-3** interact with Gal-9, HMGB1, CEACAM-1 and PtdSer. Ligand interaction triggers the dissociation of Bat3 and the binding of Fyn developing the inhibitory signals. Image adapted from Anderson *et al*., 2016 [137] and Andrews *et al*., 2019 [129]. **E. CD39** converts eATP into adenosine together with CD73 and promotes a depressive action in CD8+ T cells. Image adapted from Antonioli *et al*., 2013 [343]. Adobe Illustrator CC 2018 was used to adapt all images.

4. Current challenges to finding a cure for HIV-1

The major goal for the HIV-1 scientific community is to find a cure for the infection since the humoral and cellular responses are unable to eliminate the HIV-1-infected cells from the body. Although cART has modified the natural course of HIV-1 infection and achieved one of the most significant advances in the field, cART cannot cure HIV-1 infection. In the following section, we provide a general description of cART as well as a brief overview of the limitations of cART and other advances in the search for a cure for HIV-1.

4.1. Limitations of cART

The implementation of cART is the most important advance in the clinical management of HIV-1 infection. cART reduces the viral load to undetectable levels (<50 RNA copies/ml) and recovers CD4+ T-cell counts. In addition, the introduction of cART has dramatically reduced viral transmission, morbidity, and mortality. Indeed, the current life expectancy of HIV-1-infected individuals is similar to uninfected individuals [55]. Today, cART comprises more than 30 drugs approved for use by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA). Antiretroviral drugs are divided into six classes following their molecular targets [157]; I) non-nucleoside reverse transcriptase inhibitors (NNRTIs), II) nucleoside reverse transcriptase inhibitors (NRTIs), II) protease inhibitors (PIs), IV) fusion inhibitors, V) entry Inhibitors and VI) integrase strand transfer inhibitors (INSTIs). The standard cART involves combining two NRTIs with INSTIs or NNRTIs as the first line of treatment.

Although the introduction of cART has increased life expectancy and improved the health of people living with HIV-1, cART does not cure HIV-1 infection, and treatment is needed for decades, raising concerns about long-term health and the sustainability of treatment and care of HIV-1-infected individuals. Moreover, cART faces several challenges and drawbacks including the difficulty of providing treatment coverage worldwide [158], the potential emergence of multidrug resistance mutations [79], [159], long-term secondary effects in aging populations [160], incomplete immune restoration after cART [161] and the lack of elimination of the HIV-1 reservoir. When cART is interrupted, HIV-1 viral load usually rebounds within two to eight weeks after treatment interruption independent of the duration of cART [42], [162], [163].

Several strategies have been pursued to try to reduce the size of the viral reservoir. In particular, intensified cART using additional drugs of standard regimens has been

widely explored as a method to reduce residual viral replication. However, no study has shown suppression of residual viremia or reduction in the size of the latent reservoir by cART intensification [164]–[167]. Similarly, early cART administration has been demonstrated to be effective in reducing HIV-1 DNA levels in early treated individuals compared to chronic HIV-1-infected patients who did not receive early cART administration. In 2011, post-treatment HIV-1 control was reported in early treated HIV-1-infected individuals after cART interruption for prolonged periods [168], [169]. In the most well-known case report along these lines, the "Mississippi baby", a perinatally HIV-1-infected baby, was treated with cART within 30 hours after birth, and the virus remained undetectable for the following 27 months in the absence of cART [170]. Despite all this evidence, little is known about the mechanisms that underlie viral control after cART interruption, and this highlights the need to develop novel therapeutic strategies to eradicate HIV-1 from the body.

4.2. HIV-1 cure strategies: from virus reactivation to T-cell immunity

A cure for HIV-1 could potentially take one of two forms, either a sterilizing cure, meaning the complete eradication of the HIV-1 virus from the body, or a functional cure, meaning continuous control of HIV-1 infection by the immune system in the absence of cART. Some of the current HIV-1 cure strategies are summarized in **Figure 12**.

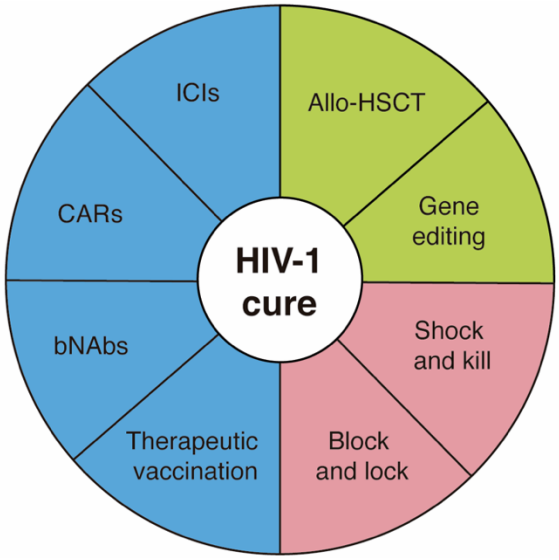

Figure 12. HIV-1 cure strategies. Allo-HSCT and gene editing (green portions) are alternative strategies currently used to achieve the complete elimination of HIV-1. Latency reversal and silencing strategies (pink portions) are curative strategies to purge or block respectively the HIV-1 reservoir. Immunotherapies (blue portions) such as vaccines, bNAbs, CARs, and ICIs are focused on enhancing the humoral and immune responses. Image created with Adobe Illustrator CC 2018.

Allogeneic hematopoietic stem-cell transplantation

The only therapeutic approach that has been shown to have fully eradicated HIV-1 so far is allogeneic hematopoietic stem-cell transplantation (allo-HSCT). Worldwide, only three HIV-1-infected individuals have experienced complete elimination of the virus without signals of infection for over one, two, and ten years after cART interruption [171]–[173]. These "Düsseldorf", "London" and "Berlin" patients went through an allo-HSCT with donor cells homozygous for the CCR5-Δ32 allele, which confers resistance to R5-tropic HIV-1 variants.

Nonetheless, not all allo-HSCT cases have been successful [174], [175]. The "Boston patients" showed a remission after transplantation but virus replication rebounded several weeks after cART interruption [175]. Concerns about implementation have been raised due to the difficulty involved in finding homozygous CCR5-Δ32 donors and the possible appearance of CXCR4 variants, and especially because allo-HSCT has only been performed in HIV-1-infected individuals with leukemia.

Gene editing

In the last few years, advances have been made in gene editing approaches that mimic CCR5-Δ32 homozygosis to induce protection from HIV-1 infection. These genome editing tools include clustered regularly interspaced short palindromic repeats (CRISPR)/ associated protein nuclease 9 (Cas9), zinc-finger nucleases (ZFN), and transcription activator-like effector nucleases (TALENs) [176]. ZFN and CRISPR-Cas9 have been tested through the generation of autologous CCR5 or CXCR4 T cells to mimic the natural CCR5-Δ32 mutation to confer resistance to HIV-1 infection [172], [177]. Aside from targeting host genes, several studies have demonstrated that targeting viral genes including the 2-LTR and Gag can disrupt viral expression [176].

Although the implementation of gene editing approaches in the clinic has shown limited effectiveness in delaying viral rebound after cART interruption [172], a recent phase I clinical trial that infused CCR5 gene-edited CD4+ T cells showed that this strategy caused a delay in the time to viral rebound and restored preexisting HIV-1-specific CD8+ T-cell responses [178]. Therefore, further studies are needed if these strategies are to be implemented as a common cure strategy.

Latency reversal strategy

One of the most prominent approaches in the search for a cure for HIV-1 has been the so-called "shock and kill" strategies. This approach involves the reactivation ("shock")

of latently HIV-1-infected CD4+ T cells using latency-reversing agents (LRAs) to enhance the recognition or death ("kill") of virally infected cells by apoptosis or by the immune system [179]. The first attempts to induce viral gene expression from latency were carried out using IL-2 in 1999. However, no changes in the latent viral reservoir were observed and toxicity was associated with the treatment [180], [181].

Today, however, the safety of LRAs has been improved and LRAs can reactivate latently HIV-1-infected CD4+ T cells by activating transcription factors or altering the chromatin structure of the integrated provirus [182]–[184]. Depending on the mechanism of action, LRAs can be categorized into several groups including epigenetic modifiers consisting of histone deacetylase inhibitors (HDACis), histone and DNA methyltransferase inhibitors (HMTis and DNMTis, respectively), protein PKC agonists, MAPK agonists, CCR5 antagonists, PI3K/Akt inhibitors, immunomodulatory molecules (TLR and IL-15 agonists) and others [185].

The most thoroughly studied LRAs are HDACis, which upregulate the transcription by reversing epigenetic silencing [184]. Several HDACis have been approved by the FDA for use in cancer treatment, including vorinostat (SAHA), panobinostat (PNBN), and romidepsin (RMD). SAHA was the first HDACi tested in suppressed chronic cART HIV-1-infected patients and showed for the first time an increase in HIV-1 transcription *in vivo* [184]. PNBN and RMD also have shown an effect in reversing HIV-1 latency [183], [186]. Moreover, LRAs have been explored in combination with other approaches including vaccine or blockade of IRs [182], [187]–[190].

Although these studies demonstrated that reactivation of HIV-1 by LRAs is possible both *in vitro* and *in vivo* [182]–[184], [190] no measurable reduction in the HIV-1 reservoir has been found to date [191]. Therefore, potential combinations of LRAs with other curative strategies will be needed to reduce the viral reservoir.

Latency silencing strategy

Contrary to latency reversal, the latency silencing or so-called "block and lock" strategy uses latency-promoting agents (LPAs) to preserve and reinforce the epigenetic mechanisms that maintain the HIV-1 in a deep latency state [192]. Whilst this method offers an alternative mechanism to the "shock and kill" strategy, its development is still in the early stages and has yet to be tested in humans. Only one study using didehydro-cortistantin A (dCA) has shown inhibition of viral reactivation and transcriptional silencing [193].

Therapeutic vaccination

In addition to the abovementioned approaches, direct interventions aiming to boost or recover antiviral immunity are also key in the search for a cure to HIV-1. The development of vaccines against HIV-1 will be an essential part of future cure therapeutics because such vaccines are optimally designed to generate a broader and multivalent antiviral immune response. Live attenuated virus, viral-like particles, and plasmid DNA vaccines are some of the prototypes developed for vaccine strategies [179]. The efficacy of a vaccine is assessed by measuring the time to viral rebound after cART interruption, by decreases in the size of the viral reservoir, and also by changes in the frequency and function of T-cell responses.

Although several studies have demonstrated some effect in terms of vaccine efficacy [194]–[197], no vaccine today has yet induced sustained long-term viral remission *in vivo* following cART interruption. Vaccination has also been explored in combination with other approaches including LRAs. In recent clinical trials, HIV-1 vaccines combined with LRA showed a reduction of viral reservoirs and enhanced viral control after cART interruption and also a recovery of immune functionality [182], [187], [198].

Broadly neutralizing antibodies

Like therapeutic vaccination, the development of broadly neutralizing antibodies (bNAbs) against HIV-1 is still underway. Extensive data from non-human primate studies have shown the potential of bNAbs to induce sterilizing protection from SIV challenge [199], [200]. For HIV-1 infection, passive immunization by transfer of bNAbs has been proposed for prevention, virological control, and also eradication [201]. Clinical studies involving bNAbs passive administration of one bNAb have demonstrated suppression of viral replication and delayed viral rebound after cART interruption [202], [203]. However, despite these promising results, viral resistant quasispecies can appear if bNAbs are given in monotherapy.

T-cell engineering of chimeric antigen receptors

T-cell gene therapy products, including T-cell engineering of chimeric antigen receptors (CARs), are currently being explored. The basic concept of CAR-based T-cell therapies involves genetic modifications of the patient's autologous T cells to express a specific CAR for the viral antigen, followed by *ex vivo* cell expansion and re-infusion back to the patient ([204]. Tools implying genetic editing are commonly used to merge protein fragments from either specific antibodies or intracellular signaling domains from TCRs

[205]. CARs, which can redirect CD8+ T cells to the HIV-1 envelope, CD4+ and CD8+ T cells, have been the subject of considerable study. However, despite promising results in treating acute lymphocytic leukemia [206], research into the use of CARs for HIV-1 is limited to a few clinical trials [207]. One study using CAR-CD8+ T cells engineered to recognize HIV-1-specific antigens did not show changes in viremia [207]. PD-1- or CTLA-4-based-CARs are also being investigated in cancer research and have shown to be effective in controlling functionality [206]. Several obstacles remain to be addressed and new CAR engineering strategies for HIV-1 are needed to enable an effective and safe clearance of the viral reservoir.

Immune checkpoint inhibitors

Together with other approaches, the use of immune checkpoint inhibitors (ICIs) or anti-IRs (αIR) is also being explored in the search for a cure for HIV-1. The use of ICIs has demonstrated their clinical efficacy in the treatment of cancer and hundreds of clinical trials have been carried out to evaluate the efficacy of ICIs as monotherapy or in combination. The use of ICIs has been demonstrated to bring about complete remission in a fraction of cancer patients [223], [224].

Over the past decade, the use of ICIs has made a breakthrough in treating solid cancers. Ipilimumab (αCTLA-4), the first ICI approved by the FDA in 2011, has been shown to enhance CD8+ T cells against melanoma [208]–[210]. Nivolumab and pembrolizumab (αPD-1), both approved by the FDA in 2014, have been confirmed to yield durable survival benefits and have become the first-line therapy to treat melanoma [211]–[215]. In addition, ICIs targeting the PD-1/PD-L1 axis, have also been approved, including atezolizumab to treat metastatic urothelial carcinoma and NSCLC [216], avelumab for metastatic Merkel cell carcinoma, and durvalumab to treat urothelial carcinoma or NSCLC [217], [218]. Also, the clinical use of combinatorial ICIs has led to successful responses in cases of melanoma, renal cell carcinoma (RCC), and NSCLC [219]–[221].

Moreover, other ICIs are under experimental and clinical development. Six human monoclonal TIGIT antibodies are currently under investigation to treat solid and hematological cancers. Of note, in one study the use of αTIGIT delayed tumor growth [222], [223]. In cases involving TIM-3 blockade, *in vitro* studies showed an effective control of tumor growth by the enhancement of TILs that was more effective in combination with αPD-1 [123], [125], [129], [153], [224]. In preclinical cancer trials, αTIM-3 resulted stabilized disease for most of the patients and partial response in one patient [225], [226]. To date, at least 13 αLAG-3 agents have been developed. αLAG-3 agents

have exhibited a modest therapeutic effect in the treatment of solid tumors with durable response in tumor growth remission and this effect is increased when αLAG-3 is combined with αPD-1 [227]. Of note, as ICIs do not directly target T cells, undesirable immune reactivity can unleash severe immune-related adverse events (irAEs), the most common being dermatological conditions [228], [229].

Although clinical trials using ICIs to treat HIV-1 infection are under investigation, the few published studies that focus on HIV-1-infected individuals are those involving the treatment of cancers [230]. This is because most cancer clinical trials exclude HIV-1-infected individuals due to the partial immune reconstitution they experience and because ICIs have been proposed as "shock and kill" agents since they can combine reactivation of provirus from latently HIV-1-infected CD4+ T cells (**Figure 13A**) and an enhancement of HIV-1-specific CD8+ Tex cells, which favors the elimination of reactivated cells (**Figure 13B**) [179], [231].

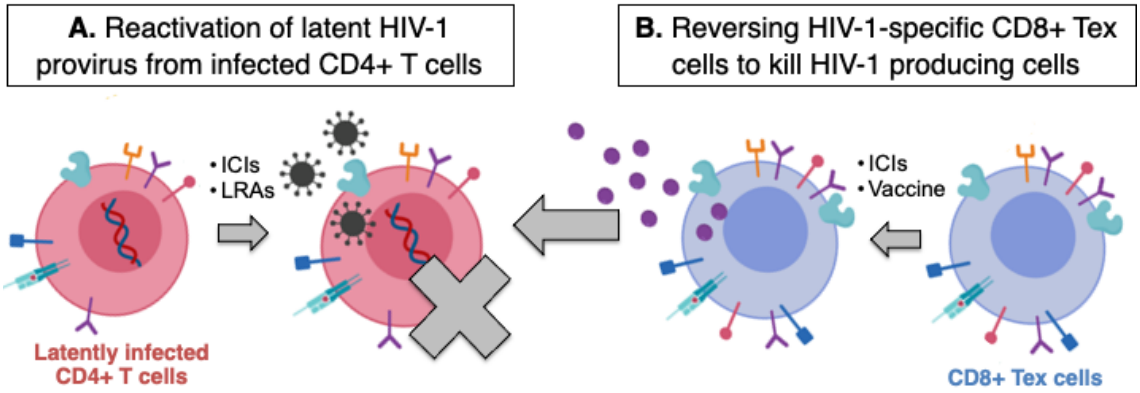

Figure 13. The action of ICIs in CD4+ and CD8+ T cells. ICIs may function as LRAs by acting on both CD4+ and CD8+ T cells. **A.** HIV-1 DNA may become transcriptionally active by IR blockade in CD4+ T cells and as a result, **B.** a potential reduction of the reservoir would be potentiated by the killing of enhanced CD8+ T cells. Image created with Biorender and Adobe Illustrator CC 2018.

Several *in vitro* and *ex vivo* studies of ICIs have shown enhancement of HIV-1-specific CD8+ T-cell responses and also an increase in viral production. **Table 2** summarizes all the studies on HIV-1 with ICIs [230]. Nevertheless, the effectiveness of ICIs to eliminate the viral reservoir is still controversial. Based on limited case reports using ICIs, results ranged from showing no changes in either HIV-1-specific CD8+ T-cell responses or viral reservoir [232], transient enhancement of responses with no variation in viral persistence [233]–[235], transient increase in viral transcription without changes in viral reservoirs [236] or depletion in HIV-1 reservoir [190], [236]. Thus, as the immunological and virological outcome of ICIs in HIV-1-infected patients remains unclear, additional data are needed to determine the use of ICIs in HIV-1 cure strategies.

Table 2. *In vitro* and *in vivo* evidence related to the impact of ICIs on HIV-1-specific T-cell responses and reservoir. Adapted from Chen *et al.* 2020 [230].

ICIs	Effect on HIV-1-specific immunity		Effect on viral reservoirs	
	in vitro	*in vivo*	*in vitro*	*in vivo*
αPD-1	• Pembrolizumab enhanced cytokine production upon antigen stimulation [237].	• Nivolumab (case study) slightly increased IFNγ in CD8+ T cells [234]. • Nivolumab or Pembrolizumab induced small HIV-1-specific CD4+ and CD8+ T-cell responses [232]. • Pembrolizumab (case study) resulted in transient increase of TNF in HIV-1-specific CD8+ T cells [235].	• Pembrolizumab reduced latency in CD4+ T cells [190], [238]. • Pembrolizumab and bryostatin enhanced HIV-1 production in CD4+ T cells [190]. • Nivolumab had no impact on viral reactivation of CD4+ T cells [239].	• Pembrolizumab (case study) induced latency reversal [238]. • Pembrolizumab reduced total and integrated HIV-1 DNA and caRNA[190]. • Nivolumab (case study) resulted in transient increase in caDNA [234]. • Nivolumab (case study) decreased caDNA[236]. • Nivolumab or Pembrolizumab caused no changes in caRNA or caDNA [232]. • Pembrolizumab (case study) resulted in transient decrease in total HIV-1 DNA [235].
αPD-L1	• αPD-L1 enhanced HIV-1-specific CD8+ T cells [100]. • αPD-L1 promoted HIV-1-specific CD4+ T-cell proliferation [240].	• BMS-936559 increased cytokine production [233].	• BMS-936559 had no impact on viral reactivation of CD4+ T cells [239].	• BMS-936559 had no effect on viral reservoirs [233].
αCTLA-4	• αCTLA-4 restored HIV-1-specific CD4+ T cell function [241]. • αCTLA-4 blocked regulatory CD4+ T cells [242].	• Ipilimumab resulted in inconsistent changes in HIV-1 RNA [243].	• Not reported	• Ipilimumab (case report) increased caRNA [244].
αTIGIT	• αTIGIT and αPD-L1 increased HIV-1-specific CD8+ T-cell responses [100]. • αTIGIT did not improve HIV-1-specific NK-cell responses [245].	• Not reported	• Not reported	• Not reported
αTIM-3	• Soluble TIM-3 and αTIM-3 promoted virus-specific T-cell proliferation [123]. • αTIM-3 enhanced cytokines and proliferation of virus-specific CD8+ T cells [237].	• Not reported	• Not reported	• Not reported

HYPOTHESIS AND OBJECTIVES

We **hypothesize** that limitations in virus reactivation and immune function need to be overcome to achieve a cure for HIV-1. Reversion of the exhausted immunological phenotype of CD8+ T cells is key in this process.

The **general objective** of this thesis is to understand the limitations of shock and kill strategies and the immunological bases of CD8+ T-cell exhaustion mediated by IR expression in the search for an effective cure for HIV-1.

Hence, the specific objectives are as follows:

Objective I: To identify the immunological barriers that prevent "shock and kill" strategies from successfully clearing the viral reservoir. This objective will be addressed in Chapter I, entitled "Antigen production after latency reversal and expression of IRs in CD8+ T cells limit the killing of HIV-1 reactivated cells".

Objective II: To delineate the expression of IRs across the landscape of CD8+ T cells and evaluate their impact in terms of functionality on HIV-1 infection and cART. This objective will be addressed in Chapter II, entitled "Selective depletion of $CD107a^{hi}$ $TIGIT^{hi}$ memory HIV-1-specific CD8+ T cells during long-term suppressive cART".

Objective III: To determine the stand-alone effect of ICIs *in vivo* as possible candidates of shock and kill strategy in HIV-1 infection. This objective will be addressed in Chapter III, entitled "Enhancement of antiviral CD8+ T-cell responses and complete remission of metastatic melanoma in an HIV-1-infected subject treated with pembrolizumab".

RESULTS

CHAPTER I. Antigen production after latency reversal and expression of inhibitory receptors in CD8+ T cells limit the killing of HIV-1 reactivated cells

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Autor's contributions: The author of this thesis contributed to the performance of shock and kill RELI model experiments with the analysis of the data. The author also contributed to the writing of the manuscript.

Abstract

The so-called shock and kill therapies aim to combine HIV-1 reactivation by LRA with immune clearance to purge the HIV-1 reservoir. The clinical use of LRA has demonstrated detectable perturbations in the HIV-1 reservoir without measurable reductions to date. Consequently, fundamental questions concerning the limitations of the recognition and killing of LRA-reactivated cells by effector cells such as CD8+ T cells remain to be answered. Here, we developed a novel experimental framework where we combine the use of cytotoxic CD8+ T-cell lines and *ex vivo* CD8+ T cells from HIV-1-infected individuals with functional assays of LRA-inducible reactivation to delineate immune barriers to clear the reservoir. Our results demonstrate the potential for early recognition and killing of reactivated cells by CD8+ T cells. However, the potency of LRAs when crossing the barrier for antigen presentation in target cells, together with the lack of expression of IRs in CD8+ T cells, are critical events to maximize the speed of recognition and the magnitude of killing of LRA-inducible provirus. Taken together, our findings highlight direct limitations in LRA potency and CD8+ T-cell functional status to succeed in the cure of HIV-1 infection.

Introduction

As mentioned in the section of current challenges to HIV-1 cure, the introduction of cART has increased life expectancy and improved the health of people living with HIV-1 infection. However, cART does not cure HIV-1 infection, and treatment is needed for decades, thus raising the alarm about long-term health and sustainability of treatment and care of HIV-1-infected individuals [246]. The current limitations of cART highlight the need for therapeutic strategies to eradicate HIV-1 from the body. A major obstacle to eradication is the establishment of the HIV-1 reservoir [247]. Besides, the reservoir is perpetuated through cellular homeostatic proliferation and clonal expansion, even after years of effective cART [248], [249].

One therapeutic approach, the so-called shock and kill strategies, has been tested in several clinical trials and proposes the use of LRAs for the transcriptional activation of HIV-1 (shock) and the clearance of reactivated cells by immune responses (kill). Although several studies have demonstrated reactivation of HIV-1 by LRA both *in vitro* and *in vivo* [182]–[184], no measurable reduction in the HIV-1 reservoir has been found to date [191]. Consequently, ensuring the immune recognition of LRA-reactivated cells by effector responses will be essential for eradication of the HIV-1 reservoir [250], [251]. Several studies have proposed CD8+ T cells as effector cells for recognition and clearance of LRA-reactivated cells [252] based on their ability to control the reservoir size in natural controllers [253], [254], their potent *in vitro* antiviral activity [255], [256], and their role in controlling viral replication despite cART [74]. Although the frequency of HIV-1-specific CD8+ T cells decays with cART [257], [258], the cells retain effector and cytotoxic properties that enable them to recognize and kill HIV-1-infected cells [74], [252], [259], [260].

However, functional barriers to CD8+ T-cell antiviral activity upon treatment with LRA can affect the success of shock and kill strategies. These barriers may be associated with CD8+ T-cell dysfunction, which is a consequence of LRA treatment itself, and with the pro-inflammatory environment driven by HIV-1 infection. Several studies suggest an immunosuppressive effect of LRAs, particularly HDACi, on CD8+ T-cell antiviral activity [261], [262]. Data remain controversial, and while some studies suggest a time-dependent or direct effect of HDACi on CD8+ T-cell function [263], others do not find a measurable impact on *ex vivo* CD8+ T-cell function after *in vivo* administration of HDACi [184]. Moreover, the chronic pro-inflammatory environment and the persistence of antigen exposure affect the functional profile of HIV-1-specific CD8+ T-cell responses [264], [265]. This pro-inflammatory environment leads to the reduction of cytotoxic

potential and the upregulation of IRs, such as PD-1, LAG-3, and TIM-3 in CD8+ T cells associated with dysfunction and Tex in HIV-1-infected individuals [96], [101], [116], [266]. In this context, fundamental questions regarding the limitations of LRA activity on target cells and CD8+ T-cell sensing in response to HIV-1 reactivation remain unanswered. In this study, we design a novel experimental framework where we combine cytotoxic HIV-1-specific CD8+ T-cell lines (CTL) and *ex vivo* CD8+ T cells from HIV-1-infected individuals with an *in vitro* model of LRA-dependent HIV-1 reactivation. In this framework, we evaluate the so-called window of opportunity between latency reversal and killing of reactivated cells by CD8+ T cells. We characterize HIV-1 protein expression upon treatment with LRA and its association with antigen presentation and delineate the kinetics of recognition and killing of HIV-1-reactivated cells by CD8+ T cells. We also analyze the functional limitations of CD8+ T cells from HIV-1-infected individuals in elimination of reactivated cells. We observed a correlation between LRA potency and the speed and magnitude of the killing of reactivated cells by CD8+ T cells. Although we found increased killing of reactivated cells by *ex vivo* CD8+ T cells in response to LRA, the magnitude of the response was highly variable across HIV-1-infected individuals and was associated with a lack of expression of IRs in CD8+ T cells. Our data highlight several limitations in the efficacy of shock and kill strategies and point to the need for a trade-off between LRA potency and CD8+ T-cell functional status in HIV-1-infected individuals if the reservoir is to be cleared.

Materials and methods

Virus and cell lines

The virus $NL43_{GFP}$ was generated by co-transfection of p83-2 and $p83\text{-}10_{eGFP}$ plasmids in MT4 cells (NIH AIDS Reagent program, USA) based on electroporation, as previously described [267], [268]. The cell-free supernatant was concentrated by spinoculation at 24,000 rpm at 4°C for 90 minutes, and stored at -80°C. The 50% tissue culture infective dose ($TCID_{50}$) was determined on TZM-bl cells (NIH AIDS Reagent program, USA) as described in Kloverpris *et al*. [269]. The HIV-1 permissive U937 cell line transfected with the HLA-B*27:05 gene (kindly provided by Paul Bowness, UK) [270] was cultured in RPMI media supplemented with 10% fetal calf serum (FCS) (R10, HyClone), L-glutamine (2 mM), penicillin (100 U/ml), streptomycin (100 µg/ml), and geneticin (0.5 mg/ml) (Invitrogen, Life Technologies). The cytotoxic HIV-1-specific CD8+ T-cell lines (CTL) used were restricted by HLA-B*27:05. The CTL1 was specific for the KK10 epitope in

Gag and CTL2 for the KY9 epitope in Pol. In addition, CTL3 was restricted by HLA-B*57:01 and specific for the KF11 epitope in Gag [269], [270].

The HIV-1 shock and kill RELI model

The U937 HLA-B*27:05 permissive cell line was infected with $NL43_{GFP}$ by magnetofection at a dose equivalent to a nominal multiplicity of infection (MOI) of 0.1, as previously reported [269], [271]. After magnetofection, cells were washed twice with phosphate-buffered saline (PBS) 1X and cultured in R10 + geneticin (0.5 mg/ml). At day three post-infection, the GFP-negative population, which included the non-productively infected cells enriched in HIV-1 integrated provirus, was sorted with a FACS Aria II Cell Sorter (BD) and cultured in the presence of ritonavir (RTV, 1 µM, Sigma, Spain). After 4 days, we obtained a culture enriched in "resting-like" cells (RELI). The mean levels of cell-associated total HIV-1 DNA in RELI-lysates were 5.1-log ± 0.19 copies/10^6 RELI as measured by droplet digital PCR previously described in Martínez-Bonet *et al*. [163]. RELI cells were treated with LRA in the presence of raltegravir (RAL, 100 nM, Sigma, Spain) to monitor HIV-1 reactivation or "shock". The LRA panel included the HDACi: panobinostat (PNBN, 30 nM, SelleckChem, USA), trichostatin A (Tricho, 250 nM, Sigma, Spain), suberoyl aniline hydroxamic acid (SAHA, 1 µM, SelleckChem, Spain), and romidepsin (RMD, 40 nM, SelleckChem, Spain), and the PKC agonist bryostatin A (Bryo, 10 nM, Sigma, Spain). RELI cells were treated for 48 hours with LRA except for RMD-treated cultures, where the drug was washed-off after 4 hours to avoid cellular toxicity.
To monitor the "shock" or HIV-1 fold induction at 48 hours after treatment with LRA, the RELI cells were harvested and stained with a Live/Dead probe (APC-Cy7, Invitrogen). After washing, cells were fixed and permeabilized with the Fix/Perm kit (A and B solutions, Thermo Fisher Scientific) for intracellular HIV-1 p24 (PE, clone KC57-RD1, Beckmann Coulter). Cells were acquired on an LSR II flow cytometer (Beckmann Coulter) and data analyzed with FlowJoV (Tree Star Inc). The HIV-1 fold induction was calculated based on the following equation: percentage of p24+ or GFP+ in CD4+ under LRA conditions / percentage of p24+ or GFP+ under untreated conditions. To proceed with the co-cultures to monitor the "killing" of reactivated cells by CD8+ T cells, the RELI cells were extensively washed with PBS 1X and left in R10 media plus RAL. Thus, we cultured LRA-reactivated RELI cells in the absence or presence of HIV-1-specific CTLs or B*27-HLA-matched *ex vivo* CD8+ T cells obtained from HIV-1-infected individuals. Cells were co-cultured to a 1:1 (effector:target) ratio for 20 hours. After 20 hours of co-culture, we simultaneously measured by flow cytometry the "shock" by intracellular p24,

and the “kill” by the reduction of p24-positive RELI cells in the absence or presence of effector cells. We also assessed LRA-related toxicity both in uninfected U937 cells and in the RELI model by measuring cellular viability using flow cytometry and staining with a Live/Dead probe (APC-Cy7, Invitrogen) at the end of the co-culture with RELI and CTL.

CD8+ T-cell killing and activation

We performed CD8+ T-cell killing experiments of LRA-reactivated cells with CTL or *ex vivo* CD8+ T cells at 20 hours after culture. We also monitored the kinetics of CD8+ T-cell killing of LRA-reactivated cells with CTL or *ex vivo* CD8+ T cells after 3, 6, and 20 hours after co-culture. We monitored CTL and CD8+ T-cell activation by expression of CD107a and production of MIP-1β and IFNγ. For analysis of HLA-class I restriction, the W6/32 antibody (BioLegend, Spain) or the isotype control LEAF purified IgG2aκ (BioLegend) was added to the co-culture at 10 µg/ml. For analysis of CD8+ T cells in contact with RELI cells, we analyzed the percentage of doublets gated cells that expressed the CD8+ surface marker in the target cells gate. For analysis of early CD107a expression, the antibody CD107a (PerCP-Cy5.5, BD) was added to the co-culture.

For the immune-phenotype, samples were collected and washed twice with PBS 1X and incubated for 3 hours in the presence of the protein transport inhibitor Golgiplug (Brefeldin A solution, BD) and Monensin A (BD Golgi STOP). Cells were then harvested, stained with a Live/Dead probe (APC-Cy7, Invitrogen). Next, cells were washed and stained to identify the surface marker CD8 (PB, clone RPA-T8, BD). After washing, cells were fixed and permeabilized with the Fix/Perm kit (A and B solutions, Thermo Fisher Scientific) for intracellular cytokine staining of MIP-1β (FITC, clone 24006, R&D Systems), IFNγ (PE-Cy7, clone 4S.B3, BD), and HIV-1 p24 (PE, clone KC57-RD1, Beckmann Coulter). Cells were acquired on an LSR II flow cytometer (Beckmann Coulter). Data were analyzed with FlowJoV (Tree Star Inc). The killing of reactivated HIV-1 cells by CTL or CD8+ T cells was calculated based on the following equation: 100 – [(percentage of p24+ in CD4+ T cells co-cultured with CD8+ T cells/ percentage of p24+ in CD4+ T cells in the absence of CD8+ T cells) x 100].

In vitro CTL exhaustion experiments

To characterize potential markers associated with CTL dysfunction in response to LRA-reactivated cells, we exposed CTL1 and CTL2 to cognate HIV-1 antigen over nine weeks. To do so, we stimulated CTL with a 1:1 mixture of irradiated autologous B-cell lines (BCLs) pulsed with cognate HIV-1 peptide (10 µg/ml) and irradiated peripheral

blood mononuclear cells (PBMCs) from three healthy HIV-1 seronegative donors weekly. We used flow cytometry to evaluate variations in the expression of PD-1, TIM-3, LAG-3, and the immune-metabolic marker CD39. Briefly, cells were taken from the culture at weeks 0, 5, 7, and 9 and stained with a Live/Dead probe (APC-Cy7, Invitrogen) and surface markers CD3 (A700, BD), CD4 (APC-Cy7, BD), CD8 (V500, BD), PD-1 (BV421, BD), TIM-3 (A647, BD), LAG-3 (PE, BD), and CD39 (FITC, BD) and incubated at room temperature (RT) for 25 minutes. Samples were washed twice with PBS 1X, fixed in formaldehyde 1%, and acquired on an LSR Fortessa. Data were analyzed with FlowJoV (Tree Star Inc). Patterns of co-expression of PD-1, LAG-3, TIM-3, and CD39 were analyzed using Pestle and SPICE v5 software [272].

Immunophenotype of HIV-1-specific CD8+ T-cell responses

The PBMCs from HIV-1-infected individuals were stimulated with HIV-1-Gag peptide pool (2 µg/peptide/ml, EzBiolab), or SEB (enterotoxin B from *Staphylococcus aureus*, 1 µl/ml, Sigma) or no stimuli in the presence of CD28/49d (1 µl/ml, BD), Monensin A (1 µl/ml, BD), and anti-human CD107a (PE-Cy5, BD) for 6 hours at 37°C in a 5% CO_2 incubator and overnight at 4°C. Stimulation cells were then washed with PBS 1X and stained for 25 minutes with the Live/Dead probe (APC-Cy7, Invitrogen). Next, cells were washed with PBS 1X and surface stained for 25 minutes with CD3 (A700, BD), CD4 (APC-Cy7, BD), CD8 (V500, BD), PD-1 (BV421, BD), LAG-3 (PE, BD), TIM-3 (A647, BD), and CD39 (FITC, BD). Subsequently, cells were washed twice in PBS 1X and fixed and permeabilized with the Fix/Perm kit (A and B solutions, Thermo Fisher Scientific) for intracellular cytokine staining of IFNγ (BV711, BD) and IL-2 (BV650, BD). After 20 minutes at RT in the dark, stained samples were washed twice with PBS 1X, resuspended in formaldehyde 1% and acquired on an LSR Fortessa. Data were analyzed with FlowJoV (Tree Star Inc). Patterns of simultaneous expression of CD107a and production of IFNγ and IL-2, or combinations of PD-1, LAG-3, TIM-3, and CD39 were analyzed using Pestle and SPICE v5 software [272].

Statistics

Statistical analyses were performed using Prism v4 (GraphPad Software, Inc.). Reported p-values were calculated using the Wilcoxon single-rank test in paired comparisons and the t-test and Mann-Whitney test for independent median comparisons. Also, we used one-way ordinary analysis of variants (ANOVA), and one-way ANOVA for multiple group comparisons adjusting the pairwise analyses by Tukey. The relationship between HIV-

1 induction measured according to the expression of p24 and GFP, and the relationship between the lack of IRs in CD8+ T cells and percentage of killing were assessed using the Spearman correlation coefficient. All statistical tests were under a significance level of 0.05.

Ethics Statement

All methods and experimental protocols of the study were approved by the Ethics Committee of Hospital Germans Trias i Pujol. For the study, both infected and healthy subjects provided their written informed consent to participate. The study was conducted according to the principles expressed in the Declaration of Helsinki.

Results

LRAs allow HIV-1 protein expression and HLA-class I antigen presentation for CD8+ T-cell recognition to increase killing of latently infected cells.

First, we developed the "resting-like" or RELI-model to evaluate HIV-1 reactivation by LRA (shock) simultaneously with the elimination of reactivated cells by cytotoxic HIV-1-specific CD8+ T-cell lines (CTL) (kill), as schematized in **Figure 14.**

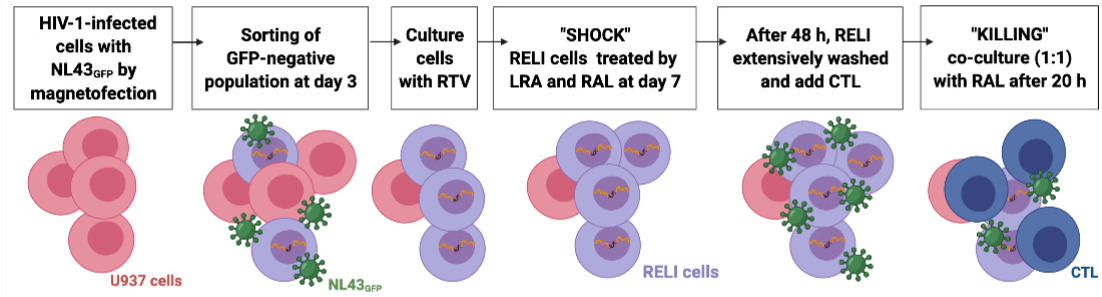
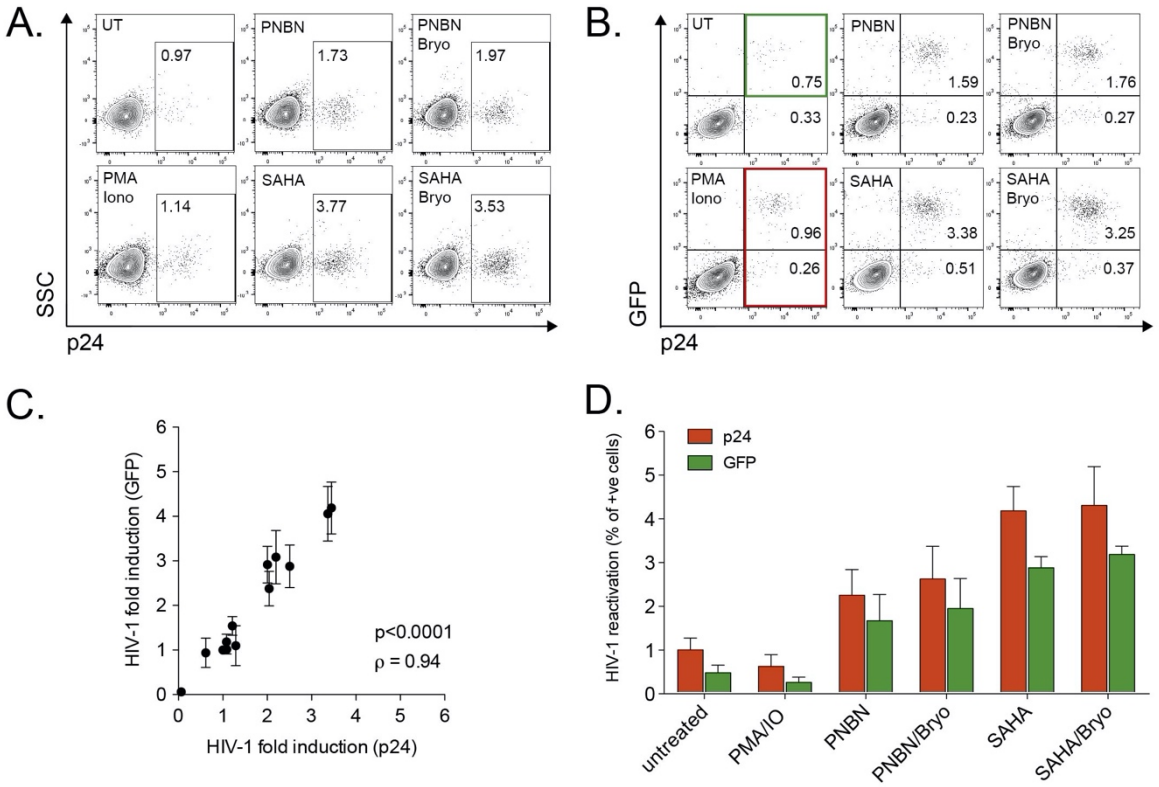


Figure 14. The HIV-1 RELI "shock and kill" model. Schematic representation of the HIV-1 RELI model for "shock and kill." RELI cells were reactivated with LRA in the presence of RAL before being extensively washed and cultured in the presence or absence of HLA-class I matched CTL. After 20 hours of co-culture, we evaluated HIV-1 reactivation and the killing of HIV-1-reactivated by CTL. Image generated by Biorender and Adobe Illustrator CC 2018.

Briefly, we infected U937-HLA-B*27:05 cells with the HIV-$1_{NL43GFP}$ reporter virus as previously reported [269], [271]. After 72 hours of infection, we sorted the GFP-negative cells and cultured them for four days in the presence of the protease inhibitor RTV. At this point, we obtained a RELI population enriched in uninfected and non-productively HIV-1-infected cells. RELI cells were then washed, treated with the HIV-1 integrase inhibitor RAL to avoid multiple rounds of infection, and exposed to LRA shock for 48 hours. We also treated RELI cells with azidothymidine (AZT) to block viral expression

from unintegrated episomal forms [273] (data not shown). LRA-treated cells were then extensively washed to uncouple the LRA effect from CD8+ T-cell function against reactivated cells [274], and co-cultured with HLA-class I matched HIV-1-specific CTLs for 20 hours (kill) (**Figure 14**). We measured HIV-1 reactivation in RELI cells in response to LRAs for 48 hours including the PNBN, RMD, Tricho, and SAHA. We also included combinations of HDACi and Bryo to evaluate potential additive effects between HDACi and protein kinase C agonists as previously reported [275]. Drug concentrations were selected based on previous clinical or *in vitro* studies [184], [186], [276], [277] and in the absence of direct cellular toxicity (**Table Supplementary 1**). The use of HIV-$1_{NL43GFP}$ enabled to monitor viral reactivation by flow cytometry through two markers the expression of p24 protein (**Figure 15A**) or the expression of GFP encoded in frame with the HIV-1 Nef protein (**Figure 15B**), as previously reported [267], [268].

Figure 15. HIV-1 reactivation measured by LRAs. A. Representative dot plots of intracellular p24 expression in RELI cells under untreated conditions (UT) compared with PMA/Ionomycin and SAHA and PNBN alone or combined with Bryo. **B.** Representative dot plots of intracellular expression of p24 and GFP from RELI cells reactivated with LRAs. The green square indicates GFP-positive cells, and the red square indicates p24-positive cells. **C.** Correlation between HIV-1 fold induction measured according to the expression of p24 or GFP in RELI cells after LRA treatment. The HIV-1 fold induction was calculated as the ratio between % of p24 or GFP-expressing cells under LRA conditions and the % of p24 or GFP expressing cells left untreated. The line indicates the fit of the data to a linear regression. The spearman correlation coefficient (ρ) and the two-tailed p value are shown. The graph represents the mean ± SEM of three independent experiments performed in replicates. **D.** HIV-1 reactivation measured by the percentage of p24 positive cells or GFP positive cells. The graph represents the mean ± SEM of three independent experiments performed in replicates.



We observed a direct correlation between HIV-1 fold induction measured by p24 or GFP ($p<0.0001$; $\rho=0.94$, **Figure 15C**). In this context, the expression of p24 was consistently higher than that of GFP (p24 vs. GFP $p=0.0031$, Wilcoxon matched pairs signed rank test) (**Figure 15D**), thus indicating its sensitivity as a marker for viral antigen expression upon reactivation for subsequent experiments. However, the use of GFP expression as a marker of HIV-1 induction will be particularly relevant given the correlation between p24 and GFP expression for the screening of large compound libraries and, thus, the increased cost-effectiveness of the method.

As shown in **Figure 16**, we compared HIV-1 reactivation after LRA treatment with Tricho, PNBN, and SAHA alone or combined with Bryo with untreated or DMSO controls. The levels of HIV-1 reactivation ranged between a 2- and 4-fold induction, with a significant increase for PNBN and SAHA alone ($p<0.05$) or combined with Bryo ($p<0.005$). The additive effects of combining HDACi/Bryo were modest or absent.

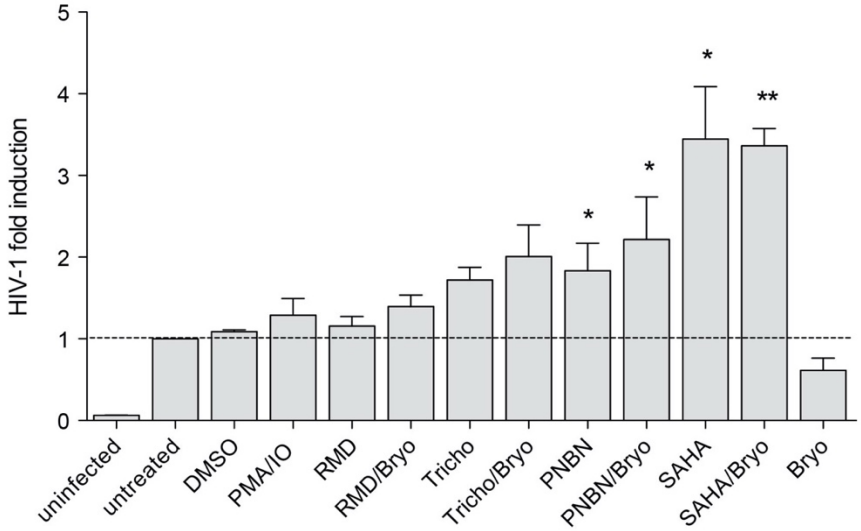


Figure 16. HIV-1 fold induction of RELI cells. The HIV-1 fold induction was calculated as the ratio between the % of p24 expressing cells under LRA conditions and the % of p24 expressing cells in the left untreated. The data represents the mean ± SEM of five independent experiments performed in replicates. p values were calculated using the one-sample t-test. Significant values are shown (*$p<0.05$, **$p<0.005$).

Next, we assessed whether LRA treatment enabled HIV-1 antigen presentation in the context of HLA-class I molecules for their recognition and killing by cytotoxic HIV-1-specific CTLs. For this purpose, we used CTL1 and CTL2, which recognized HLA-B*27:05 restricted epitopes (Gag KK10 and Pol KY9, respectively), as previously described [269]. Briefly, we exposed RELI cells to PNBN and SAHA alone or combined with Bryo and co-cultured them with CTLs restricted by HLA-B*27:05. After 20 hours of co-culture, we assessed killing by measuring the frequency of p24-expressing cells in the absence or presence of CTL1 (**Figure 17A** and **Figure Supplementary 1A**). As shown in **Figure 17B**, we observed significantly increased killing by CTL1 and CTL2

after LRA treatment ($p < 0.0005$, one-way ANOVA). In fact, CTL1 and CTL2 were highly functional and had similar 50% effective concentrations (EC_{50}, -4.8 and -5.5, respectively), despite differences in epitope specificity and a higher response for CTL1 at low HIV-1 antigen concentrations (**Figure Supplementary 1B-C**).

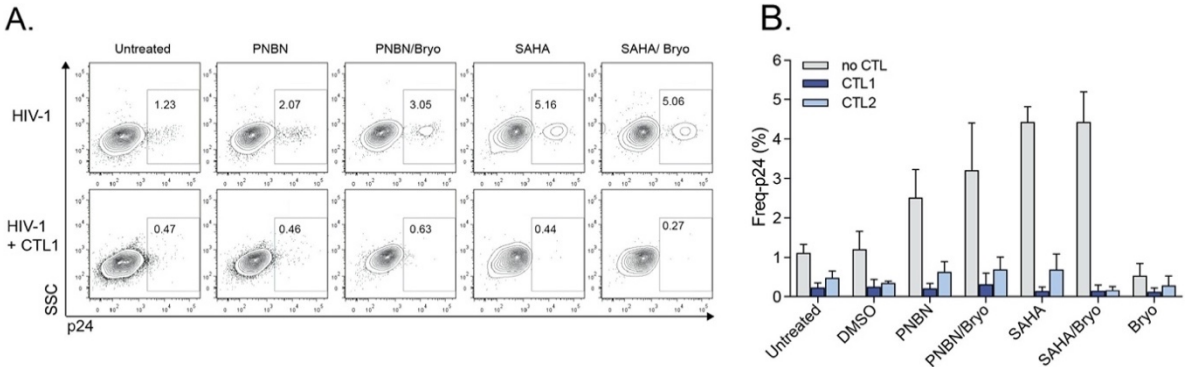


Figure 17. LRA treatment increased the magnitude of killing of HIV-1-reactivated cells by CD8+ T cells. A. Representative dot plots showing the frequency of p24 cells from live HIV-1-reactivated cells in the absence (top) or in the presence of CTL1 for 20 hours. **B.** Frequency of p24 cells from HIV-1-reactivated cells in the absence or presence of CTLs (CTL1 and CTL2). The graph represents the mean ± SEM of three independent experiments performed in duplicate.

In addition, we added an anti-HLA-A/B/C (W6/32) antibody or an HLA-mismatched (MM) CTL3 to demonstrate that, upon viral reactivation, CTL killing was mediated through contacts with HLA-class I HIV-1 antigen complexes. The elimination of reactivated cells was significantly abolished both by means of the W6/32 antibody and by impeding the recognition of the HLA-class I peptide complexes with an HLA-MM co-culture ($p<0.05$, one-way ANOVA) (**Figure 18A**).

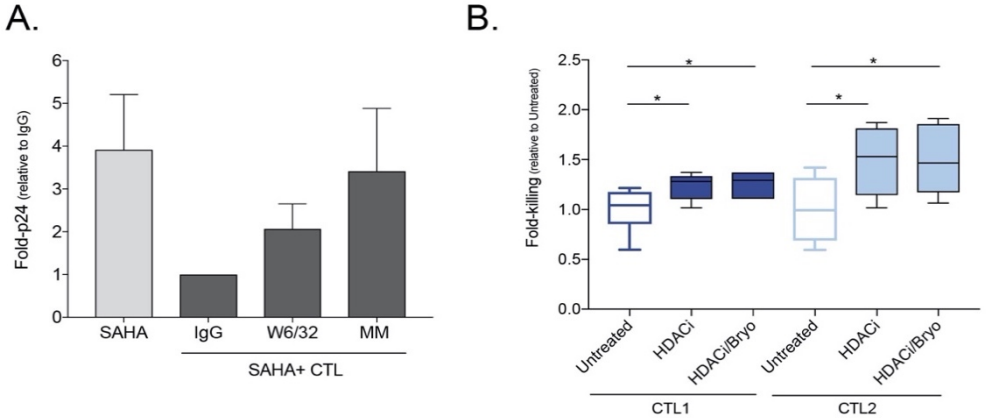


Figure 18. Antigen presentation in HLA-class I and compiled analysis. A. Fold-p24 expression relative to SAHA-reactivated cells in the presence of HLA-matched CTLs treated with isotype control antibody IgG. SAHA-reactivated cells in the absence or presence of HLA-matched CTLs (CTL1, CTL2), treated isotype control antibody IgG (IgG), and HLA-blocking antibody (W6/32), and in the presence of HLA-mismatched CTL3 (MM). The graph represents the mean ± SD of three experiments performed in duplicate. **B**. Relative killing of RELI cells by untreated CTL1 and CTL2 and presence of HDACi (including PNBN and SAHA) alone and combined with Bryo. Box and whisker plots included data from four independent experiments performed in duplicate. The p-values were calculated using the Mann-Whitney test. Only significant values are shown in the figure (*$p<0.05$).

Thus, the compiled analysis of all LRAs tested showed a significant increase in the magnitude of killing of cells latently infected by HIV-1 after LRA treatment in both CTL ($p<0.05$, **Figure 18B**). However, we did not observe any additional benefit in the frequency of reactivated cell killing through the combination of HDACi with Bryo (**Figure 18B**). These data confirm that inducible reactivation of HIV-1 by LRAs enables antigen presentation in the context of HLA-class I molecules to increase killing of latently infected cells by CTL.

LRA potency modulates the speed of HIV-1 antigen recognition and the magnitude of the killing of reactivated cells by CD8+ T cells.

It is essential to investigate the kinetics of recognition and killing of LRA-reactivated cells and to establish a window of opportunity between shock and kill by CD8+ T cells for therapeutic optimization.

By using the RELI model, we monitored the kinetics of recognition and elimination of HIV-1-reactivated cells by CTLs at 3, 6, and 20 hours post co-culture. We measured changes in the frequency of p24-positive cells from time 0 by flow cytometry at 3, 6 and 20 hours after co-culture of untreated and LRA-treated RELI cells with CTLs (**Figure 19A**). We observed a reduction in the frequency of p24 expressing cells over time in culture with CTLs (**Figure 19B**). HIV-1-reactivated cells were eliminated rapidly after 3 hours of co-culture in SAHA, PNBN, SAHA/Bryo, and PNBN/Bryo conditions compared with untreated for both CTLs (untreated vs. HDAC± Bryo; $p<0.0001$) (**Figure 19C**).

These differences were sustained at 6 hours after co-culture (untreated vs. HDAC ± Bryo; $p<0.0005$) and 20 hours after co-culture (untreated vs. HDAC± Bryo; $p<0.005$) (**Figure 19D, E**). These data indicate a general increase in the speed of recognition and killing of reactivated cells by CTLs starting at 3 hours in the presence of PNBN, PNBN/Bryo, SAHA and SAHA/Bryo. although our results do not reveal a hierarchy for LRAs. The data suggest a prolonged effect over the first 6 hours by PNBN/Bryo, SAHA and SAHA/Bryo compared with untreated cells (**Figure 19D,E**) (untreated vs. PNBN/Bryo; $p<0.05$, untreated vs. SAHA; $p<0.005$; and untreated vs. SAHA/Bryo; $p<0.005$). By contrast, the use of Bryo alone did not significantly increase the speed or magnitude of killing compared with untreated condition at 3, 6 or 20 hours of co-culture (**Figure 19C-E**). Although we observed a marginal augment in the killing at early time points, suggesting an indirect effect of Bryo in CTL activation despite extensive washout of the drug as previously reported [274].

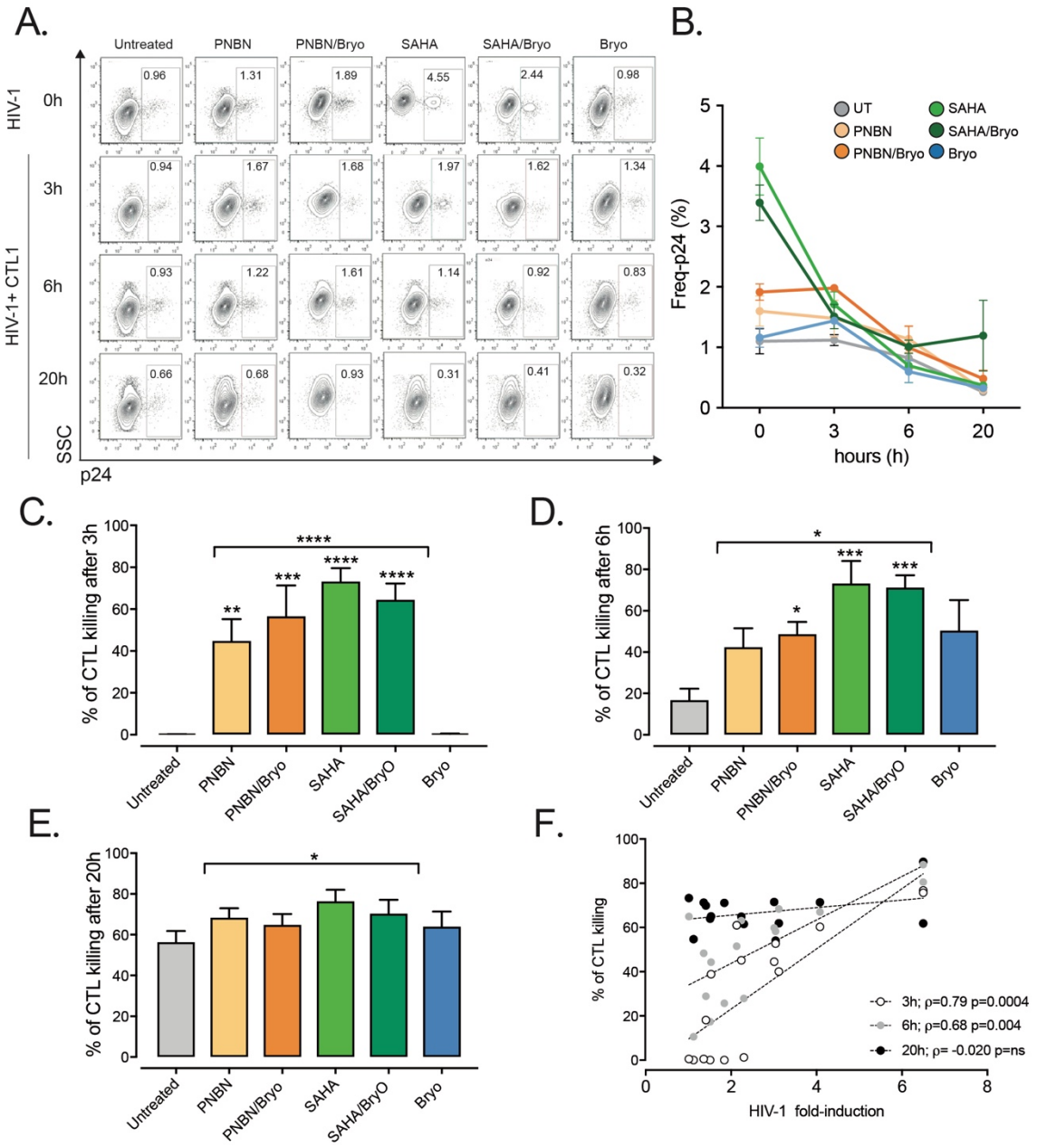


Figure 19. Kinetics of killing in response to HIV-1 reactivation by CTL. A. Representative dot plots showing the percentages of intracellular p24 from HIV-1 RELI cells. The top line indicates the level of HIV-1 reactivation of RELI cells at baseline (0 hours) in untreated and LRA-treated conditions and 3, 6, and 20 hours after co-culture with CTL1. **B.** Kinetics of elimination of HIV-1-reactivated by CTL. The graph shows the mean ± SEM of two independent experiments for CTL1. **C.** Kinetics of killing of HIV-1-reactivated cells by CTLs at 3 hours, **D.** at 6 hours, and **E.** at 20 hours after co-culture. Values in graphs C to E correspond to the mean ± SEM of three independent experiments for CTL1 and three independent experiments for CTL2 in replicates. The p-values were calculated using the unpaired t-test for comparisons between untreated cells and cells treated with HDACi (top line) and one-way ANOVA for multiple comparisons (asterisks above the bars). Only significant p-values are represented in the figure (*p<0.05, **p<0.005, ***p<0.0005, ****p<0.00005). **F.** Correlation between HIV-1 fold induction and frequency of killing mediated by CTLs. The line indicates the fit of the data to a linear regression. The Spearman correlation coefficient (ρ) and the two-tailed p-value are indicated. The graph represents the frequency of killing from three independent experiments for CTL1 in replicates and three independent experiments for CTL2 in replicates.

Interestingly, as shown in **Figure 19F**, we found a direct correlation between the magnitude of CTL killing and the levels of HIV-1 inducible reactivation by LRA at early time points (3 hours $p<0.0005$, $\rho=0.74$; 6 hours $p<0.005$; $\rho=0.68$), thus indicating that the potency of the LRAs for HIV-1 antigen expression dictates the speed of CTL recognition. Based on these data, we propose a threshold of >2-fold HIV-1 induction upon LRA treatment to allow antigen presentation and engage rapid recognition and killing of HIV-1-reactivated cells by CTLs.

Besides, we investigated whether HIV-1 killing by CTLs can be monitored by changes in cytokine production and degranulation upon antigen recognition using CTLs as biosensors [278], we measured CD107a/MIP-1β and IFNγ (**Figure 20A**, dot plots). Despite the increase of HIV-1 expression by LRAs, the use of SAHA or PNBN did not alter the kinetics of CD107a/MIP-1β or IFNγ in the tested CTLs, as compared with untreated conditions. By contrast, Bryo indirect effects increased both CD107a/MIP-1β and IFNγ by CTLs at early time points (**Figure 20B**). We also found different profiles for CD107a/MIP-1β and IFNγ over time, with increasing frequencies for CD107a/MIP-1β and decreasing frequencies for IFNγ. Thus, the kinetics of cytokine production and degranulation by CTLs did not mirror changes associated with HIV-1 inducible reactivation or killing.

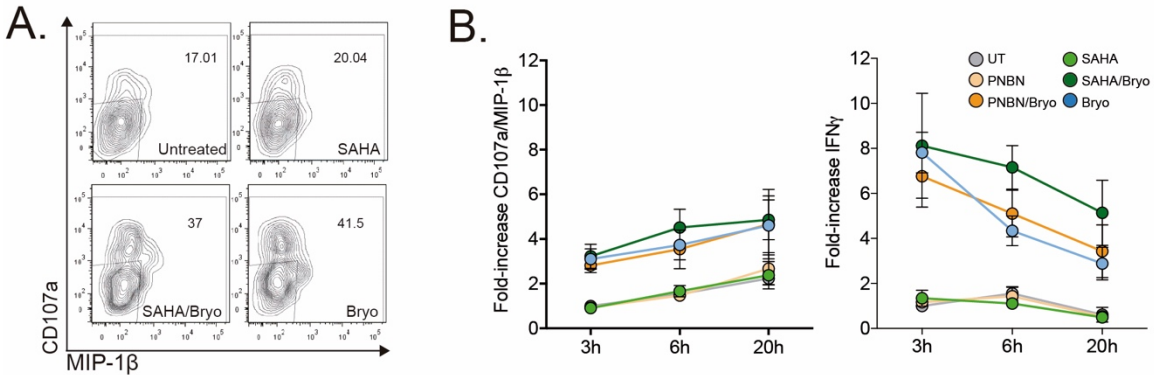


Figure 20. CD107a/MIP-1β expression and IFNγ production from CTLs after co-culture. A. Representative dot plots showing percentages of CD107a/MIP-1β from CTL1 after co-culture with HIV-1-reactivated RELI cells. **B.** Kinetics of CD107a/MIP-1β and IFNγ from CTLs after co-culture with HIV-1-reactivated RELI cells. The graphs show the mean ± SEM of two independent experiments for CTL1 and two independent experiments for CTL2 in replicates.

Overall, these data indicate a direct relationship between the potency of inducible reactivation of HIV-1 by LRAs and the speed of recognition and killing of reactivated cells by CD8+ T cells. Therefore, the potency of LRAs to cross the threshold of HIV-1 antigen presentation in reactivated cells is a crucial event to shortening the window of opportunity for antigen recognition, thus maximizing CD8+ T-cell sensing for elimination of HIV-1-reactivated cells.

Inter-individual divergences in *ex vivo* CD8+ T-cell recognition and killing of LRA-reactivated cells

Although the use of CTLs supports rapid recognition of HIV-1-reactivated cells based on LRA potency, these CTLs are highly functional and sensitive to HIV-1 antigen presentation and may not fully recapitulate the diversity of HIV-1-specific CD8+ T cells in infected individuals. To delineate the kinetics of recognition and killing of LRA-reactivated cells by CD8+ T cells, we initially co-cultured SAHA-treated cells with *ex vivo* CD8+ T cells obtained from three of the eight HIV-1-infected individuals (**Table 3**).

Table 3. Participant characteristics.

Participant ID	Age at sample	Gender, Ethnicity	cART regimen	HLA-B serotype	VL (copies/ml)
PT1	34	M, CAU	TDF/FTC/RAL	B*27:05	<40
PT2	43	F, CAU	Naive	B*27:02	<40
PT3	36	M, CAU	ABC/3TC/DTG	B*27:05	<40
PT4	38	M, CAU	TDF/FTC/RAL	B*27:05	<40
PT5	36	M, CAU	Naive	B*27:05	44,000
PT6	40	M, CAU	TDF/FTC/ELV/COB	B*27:05	<40
PT7	37	F, CAU	Naive	B*27:05	982
MM	47	M, CAU	AZT/ddC <50	no-B*27:05	<50

VL, Viral load; Und-VL, Undetectable viral load; TDF, Tenofovir; FTC, Emtricitabine; RAL, Raltegravir; ABC, Abacavir; 3TC, Lamivudine; DTG, Dolutegravir; ELV, Elvitegravir; COB, Cobicistat; AZT, Zidovudine; ddC, Zalcitabine.

As shown in **Figure 21A**, we observed variation in the kinetics of recognition and killing of reactivated cells by *ex vivo* CD8+ T cells. While we observed killing in response to SAHA at 3 hours for PT1, we did not observe elimination of reactivated cells for PT2; in addition, elimination of reactivated cells was only observed after 20 hours of co-culture for PT3. These divergences suggest functional differences between CD8+ T cells from HIV-1-infected individual associated a particular profile of recognition and killing of LRA-reactivated cells [279]. Next, we extended our initial analyses to an additional group of HIV-1-infected individuals (**Table 3**). As in the previous experiments, we found high inter-individual variability in the killing capacity of HIV-1-reactivated cells by CD8+ T cells ranging from 2% (PT2 and PT7) to 70% (PT4 and PT5) (**Figure 21B**). The killing observed was specific for CD8+ T-cell recognition in the context of HLA-class I presentation, as supported by the lack of killing observed by mismatch CD8+ T cells (MM). Despite this biological divergence, we observed a significant increase in the killing of SAHA-treated cells by CD8+ T cells ($p<0.05$, **Figure 21C**). Moreover, we were able to enhance the magnitude of CD8+ T-cell killing *ex vivo*, either by increasing the concentration of SAHA 2-fold or the effector-to-target ratio 3 times (**Figure 21D**). These results demonstrate the possibility of modulating and improving the efficacy of

CD8+ T-cell killing in response to HIV-1 inducible expression by higher LRA dosage or higher frequency of effector cells.

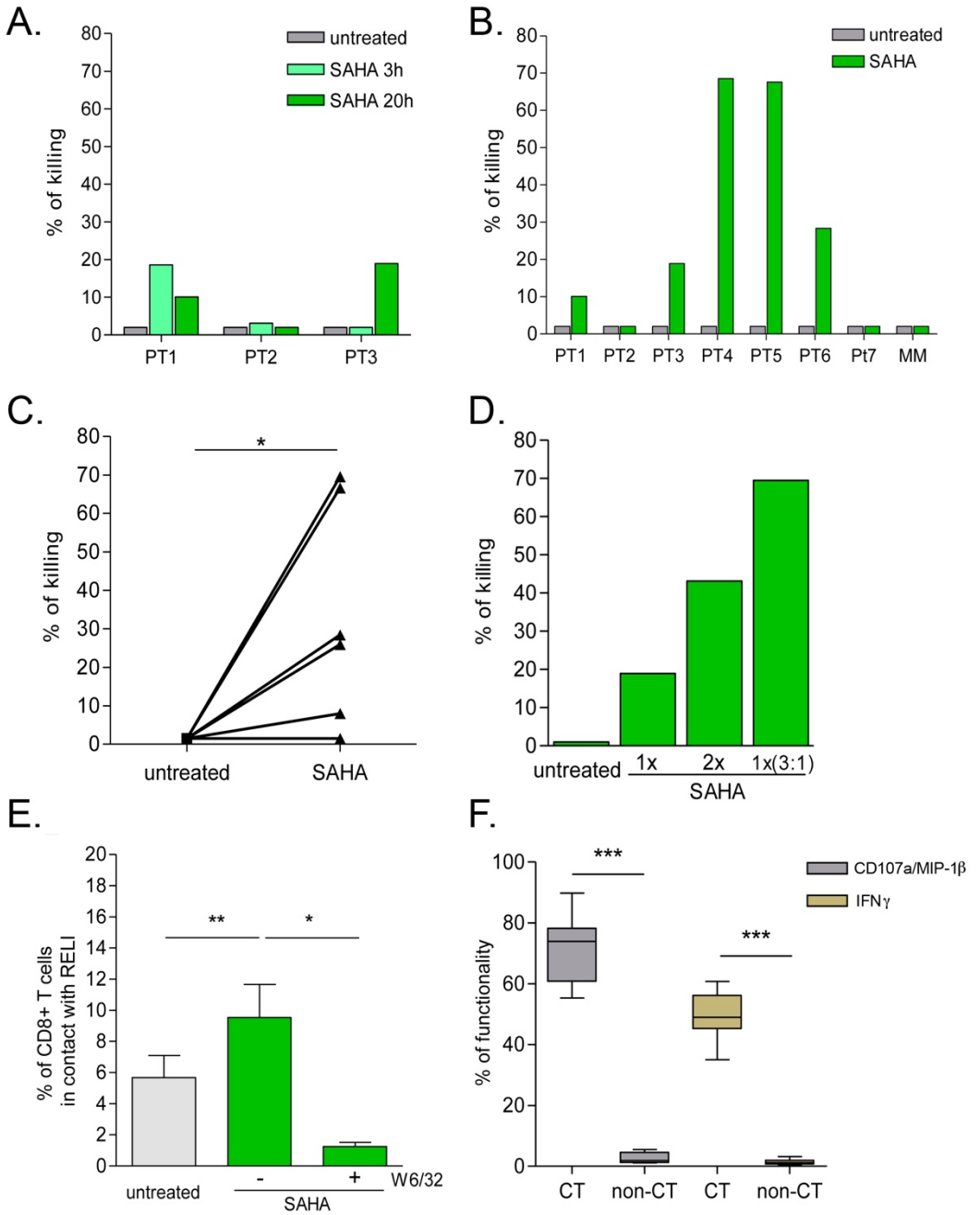


Figure 21. Killing of HIV-1-reactivated cells by *ex vivo* CD8+ T cells. A. Percentage of killing of untreated or SAHA-treated HIV-1 RELI cells at 3 and 20 hours by *ex vivo* CD8+ T cells from HIV-1-infected individuals (n=3). **B.** Percentage of killing of untreated or SAHA-treated HIV-1 RELI cells by *ex vivo* CD8+ T cells at 20 hours (n=7). **C.** Paired comparison of the percentage of killing of untreated or SAHA-treated HIV-1 RELI cells by *ex vivo* CD8+ T cells shown in B. The graph represents the percentage of the killing of RELI cells. The p-values were calculated using the paired t-test. **D.** Percentage of killing of untreated or SAHA-treated RELI cells by *ex vivo* CD8+ T cells from PT3. Conditions with a 1X SAHA and 2X SAHA and co-cultures at an E:T ratio of 3:1 in 1X SAHA conditions are shown. **E.** Percentage of CD8+ T cells in contact with untreated or SAHA-treated HIV-1 RELI cells in the absence or presence of a W6/32 antibody after 20 hours of co-culture. The p-values were calculated using the paired t-test. **F.** Percentage of CD107a/MIP-1β and IFNγ of CD8+ T cells after 20 hours in contact or not (CT/non-CT) with SAHA-reactivated cells. The p–values were calculated using the paired t-test. Only significant values (*p<0.05, **p<0.005 and ***p<0.0005) are shown.

We also carried out a detailed study of cell-to-cell interactions in our co-culture system. We observed an increase in the number of CD8+ T-cell contacts with target cells upon reactivation of SAHA ($p<0.005$). These contacts were specifically blocked by the addition of an anti-HLA-A/B/C antibody ($p<0.05$) (**Figure 21E**), thus demonstrating the dependency of cell-to-cell contact on the interactions between TCR and HLA-class I/peptide complexes after LRA treatment. In addition, CD8+ T cells that established contact were highly functional, as measured by the levels of CD107a/MIP-1β and IFNγ when compared with CD8+ T cells lacking contacts with target cells ($p< 0.0001$) (**Figure 21F**).

These findings support an overall increase in the killing of HIV-1-infected cells by CD8+ T cells upon LRA induction of viral reactivation despite inter-individual divergences. Moreover, our data point to the opportunity to optimize the immune clearance of HIV-1-reactivated target cells by CD8+ T cells by increasing the LRA dosage and the frequency of effector CD8+ T cells.

Expression profiles of IRs in HIV-1-specific CD8+ T cells modulates the killing of HIV-1-reactivated cells

Inter-individual differences in functional ability to recognize and eliminate HIV-1-infected cells *ex vivo* by CD8+ T cells may account for discrepancies in time to initiation of cART [280], in the persistence of antigen exposure and in the chronic pro-inflammatory environment despite cART [266]. The pro-inflammatory environment and the long-term exposure to antigen lead to dysfunctional HIV-1-specific CD8+ T-cell responses and eventually to Tex.

To delineate the determinants of CD8+ T-cell dysfunction in response to LRA-reactivated cells, we cultured CTLs with HIV-1 cognate peptide for 9 weeks with weekly rounds of peptide re-stimulation to simulate *in vitro* long-term antigen exposure (**Figure 22A**). At weeks 0, 5, 7, and 9 and performed an immune phenotype of IRs (PD-1, LAG-3 and TIM-3) and the immune-metabolic marker CD39. All these markers have been previously associated with HIV-1-specific CD8+ Tex [96], [126]. As shown in **Figure 22B**, we found a significant reduction in the killing of LRA-reactivated cells by CTLs after nine weeks of continuous antigen exposure ($p<0.005$). Despite the overall reduction in the killing by CTLs after 9 weeks, we observed an increase in killing between untreated and SAHA conditions (35% - 58%) (**Figure 22B**).

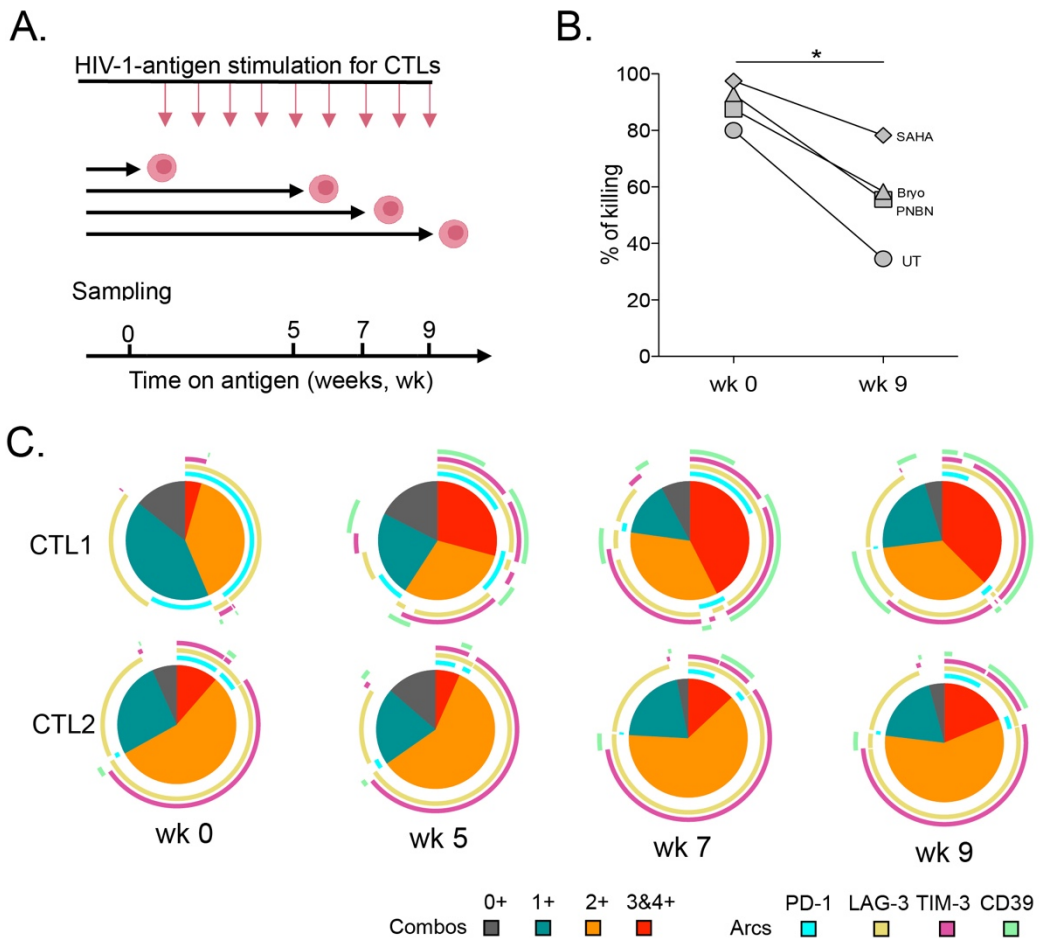


Figure 22. CD8+ T-cell dysfunction impairs clearance of HIV-1-reactivated cells associated with an increase in the co-expression of IRs. A. Schematic representation of the *in vitro* exhaustion process, where CTLs were exposed to HIV-1 cognate peptide over a period of 9 weeks. **B.** Percentage of killing of HIV-1-reactivated cells by CTLs after antigen stimulation at week 0 and at 9 weeks. The graph includes one representative experiment. The p-value was calculated using the paired t-test. Statistical significance is shown in the figure (*$p < 0.05$). **C.** Pie charts representing the fraction of cells expressing different combinations of PD-1, LAG-3, TIM-3, and CD39 at weeks 0, 5, 7, and 9 after antigen stimulation.

We also monitored phenotypic changes in CTLs and found a contraction in the frequency of subpopulations without expression or expressing a single IR concomitant with the expansion of cells co-expressing three or four IRs over time (**Figure 22C**, pie charts). This phenotype of expansion/contraction was coincident between CTL1 and CTL2, which vary in HIV-1 antigen specificity. However, the specific combinations of IRs diverge between CTL lines and time, thus highlighting the diversity of T-cell inhibitory pathways. Also, CD39+ subpopulations appeared after 5 weeks in cells already expressing two or more IRs (**Figure 22C**, arcs). These data suggest the presence of terminally CD8+ Tex cells identified by CD39 expression, as previously described [126].

Based on these data and the biological diversity found in the recognition and killing of LRA-reactivated cells by *ex vivo* CD8+ T cells, we measured HIV-1-specific CD8+ T-cell

function in response to a pool of Gag overlapping peptides. We monitored CD107a degranulation, IFNγ, and MIP-1β cytokines, and PD-1, LAG-3, TIM-3, and CD39 expression by multiparametric flow cytometry. As shown in **Figure 23A**, we observed variation in the profile of cytokine production among individuals in response to HIV-1. Meanwhile, the functional response to SEB control stimuli was very homogeneous, as expected. Furthermore, we did not find a correlation between the frequency of polyfunctional cells among the HIV-1-specific CD8+ T cells and the killing in response to LRA-reactivated cells, as it has previously been associated with spontaneous control of HIV-1 replication [281], [282]. Importantly, individuals harboring HIV-1-specific CD8+ T cells lacking expression of IRs retained the highest suppressive capacity against HIV-1 LRA-reactivated cells (**Figure 23A**). Thus, PT5 responded to LRA treatment with a 68% *in vitro* suppressive capacity and 63% of HIV-1-specific CD8+ T cells lacking IRs. Participants PT3, PT1, and PT7 with 36, 35, and 24% of HIV-1-specific CD8+ T cells lacking IRs had suppressive capacities achieving 19, 10, and 2% of killing of SAHA-reactivated cells. Although limited by sample size, our data delineate a correlation between the percentage of HIV-1-specific CD8+ T cells lacking IRs and their immune responsiveness to killing LRA treatment ($p<0.005$, **Figure 23B**). According to these data, changes in the profile of IRs in HIV-1-specific CTL and primary CD8+ T cells modulate their antiviral potency. Therefore, the levels of IR co-expression in CD8+ T cells can limit the magnitude of the killing of HIV-1-reactivated cells.

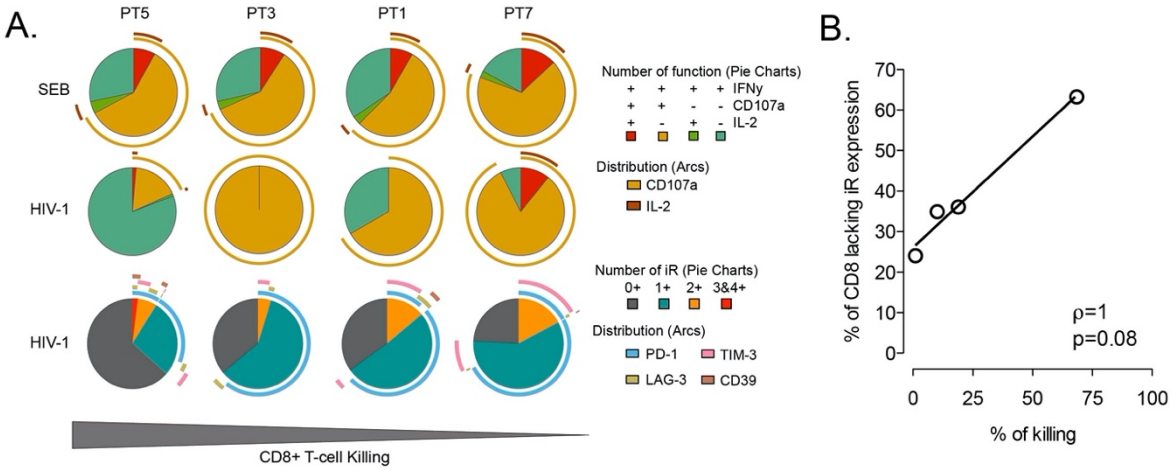


Figure 23. Functional profiles and IRs in HIV-1-specific CD8+ T cells. A. Pie charts represent functional profiles associated with CD107a, IFNγ and IL-2 (upper panel) and IR expression in HIV-1-specific CD8+ T cells from infected individuals taken from IFNγ positive cells (bottom panel). Data from four HIV-1-infected individuals following 6 hours of stimulation with SEB or HIV-1 Gag peptides are shown. For both representations, the pie slices represent the frequency of cells with expression of one or more markers, and the pie arcs represent the specific markers expressed. The gray gradient below indicates the magnitude of killing of HIV-1-reactivated cells by four HIV-1-infected individuals. **B.** Correlation (ρ) of the frequency of HIV-1-specific CD8+ T cells lacking IR expression and the magnitude of SAHA-reactivated cell killing. The line indicates the fit of the data to a linear regression.

Discussion

Clinical use of LRAs revealed detectable perturbations in the HIV-1 reservoir with no measurable impact on reservoir size [182]–[184], [191]. Therefore, the potential of shock and kill strategies to purge HIV-1 reservoirs should be carefully evaluated to delineate current therapeutic limitations. On the one hand, LRAs should be able to induce HIV-1 latency reversal for recognition by immune effector cells. On the other, the functional characteristics of CD8+ T cells should ensure the effective clearance of reactivated cells. Here, we report the barriers that account for the immune recognition of latently HIV-1-infected cells by CD8+ T cells in the context of shock and kill cure strategies. Our data demonstrate the potency of LRAs for speeding up the recognition of reactivated cells and CD8+ T-cell dysfunction associated with the expression of IRs as limitations to ensure the killing of HIV-1-reactivated cells. Thus, we characterize the level of HIV-1 protein expression upon treatment with LRAs as a mechanism that is essential for rapid and potent killing of reactivated cells by CD8+ T cells. Moreover, our findings suggest high inter-individual variation in CD8+ T-cell responsiveness to HIV-1 reactivation, where the expression of IRs modulates the magnitude of killing.

As shown by previous reports, we found how reactivation of latently infected cells by LRAs enables HIV-1 antigen presentation for recognition and killing by CD8+ T cells [184], [283]. Our data are consistent with those from previous studies and suggest that once LRAs induced HIV-1 antigen production, there was no interference in antigen presentation in the context of HLA-class I molecules for recognition by CD8+ T cells [262]. In addition, we were able to characterize in detail the characteristics of the so-called window of opportunity between reactivation and killing for CD8+ T cells. In our experimental framework, the killing of reactivated cells can happen as early as 3 hours after co-culture, under conditions where LRA treatment achieves the highest reactivation level. The correlation between LRA potency measure as HIV-1-fold induction, and the speed of recognition by CD8+ T cells uncover the presence of a threshold of antigen production after administration of LRAs for killing. Although our experimental data propose a threshold above a 2-fold induction of HIV-1 reactivation for CD8+ T-cell killing, we need to remember that the data were obtained in the context of two particular CTLs. Our results constitute a proof of principle on the need for potent LRAs to ensure the rapid recognition of HIV-1-reactivated cells for effective shock and kill strategies, even in the context of fully functional CD8+ T-cell responses. However, we cannot extrapolate the current findings as this theoretical threshold may vary across HIV-1-infected individuals with differences in TCR affinities [269], [284]. It is necessary

to perform further experiments that evaluate diversity in TCR affinities between CD8+ T-cell clonotypes in HIV-1-infected individuals in the context of LRAs in order to ensure accurate delineation of the early reactivation events and broad immune recognition of LRA-reactivated cells. The consistency of our data across CTLs may indicate that clearance of reactivated cells is independent of CTL antigen specificity when an adequate threshold of reactivation is reached.

Furthermore, we did not find differences in the levels of functional profiles by CTLs between untreated and HDACi-treated conditions, which may constrain their use as biosensors of HIV-1-reactivated events, as previously suggested [278]. As mentioned, the use of highly sensitive CTLs in our experiments may limit their capacity to detect small differences in the number of antigens presented between the conditions tested. Thus, we propose to explore additional markers of CD8+ T-cell activation to validate their use as biosensors of HIV-1 reactivation. We also detected a significant increase in cytokine production, degranulation and killing by CTLs under Bryo conditions in the absence of HIV-1 reactivation [262], [274]. Such a pre-activation status driven by residual Bryo despite extensive washout, or by the direct effect of Bryo on target cells did not show a measurable benefit concerning faster or better killing when compared with HDACi alone. These data alert to the potential effect of using Bryo, which leads to undesirable and non-specific CD8+ T-cell activation and cytokine production, as suggested in other studies [261], [274].

Together with LRA potency, CD8+ T-cell functional status is essential to guaranty the efficacy of shock and kill strategies to clear the HIV-1 reservoir. Our data indicate biological diversity across *ex vivo* CD8+ T cells from HIV-1-infected individuals in response to LRA-reactivated cells. In our experimental framework, we can exclude some of the barriers for CD8+ T-cell function, including viral escape [259], anatomical compartmentalization [285], and lack of viral protein translation [260]. Therefore, the differences observed here are mainly due to functional differences between CD8+ T cells from HIV-1-infected individuals. Although we demonstrate an increase in the killing of reactivated cells by *ex vivo* CD8+ T cells after treatment with SAHA, we found differences in the kinetics and the total magnitude of killing between individuals. These differences in response to LRA treatment reflect the heterogeneity of the HIV-1-specific CD8+ T-cell responses, even in the context of the HLA-B*27 allele expressed in our target cells, which has been associated with strong antiviral responses and elite control of HIV-1 [253].

Multiple factors account for the functional diversity of HIV-1-specific-CD8+ T-cell responses irrespective of the clinical stage of HIV-1, including the low frequency of HIV-1-specific CD8+ T cells upon antigen clearance by long-term cART and continuous immune CD8+ T-cell activation despite cART [257], [258]. Moreover, HIV-1-specific CD8+ T-cell dysfunction is associated with a high level of expression of IRs including PD-1, TIM-3, LAG-3, and TIGIT [101], [116], [265]. These markers have been associated with CD8+ T-cell dysfunction and Tex in chronic infections [264], [266]. Our *in vitro* data demonstrate that upon continuous exposure to HIV-1 antigens, there is a contraction of subpopulations lacking IRs. This is concomitant with the expansion of CTL subpopulations co-expressing at least three IRs, including PD-1, LAG-3, TIM-3, and the metabolic marker CD39 [126]. In our experimental setting, the expansion of CTL co-expressing IRs over time diminished the magnitude of the killing of LRA-reactivated cells. However, our *in vitro* model does not account for the pro-inflammatory environment of HIV-1 infection. Further studies including the use of pro-inflammatory cytokines and characterization of the expression of IRs in tetramer-sorted HIV-1-specific cells expose to HIV-1 peptides will help to clarify whether our findings can be extended to HIV-1-specific circulating CD8+ T-cells.

Despite the limitations mentioned, our findings were consistent with the direct use of *ex vivo* CD8+ T cells, where the frequency of HIV-1-specific CD8+ T cells lacking IRs directly correlated with the magnitude of killing of LRA-reactivated cells. This is the first study to our knowledge that suggest an inter-dependency between CD8+ T-cell function and the expression patterns of IRs in response to HIV-1 reactivation [96].

The identification of novel IRs as markers of HIV-1-specific CD8+ T-cell dysfunction has increased in recent years. The interest has risen due the unprecedented success of clinical trials targeting IRs in cancer regression [286], [287]. Consequently, additional efforts are needed to clarify the impact of ICIs in HIV-1-infected individuals. Immunotherapy based on blockade of IRs has proven effective in various types of cancer. The use of PD-1 inhibitors have demonstrated to be safe and effective in HIV-1-infected individuals with lung cancer [288]. Moreover, the use of an αPD-L1 in HIV-1-infected individuals has already been evaluated in a Phase I clinical trial [233], in which αPD-L1 had a limited effect on the recovery of HIV-1-specific CD8+ T-cell responses (2 out of 6 patients). Consequently, the delineation of the functional characteristics of HIV-1-specific CD8+ T cells behind successful shock and kill strategies will play a key role in the assessment of treatment efficacy. The efforts to define these traits should accompany the development of ultrasensitive technologies for the monitoring of small

perturbations in the size of the HIV-1 reservoir in response to low fractions of reactivated cells killed by CD8+ T cells in blood or tissues. These technologies will be essential to guide the outcome of curative strategies.

In summary, our study suggests the need for a strong flush of latent virus to speed up recognition of HIV-1-reactivated cells and an adequate functional capacity of HIV-1-specific CD8+ T cells to maximize elimination of LRA-reactivated cells. An adequate trade-off between shock and kill is essential to ensure of potential curative strategies. The immune characterization of HIV-1-specific CD8+ T-cell responses, including patterns of IRs associated with the killing of reactivated cells, will allow the design of novel immune-monitoring and immune based therapeutics to ensure effective antiviral responses before latency reversal in HIV-1-infected individuals.

Acknowledgements, funding, and conflict of interest

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Supplemental Material

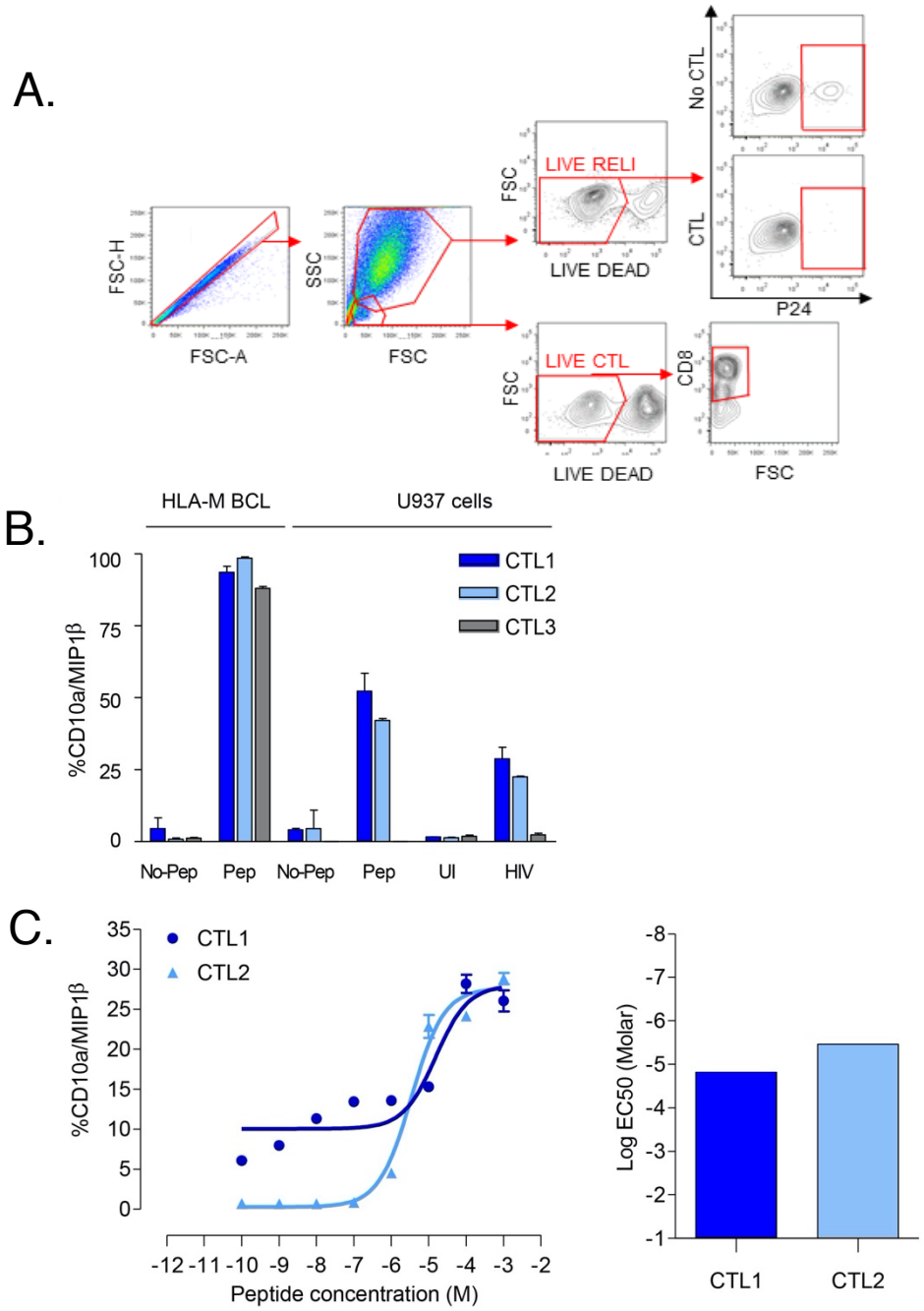

Figure supplementary 1. RELI model gating strategy and CTL functional profiles. A. The "shock" in target-RELI cells was measured by intracellular p24 and GFP expression levels by flow cytometry. The "kill" was evaluated with the reduction of p24-positive cells in the absence or presence of CTLs. Cellular viability was monitored both in target and effector cells. **B.** CD107a/MIP-1β from CTL1, CTL2 and CTL3 after 3 hours of co-culture with different target cells: peptide-pulsed HLA-matched B-cell lines (HLA-M BCL), and the HLA-B*27 U937 cell line, peptide-pulsed or not, and HIV-1-infected or not. CTL1 and CTL2 matched with HLA-B*27 U937 cells, while CTL3 is a mismatch for this cell line. **C.** Curves of CD107a/MIP-1β from CTL1 and CTL2 after co-culture with U937 cells pulsed with serially diluted cognate peptide. U937 cells were pulsed with the indicated Log10 serial peptide dilutions for one hour at 37ºC before co-culture with CTL1 or CTL2 for 3 hours. The CD107a/MIP-1β profile was determined by flow cytometry. Log EC50 values were calculated and plotted for CTL1 and CTL2.

Table supplementary 1. Cellular toxicity associated with LRA treatment.

			Viability (mean± SD)	
Drug		Concentration	RELI	CTL
Untreated			84.9 ± 11.0	70.2 ± 5.3
DMSO		0.2 %	82.5 ± 10.5	66.2 ± 9.0
PMA/Ionomicin	PMA/IO	8 nM / 2 µM	79.0 ± 2.8	75.6 ± 8.6
Panabinostat	PNBN	30 nM	80.2 ± 10.4	69.4 ± 10.5
Romidepsin	RMD	40 nM	72.9 ± 15.2	67.1 ± 14.2
Trichostatin A	Tricho	250 nM	71.7 ± 16.7	73.2 ± 6.9
Vorinostat	SAHA	1 µM	63.7 ± 17.7	72.9 ± 9.1
Bryostatin A	Bryo	10 nM	71.8 ± 22.8	68.0 ± 9.5
Panabinostat + Bryo	PNBN/Bryo	30 nM / 10 nM	86.9 ± 8.6	64.8 ± 13.4
Romidepsin + Bryo	RMD/Bryo	40 nM / 10 nM	65.9 ± 19.1	69.4 ± 10.8
Trichostatin A + Bryo	Tricho/Bryo	250 nM / 10 nM	72.8 ± 26.3	69.4 ± 12.2
Vorinostat + Bryo	SAHA/Bryo	1 µM / 10 nM	66.2 ± 19.0	64.9 ± 13.1

CHAPTER II. Selective depletion of CD107a^{hi} TIGIT^{hi} memory HIV-1-specific CD8+ T cells during long-term suppressive cART

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Autor's contributions: The author of this thesis contributed by measuring the surface and functional immunophenotyping of CD8+ T cells by flow cytometry. Moreover, measuring the effect of IR blockade. The author also contributed in the designing of the study, analyzing single-cell data, interpreting the results, and writing the manuscript.

Abstract

The functional regulation of CD8+ T cells through the expression of IRs remain elusive in health and disease. During continuous antigen stimulation such as HIV-1 infection and cancer, the upregulation of IRs can lead to the loss of cellular functions and T-cell exhaustion (Tex). We evaluate the combinatorial IR expression and functional markers across the landscape of CD8+ T cells in 24 longitudinal HIV-1-infected individuals in cART to characterize the immunological parameters of Tex. We use high-dimensional cytofluorimetrics and comparative unsupervised net-SNE single-cell analyses. Alterations in the immune profile based on IR expression become irreversible despite cART in HIV-1 infection. Our data reveal that high TIGIT+ expression in TM-CM CD8+ T cells persists after long-term cART. In addition, TIGIT+ in total CD8+ T cells inversely correlates with CD4+ T-cell counts. Single-cell analysis unravel a wide heterogeneity and complexity of combinatorial IR expression across total, TCR-activated polyclonal and HIV-1-specific CD8+ T cells. Cluster deconvolution in total CD8+ T cells discloses contractions of CM-TM-like clusters lacking IRs concomitant with expansions of Eff-like with IR poly-expression. Polyclonal CD8+ T cells show similar homeostatic changes. HIV-1-specific single-cell reveals a unique cluster of memory-like $TIGIT^{hi}CD107a^{hi}$ with depletion in cART. TIGIT+ CM HIV-1-specific CD8+ T cells impacted the CD107a profile, and this defect was recovered by short-term TIGIT blockade. In summary, this study characterizes the expression of TIGIT as a biomarker of Tex in CD8+ T cells. We propose targeting TIGIT to regain the lost degranulation function of HIV-1-specific CD8+ T cells in cART.

Introduction

The expression of IRs plays a critical role in cellular homeostasis and self-tolerance, driving the regulation of T-cell activation until the resolution of acute viral infections [105], [106], [134], [137], [138]. IRs are transiently expressed on effector and memory CD8+ T cells until the viral antigen is cleared [55], [230]. However, in the face of persistent antigen stimulation in chronic infections and immune activation, CD8+ T cells become dysfunctional and exhausted [103]–[106]. The progressive loss of effector function defines Tex. Tex is linked to persistent expression of IRs together with changes in transcriptional, epigenetic, and metabolic profiles [113], [289]–[291].

In HIV-1 infection, CD8+ Tex cells limit the complete elimination of latently HIV-1-infected cells and are currently one of the significant barriers to developing effective therapeutics to cure HIV-1 infection [292], [293]. The introduction of cART normalizes the levels of CD4+ and CD8+ T-cell counts and potentially preserves immune function in HIV-1-infected individuals [294]–[297]. However, persistent immune activation through residual viral replication at sanctuary sites contributes to Tex in CD8+ T cells. Several studies have shown that despite prolonged periods of cART, the expression of IRs including TIGIT, PD-1 and LAG-3 is higher in HIV-1-infected individuals than uninfected controls [95]–[101]. Indeed, these markers alone or in combination with other IRs are associated with clinical HIV-1 progression [293], suggesting that morbidity may increase after long-term cART as CD8+ Tex cells fail to eliminate residual HIV-1-infected cells.

In the past years, the use of IR blocking antibodies or ICIs has represented an unprecedented success in the cancer field, affording complete clinical remission in a fraction of patients [100], [101], [123], [298]. In chronic viral infections, the use of ICIs have been proposed to reverse Tex of antigen-specific CD8+ T cells [179], [231]. Several studies demonstrated the expansion of HIV-1-specific CD8+ T cells, prolonged cell survival, and increased production of cytokine and cytotoxic molecules after PD-1/PD-L1 blockade, among others pathways [190], [233]–[236]. Interestingly, the combinatory use of ICIs has revelated synergistic effects to reverse dysfunctional HIV-1-specific CD8+ cells [299]. However, the role of IRs co-expression in HIV-1-specific CD8+ T cells and the impact on their functional status is not fully understood in the course of the infection and particularly after long-term cART. Limitations of previous studies include the focus of IR expression in total CD8+ T cells, combinatorial analyses of IRs together

with functional markers in CD8+ T cells, and lack of longitudinal information about immune IR profiling in long-term cART [95], [98], [100], [126], [298].

To overcome previous study limitations, we used multidimensional comparative and unsupervised single-cell analyses of IRs and functional markers across the landscape of CD8+ T cells. We studied longitudinally 24 HIV-1-infected individuals over ten years in fully suppressive cART, early HIV-1-infected individuals, and healthy donors to gain precision in the discovery of biomarkers and immunotherapeutic targets of Tex. The frequency of TIGIT+ and TIGIT+TIM-3+ in memory and effector CD8+ T-cell subsets increased in cART. Levels of TIGIT+ in total CD8+ T cells negatively correlated with CD4+ T-cell counts. Unsupervised single-cell analysis revealed cellular clustering with wide heterogeneity in IR expression among total, polyclonal, and HIV-1-specific CD8+ T cells. At the total CD8+ T-cell level, clustering analysis demonstrated homeostatic contraction of memory-like clusters without IR expression and expansions of Eff-like clusters with IR poly-expression in cART. At the TCR-activated polyclonal CD8+ T-cell level, we identified a similar homeostatic contraction and expansion of clusters linked with IR co-expression after long-term cART. Single-cell analysis in HIV-1-specific CD8+ T cells revealed a selective depletion of $TIGIT^{hi}CD107a^{hi}$ memory-like clusters. Short-term TIGIT blockade recovered degranulation activity of HIV-1-specific CD8+ T cells in cART. These results led us to propose TIGIT as relevant marker of Tex and as immunotherapeutic target to recover degranulation functional activity of HIV-1-specific CD8+ T cells in cART.

Materials and methods

Study group

This retrospective study analyzed clinical data and biological sample availability from a total of 3,000 patients assigned to the HIV-1 clinical unit of the Germans Trias i Pujol University Hospital. We excluded individuals with integrase inhibitors, cART as monotherapy, cART with mitochondrial toxicity including Trizivir, d4T, ddI,, AZT and blips over the cART period. We included individuals with cryopreserved PBMCs available in our collection. We identified 24 chronically HIV-1-infected individuals who had been treated mainly with a combination of NNRTI and NRTI for more than ten years with sustained virological suppression (<50 HIV-1 RNA copies/ml) and with longitudinal samples at timepoint 1 (S1, median of 2 years on fully suppressive cART) and at timepoint 2 (S2, median of 10 years on fully suppressive cART) (**Figure 24A, Table Supplementary 1**). For comparative purposes, we included 24 early HIV-1-infected

individuals (Ei) defined in a window of two years after seroconversion in the absence of cART and 24 healthy seronegative controls (HC) (**Figure 24A**). The groups were balanced by age to the S2 samples to avoid confounding effects on IR expression (**Table Supplementary 1**). The Ethics Committee of Hospital Germans Trias i Pujol approved all experimental protocols (PI14-084). For the study, subjects provided their written informed consent for research purposes of biological samples taken from them. The study was conducted according to the principles expressed in the Declaration of Helsinki (Fortaleza, 2013).

Immunophenotyping of total, TCR-activated and HIV-1-specific CD8+ T cells

Cryopreserved PBMCs from HC, Ei, S1, and S2 groups were thawed and rested overnight at 37ºC in a 5% CO_2 incubator. The following day, PBMCs were incubated for six hours at 37ºC in a 5% CO_2 incubator under RPMI complemented medium 10% FBS in the presence of CD28/49d co-stimulatory molecules (1µl/ml, BD), Monensin A (1µl/ml, BD Golgi STOP), and anti-human antibody for CD107a (PE-Cy5, clone H4A3, Thermo Fisher Scientific). PBMCs were left unstimulated, stimulated with SEB for polyclonal TCR-activation (1µg/ml, Sigma-Aldrich), and stimulated with HIV-1-Gag peptide pool (2µg/peptide/ml, EzBiolab). After six hours of stimulation, cells were rested overnight at 4ºC as previously described [235]. The next day, PBMCs were washed with PBS 1X and stained 25 minutes with the Live/Dead probe (APC-Cy7, Thermo Fisher Scientific) at RT to discriminate dead cells. Cells were washed with PBS 1X and surface stained with antibodies for 25 minutes at RT. We used CD3 (A700, clone UCHT1, BD), CD4 (APC-Cy7, clone SK3, BD), CD8 (V500, clone RPA-T8, BD), CD45RA (BV786, clone HI100, BD), CCR7 (PE-CF594, clone 150503, BD), CD27 (BV605, clone L128, BD), TIGIT (PE-Cy7, clone MBSA43, Labclinics SA), PD-1 (BV421, clone EH12.1, BD), LAG-3 (PE, clone T47-530, BD), TIM-3 (A647, clone 7D3, BD) and CD39 (FITC, clone TU66, BD) antibodies. Afterward, cells were washed twice in PBS 1X, fixed and permeabilized with Fix/Perm kit (A and B solutions, Thermo Fisher Scientific) for intracellular cytokine staining with anti-human antibodies of IFNγ (BV711, clone B27, BD) and IL-2 (BV650, clone MQ1-17H12, BD). Finally, stained cells were washed twice with PBS 1X and fixed in formaldehyde 1%.

TIGIT and TIM-3 short-term antibody blockade experiments

We selected cryopreserved PBMCs from S1 (n=10) and S2 (n=10). Samples were previously characterized by the expression of TIGIT and TIM-3 on total CD8+ T cells.

PBMCs were thawed and rested for four hours at 37ºC in a 5% CO_2 incubator. Next, cells were incubated under RPMI complemented medium 10% FBS with 1µl/ml of anti-CD28/CD49d and 1µl/ml of Monensin A overnight at 37ºC in a 5% CO_2. PBMCs divided into three conditions; 1) unstimulated, 2) SEB (1µg/ml, Sigma-Aldrich) and 3) HIV-1-Gag peptide pool (2µg/peptide/ml) in the absence or presence of αTIGIT and/or αTIM-3 and its respective isotype antibodies. For the single blockade of TIGIT (αTIGIT), we included Ultra-LEAF™ purified anti-human TIGIT antibody (10µg/ml, clone A15153G, Biolegend) or its control isotype Ultra-LEAF™ purified mouse IgG2a antibody (10µg/ml, MOPC-173, Biolegend). For single TIM-3 blockade (αTIM-3), we used Ultra-LEAF™ purified anti-human TIM-3 antibody (10µg/ml, clone F38-2E2, Biolegend) or its respective isotype Ultra-LEAF™ purified mouse IgG1 antibody (10µg/ml, MOPC-21, Biolegend). Finally, we included αTIGIT+αTIM-3 or their respective IgG2+IgG1 isotypes for a combinational blockade. The next day, PBMCs were surface and intracellularly stained with the panel of antibodies and the methodology described in the section above.

Flow cytometry analysis

Stained PBMCs were acquired on an LSR Fortessa cytometer using FACSDiVa software (BD). Approximately 1,000,000 events of PBMCs were recorded per specimen. Antibody capture beads (BD) were used for single-stain compensation controls. Flow cytometry data were analyzed with FlowJo software v10.6.1, and fluorescence minus one (FMO) was used to set manual gates. We analyzed CD8+ T cells by excluding dump and CD4+ T cells. As previously described, IRs were measured in CD8+ T-cell subsets including Naïve, CM, TM, EM, Eff CD8+ T cells [95], [235]. For polyclonal TCR-activated and HIV-1-specific CD8+ T-cell cytokine production, we subtracted the background of CD107a, IFNγ, and IL-2 from unstimulated PBMCs.

Single-cell immunophenotype data analysis

The phenotypic and functional characterization of cellular populations was analyzed by using t-Distributed Stochastic Neighbor Embedding (t-SNE) [300] and net-SNE [301] dimensionality reduction algorithms to visualize single-cell distributions in two-dimensional maps. Briefly, cell intensity was z-normalized, and a randomly selected subset of cells, at least more than 1,000 cells per sample, was passed through the t-SNE algorithm. The resulting t-SNE dimension was then used to predict the position of all remaining cells acquired per sample by using the net-SNE algorithm based on neural networks. All available CD8+ T cells of each sample from each group of individuals were

merged and mapped into net-SNE maps. For functional analysis, we selected TCR-activated polyclonal and HIV-1-specific CD8+ T cells producing at least CD107a, IFNγ or IL-2 under SEB or HIV-1 conditions, respectively. In addition, an unsupervised KNN algorithm was implemented for cell clustering by phenograph algorithm and Louvain method [302], [303]. Each cluster composition in the heatmap was represented using the means of cellular clusters and markers of interest. The color scale was represented as the median intensity of each marker on a biexponential scale. For each sample, we calculated quantitative assessments of cellular clusters in the percentage of cells to analyze and compare the distribution between HC, Ei, S1, and S2 groups in a similar way to the classical flow cytometry analysis. Clusters with high similitudes in marker expression and between groups were merged to increase the number of cells and obtain robust data.

Statistical analysis

Univariate analysis was conducted using nonparametric methods as follows: Mann-Whitney U test for independent median comparison between groups, signed-rank test for paired median changes over time, permutation test for composition distribution between groups, Kruskal-Wallis test for comparison between more than two groups, and spearman linear correlation coefficient to study the association between continuous variables. All statistical tests were under a significance level of 0.05, and statistics were programmed and performed using the R statistical package [304]. Moreover, pattern distribution and graphical representations of all possible Boolean combinations for IR poly-expression and functional markers were conducted using the data analysis program Pestle v2.0 and SPICE v6.0 software [272]. Graph plotting was performed by GraphPad Prism v8.0 software.

Results

Elevated TIGIT+ and TIGIT+TIM-3+ expression persists in CD8+ T-cell subsets after cART suppression

Initially, we assessed longitudinal changes in CD8+ T-cell phenotype at the level of classical gating strategy in the study groups. We monitored frequencies of PD-1, TIGIT, LAG-3, TIM-3, and CD39 expression in total and CD8+ T-cell subsets (**Figure Supplementary 1A to C**) and combinatorial IR expression between the study groups (**Figure 24A to C**).

We found a preferential distribution of TIGIT, PD-1, and CD39 expression across Eff, TM, and CM CD8+ T cells, respectively (**Figure Supplementary 1B and C**). Levels of TIGIT in TM, LAG-3 in CM, and TIM-3 in EM and Eff CD8+ T cells were significantly higher in Ei and S1 compared to HC ($p<0.05$ for all comparisons). Although most of the markers normalized with time on cART, TIGIT remained elevated in TM during S2 (HC vs. S2; $p<0.05$) (**Figure Supplementary 1C**). Combinatorial IR profiling revealed significant perturbations in CM and TM CD8+ T cells (HC vs. Ei; HC vs. S1; $p<0.05$) that persisted overtime only in CM in S2 (HC vs. S2; $p<0.05$) (**Figure 24B**). These alterations were marked by a reduction of CM lacking IRs and an increase of CM expressing one IR during cART (HC vs. S1 and S2; $p<0.05$) (**Figure 24C**). In addition, we observed increasing of TM, EM, and Eff CD8+ T cells expressing >3 IR combinations in early HIV-1 infection and S1 (HC vs. Ei and S1; $p<0.05$) (**Figure 24C**).

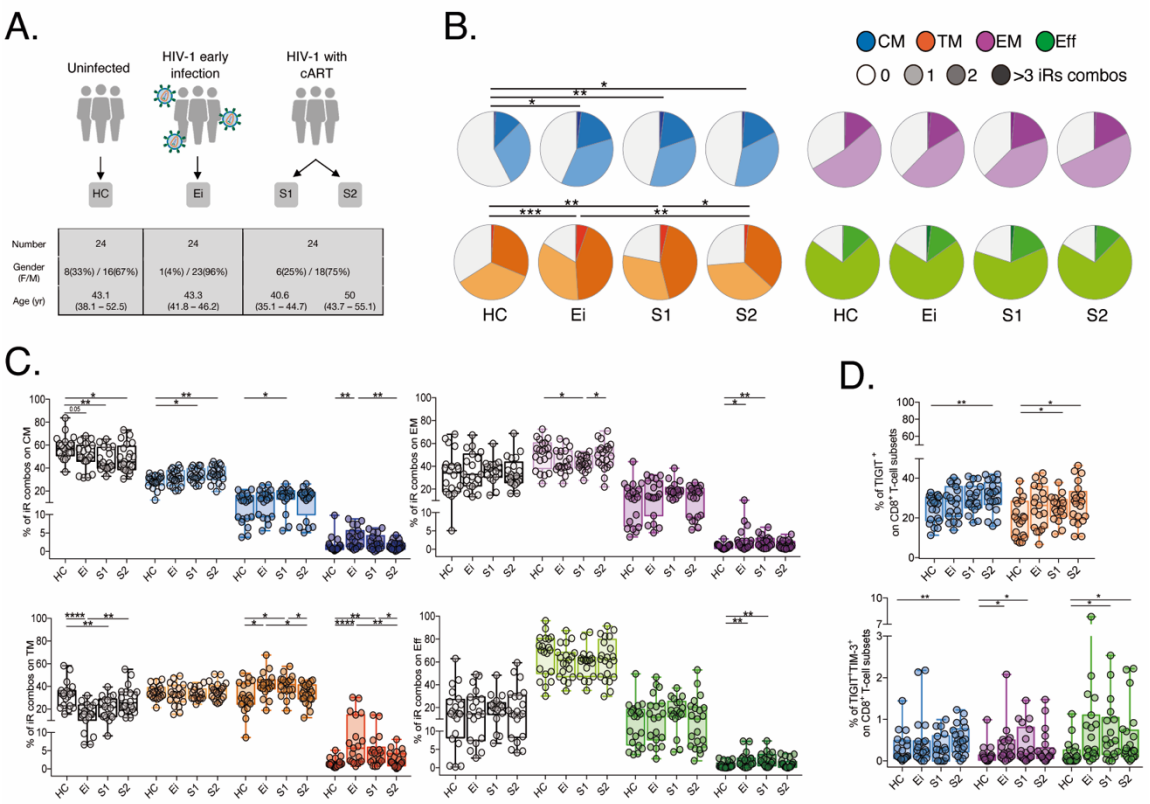

Figure 24. Combinatorial profiling of IRs. A. Overview of study groups, healthy controls (HC), early HIV-1-infected individuals (Ei), and HIV-1-infected individuals on fully suppressive cART in S1 and S2. **B.** The expression of IRs is summarized in the pie-chart as none, one, two, or more than three IRs. For statistical analysis, we used permutation tests using SPICE software. **C.** Scatter plots showing the median and interquartile ranges of IR combinations. **D.** Scatter plots of single TIGIT+ and TIGIT+TIM-3+ frequencies. (**B-D**) IR combinations are color-coded as a gradient scale. Each subset is colored as indicated in the legend. Naïve CD8+ T cells are not shown. We used the Mann-Whitney test in unpaired samples and the Wilcoxon test in paired samples for statistical analysis. P-values: *<0.05, **<0.005 and ***<0.0005.

Next, we investigated the 32 possible combinations of IR expression at the level of CD8+ T -cell subsets (**Figure Supplementary 2**). We found a significant expansion of single TIGIT+ in CM and TM after long-term cART (HC vs. S2; p<0.05). Similarly, the expression of TIGIT+ combined with TIM-3+ was increased after long-term cART in CM and Eff CD8+ T cells (HC vs. S2; p<0.05) (**Figure 24D**).

Next, we analyzed the association between the frequency of total and CD8+ T-cell subsets expressing IRs and CD4+ T-cell counts in Ei, S1, and S2 groups (**Figure 25**). We found negative correlations in single TIGIT+ expression (p=0.0157, r=-0.58) and >1 IR expression (p=0.0386, r=-0.51) in total CD8+ T cells between CD4+ T-cell counts in S2 (**Figure 25**). Similarly, the expression of >1 IR in CM and TM CD8+ T cells negatively correlated with CD4+ T-cell counts in S1 (p=0.0087, r=-0.61) and S2 (p=0.0346, r=-0.52), respectively. CM CD8+ T cells also were negatively correlated in the expression of two combinations of IRs in both timepoints S1 (p=0.0054, r=-0.64) and S2 (p=0.0072, r=-0.64). Of note, we found a positive correlation between the expression of TIGIT+TIM-3+ in EM CD8+ T cells in S1 (p=0.033, r=0.53) (**Figure 25**). These data support an increase of single TIGIT+ and TIGIT+TIM-3+ co-expression in CD8+ T-cell subsets initiated in early HIV-1 infection and persisted despite long-term suppressive cART. Of note, the high frequency of CD8+ T cells expressing single TIGIT+ was associated with low numbers of CD4+ T cells despite long-term suppressive cART.

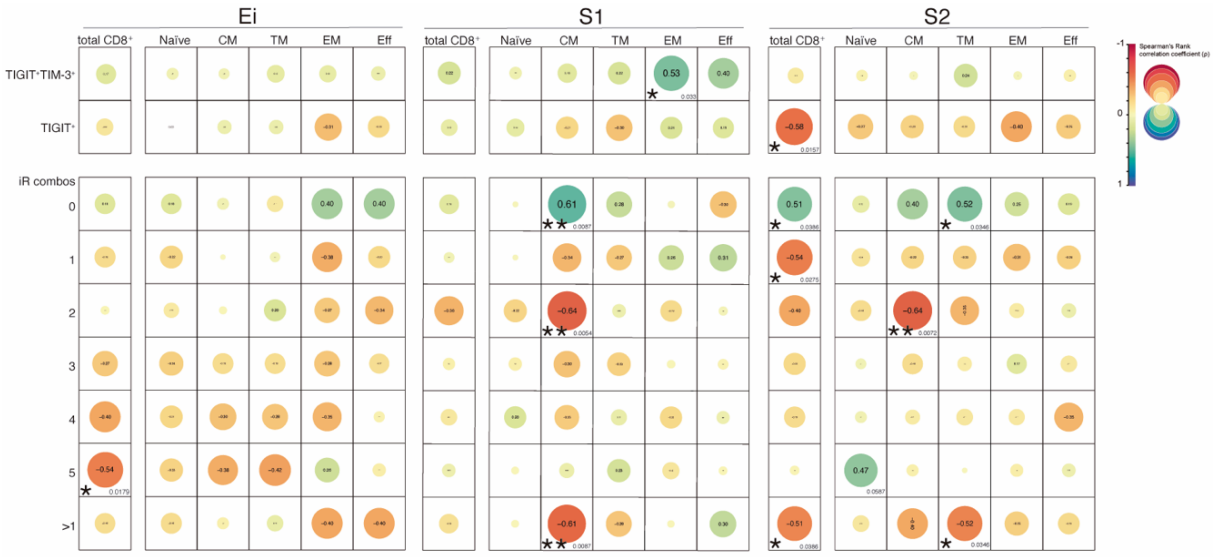

Figure 25. Immune correlations of IR expression in CD8+ T cells. Correlations between CD4+ T-cell counts as a function of single TIGIT+, TIGIT+TIM-3+ and combinations of IRs from total CD8+ T cells and subsets in Ei, S1, and S2. Asterisks mark statistically significant values. The correlations of the 32 boolean IR combinations are not shown. P-values: *<0.05, **<0.005 and ***<0.0005.

cART leads to an increase of total CD8+ T cells with high heterogeneity and complexity in IR co-expression

Next, we sought to characterize IR signatures in total CD8+ T cells using an unsupervised single-cell net-SNE analysis between the study groups. We concatenated 1,988,936 total CD8+ T cells, and we analyzed the phenotypes with the topographical regions of each surface marker (**Figure 26A and B**). Cells were classified into 38 cellular clusters distributed according to the relative marker expression of 14-parameters (**Figure 26C to D**).

Out of the 38 clusters identified, we observed 15 cellular clusters with statistical differences in frequency between the study groups (**Figure 26D**)**.** Of note, we merged the clusters with high similitudes (#5, #6, and #7), (#9 and #10), and (#12, #13, and #14) to increase the number of cells in the quantitative assessments (**Figure 26E and F**). Of these 15 cellular clusters, #1, (#5-7), #8, (#9-10), #11, and (#12-14) markedly altered in composition and proportion in early HIV-1 infection (HC vs. Ei; $p<0.05$) and #1 to #15 after cART (HC vs. S1 or S2; $p<0.05$) (**Figure 26E and F**). While low differentiated CD8+ T-cell clusters lacking IRs decreased in frequency, Eff-like clusters with co-expression of IRs increased in cART. Indeed, naïve, CM, or TM-like CD8+ T-cell clusters (#1 to #7) expressing low numbers of IRs decreased the cell's frequency in cART suppressed individuals (HC vs. S1 or S2; $p<0.05$) (**Figure 26E**). PD-1 was the most expressed IR in clusters #1, #2, and #3. By contrast, Eff or EM-like CD8+ T-cell clusters (#8 to #15) with co-expression of IRs increased in frequency on cART (HC vs. S1 or S2; $p<0.05$) (**Figure 26F**). We observed predominant co-expression of TIGIT, LAG-3, and TIM-3. Notably, we identified in cluster #15 an expansion of naïve-CM-like CD8+ T cells expressing $TIGIT^{hi}$ after long-term cART (HC vs. S1 and S2; $p<0.05$) (**Figure 26F**). These data support previous observations by classical gating.

These results identify homeostatic changes of contraction and expansion of total CD8+ T-cell clusters in the course of HIV-1 infection and after long-term cART. Indeed, the cART period was characterized by expanding CD8+ T cells with Eff-like properties and high heterogeneity and complexity in IR co-expression.

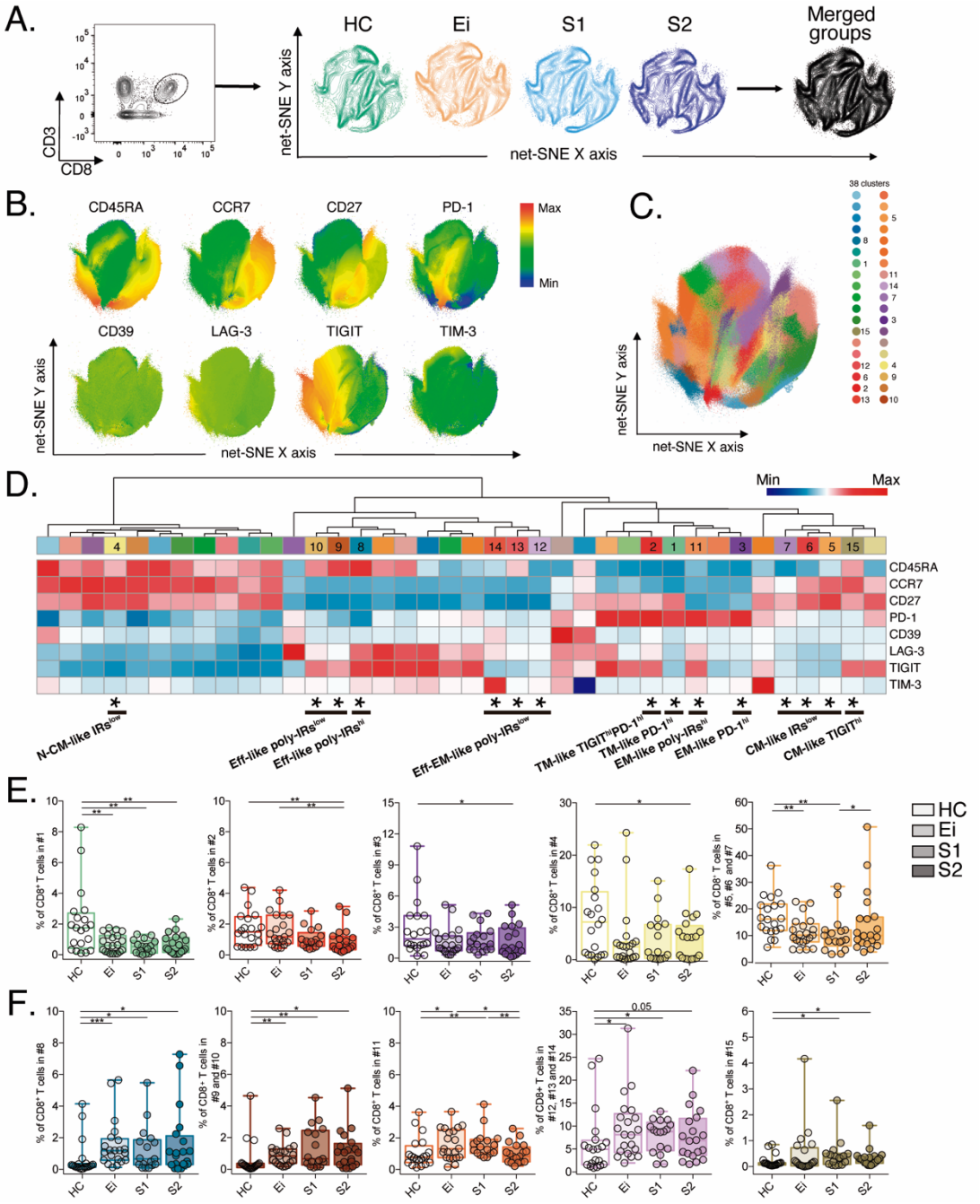

Figure 26. Unsupervised net-SNE and cellular clustering of total CD8+ T cells. A. Gating strategy for the selection of total CD8+ T cells and concatenation of 1,988,936 events between HC (green), Ei (orange), S1 (light blue), and S2 (dark blue) groups. Merged groups are shown in black. **B.** Representative net-SNE visualization of surface markers. The color gradient displays the relative marker expression. **C.** Unsupervised KNN algorithm of 38 clusters colored according to the legend. Only clusters with statistical differences are represented in the legend. **D.** Heatmap of the median biexponential-transformed marker expression normalized to a -3 to 3 range of respective markers. Asterisks represent the clusters with statistical differences to HC (HC vs. Ei, S1, and S2; $p<0.05$). The phenotype-like of the significant clusters is indicated at the bottom of the heatmap. **E** and **F**. Scatter plots showing the median and interquartile ranges of cell's frequency with contraction (**E**) and expansion (**F**) in cART (HC vs. S1 or S2; $p<0.05$). The gradient of colors represents the study groups. Clusters with high similitudes (#5, #6, and #7), (#9 and #10), and (#12, #13, and #14) were merged to increase the number of cells in the quantitative assessments. We used the Mann-Whitney test in unpaired samples and the Wilcoxon test in paired samples for statistical analysis. P-values: =0.05, *<0.05, **<0.005, and ***<0.0005.

cART leads to an increase of CD8+ T cells susceptible to TCR-activation with IR co-expression and Eff-like characteristics

Next, we sought to characterize further functional immune signatures in polyclonal TCR-activated CD8+ T cells using the unsupervised single-cell net-SNE analysis. PBMCs were stimulated with SEB, and 253,021 polyclonal TCR-activated CD8+ T cells producing at least CD107a, IFNγ, or IL-2 were concatenated among study groups (**Figure 27A and B**).

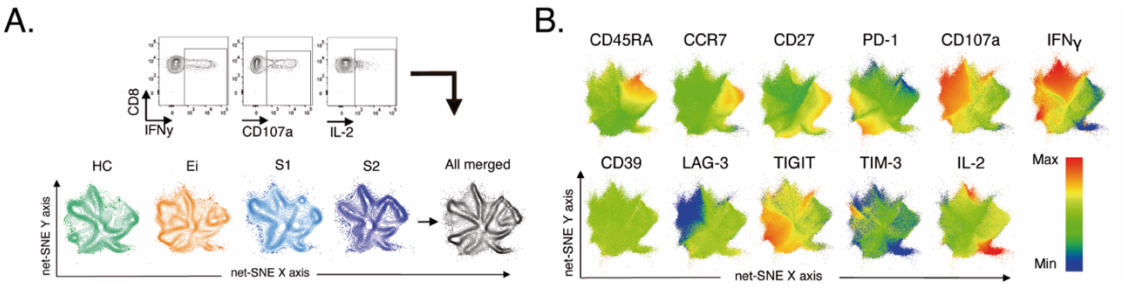

Figure 27. net-SNE representation for polyclonal CD8+ T cells. A. Gating strategy for the selection of polyclonal TCR-activated CD8+ T cells and concatenation of 253,021 events between HC (green), Ei (orange), S1 (light blue), and S2 (dark blue). Merged groups are shown in black. **B.** Representative net-SNE visualization of IR expression, lineage, and functional markers. The color gradient displays relative marker expression.

Polyclonal TCR-activated CD8+ T cells were classified into 29 unique clusters distributed according to the relative marker expression of 14-parameters (**Figure 28A**). Among those, we observed a wide diversity of both IR expression and functional markers with differential frequencies in clusters between HC, Ei, S1, and S2 groups (**Figure 28B to D**). Out of the 29 clusters, we observed 14 polyclonal TCR-activated clusters with statistical differences in frequency between the study groups (**Figure 28B).** Of note, we merged the polyclonal clusters with high similitudes (#1 and #2), (#4 and #5), and (#8, #9 and #10) to increase the number of cells in the quantitative assessments (**Figure 28C and D**). From this, (#1-2), (#4-5), #7, and #14 polyclonal clusters were significantly altered in proportion in early HIV-1 infection (HC vs. Ei; $p<0.05$) and #1 to #14 in cART (HC vs. S1 or S2; $p<0.05$) (**Figure 28C and D**). Similarly to total CD8+ T-cell analysis, we observed a contraction and an expansion of polyclonal TCR-activated CD8+ T-cell clusters in cART, indicating homeostatic cellular processes in early HIV-1-infection and after long-term cART. While polyclonal CM, TM-like CD8+ T-cell clusters (#1 to #6) with co-expression of IRs showed a contraction in frequency, polyclonal Eff-like CD8+ T-cell clusters (#8 to #13) co-expressing IRs increased the cell's frequency in cART. IL-2^{hi} was the most functional marker in clusters (#4-5) and #6 and IFNγ^{hi}CD107a^{hi} in #8 to #13.

These observations support that high numbers of IRs in memory phenotypes may be involved in Tex by reducing polyclonal cell frequency after long-term cART. By contrast, the co-expression of IRs in the effector phenotypes may involve activation through the expansion and persistence of polyclonal cell frequency after long-term cART.

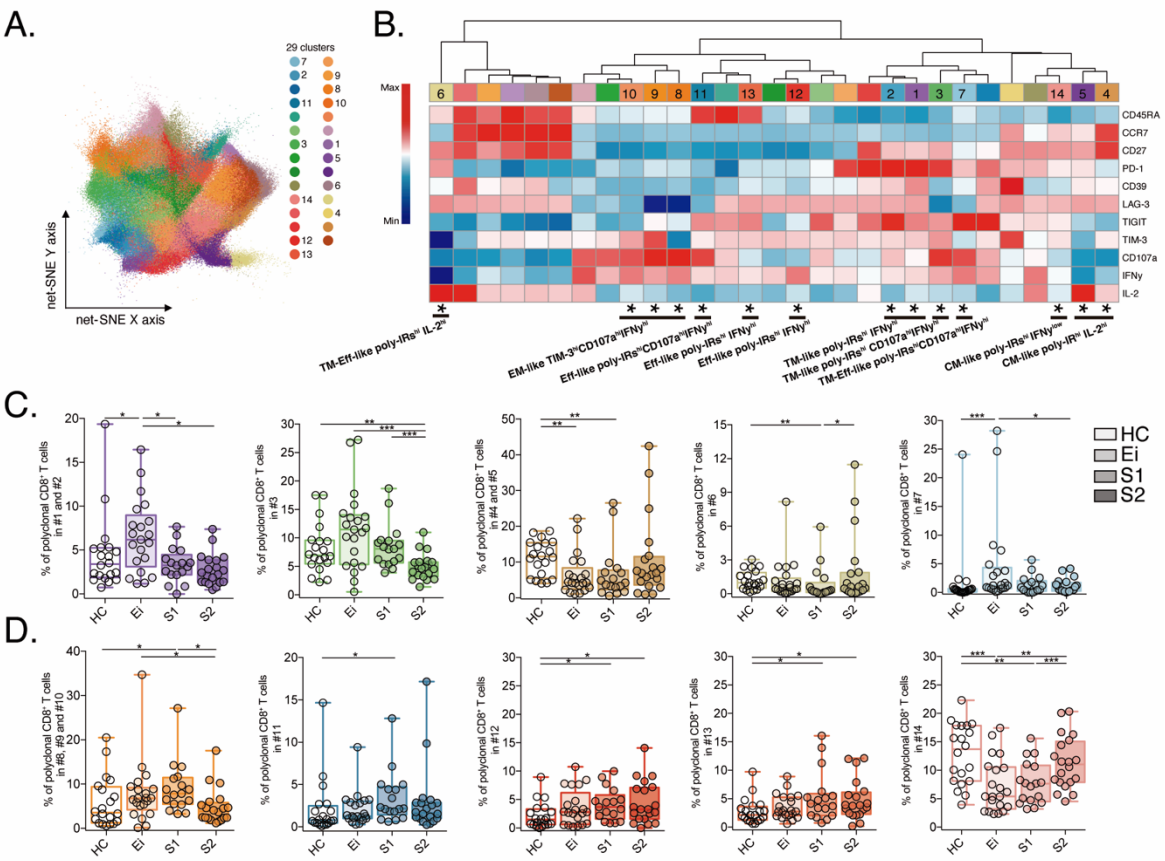

Figure 28. Phenograph and dynamics of polyclonal TCR-activated CD8+ T cells. A. Unsupervised KNN algorithm for 29 polyclonal clusters color-coded according to the legend. Clusters with statistical differences between groups are represented in the legend. **B.** Heatmap of the median biexponential-transformed marker expression normalized to a -3 to 3 range of respective markers. Asterisks represent the clusters with statistical differences to HC (HC vs. Ei, S1, and S2; p<0.05). The phenotype-like of significant clusters is indicated at the bottom of the heatmap. **C** and **D**. Scatter plots showing the median and interquartile ranges of polyclonal cell frequency with contraction (**C**) and expansion (**D**) in cART (HC vs. S1 or S2; p<0.05). The gradient of colors represents the study groups. Clusters with high similitudes (#1 and #2), (#4 and #5), and (#8, #9, and #10) were merged to increase the number of polyclonal cells in the quantitative assessments. We used the Mann-Whitney test in unpaired samples and the Wilcoxon test in paired samples for statistical analysis. P-values: *<0.05, **<0.005, and ***<0.0005.

TIGIT^{hi} and CD107a^{hi} HIV-1-specific memory-like CD8+ T cells decrease in frequency after long-term cART

We next sought to characterize changes in IR expression and functional markers at the level of HIV-1-specific CD8+ T-cells. We evaluated changes from 53,751 events of HIV-1-infected groups based on selecting HIV-1-specific CD8+ T cells producing at least CD107a, IFNγ, or IL-2 (**Figure 29A and B**).

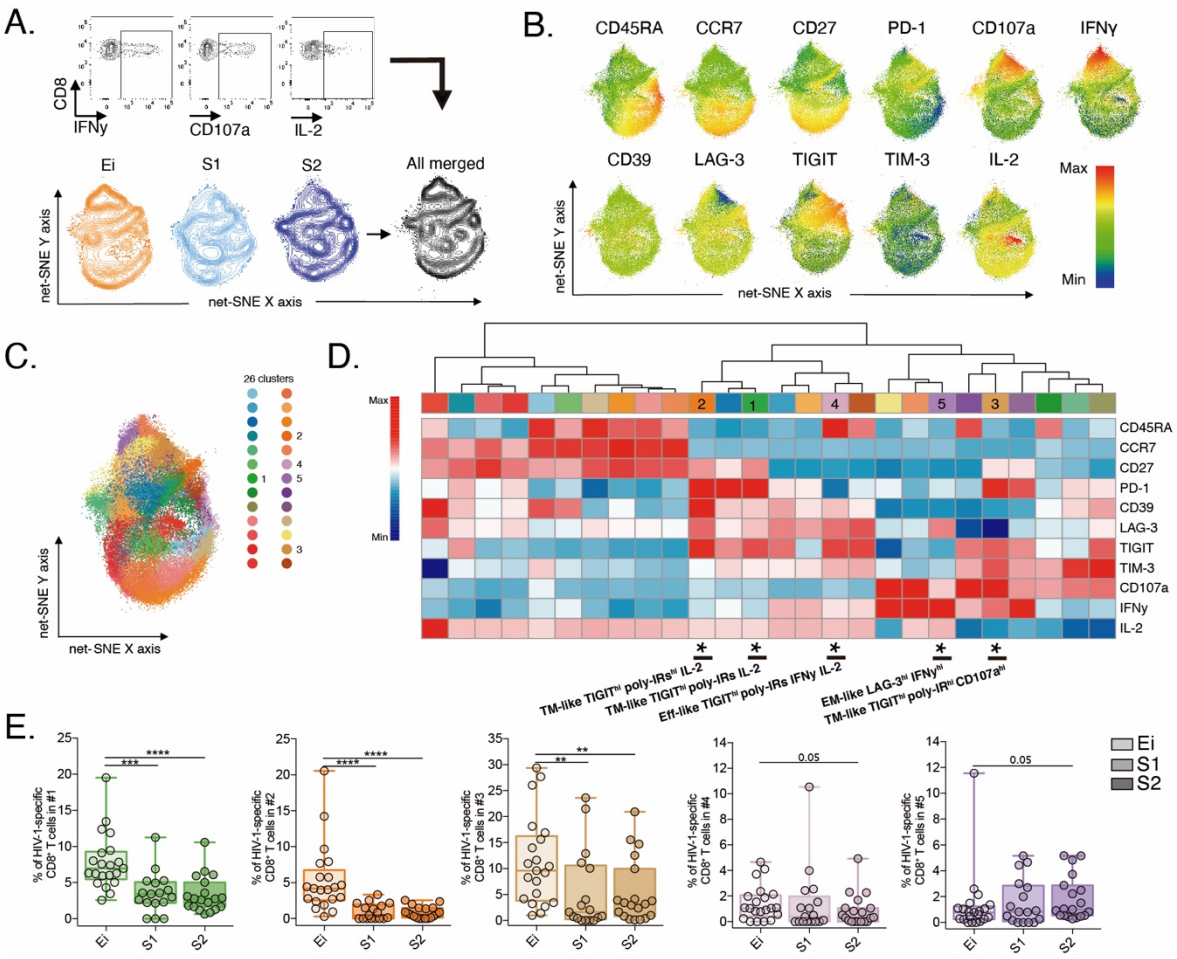

Figure 29. Unsupervised single-cell of HIV-1-specific CD8+ T cells. A. Selection of HIV-1-specific CD8+ T cells producing at least CD107a, IFNγ, or IL-2 and concatenation for Ei (orange), S1 (light blue), and S2 (dark blue). 53,751 events for HIV-1-specific CD8+ T cells were acquired and merged between the groups (black). **B.** Different net-SNE plots with the expression of surface and functional markers. The color gradient displays the relative expression. **C.** Unsupervised KNN algorithm for 26 HIV-1-specific clusters color-coded according to the legend. Only clusters with statistical differences are represented in the legend. **D.** Heatmap of the median biexponential-transformed marker expression normalized to a -3 to 3 range of respective markers. Asterisks represent the clusters with statistical differences to Ei (Ei vs. S1 and S2; $p<0.05$). The phenotype-like of the significant clusters is indicated at the bottom of the heatmap. **E.** Scatter plots showing the median and interquartile ranges of HIV-1-specific CD8+ T cells. The gradient of colors represents the study groups. We used the Mann-Whitney test in unpaired samples and the Wilcoxon test in paired samples for statistical analysis. P-values: =0.05, *<0.05, **<0.005, ***<0.0005, and ****<0.0001.

High-dimensional cellular mapping revealed 26 clusters classified according to the relative marker expression of 14-parameters in HIV-1-specific CD8+ T cells (**Figure 29C and D**). Out of these 26 clusters, only five showed significant differences between HIV-1-infected groups. Meanwhile, cluster #5 increased in cART (Ei vs. S2; p=0.05), four clusters (#1 to #4) were reduced in cART (Ei vs. S1 or S2; $p<0.05$) (**Figure 29E**). Phenotypically, clusters #1, #2, and #3 shared common cellular characteristics, including IR co-expression and particularly TIGIT^{hi} in TM-like HIV-1-specific CD8+ T cells (**Figure 29D**). IL-2^{low} was the common functional marker in clusters (#1 to #4) and

CD107a^{hi}IFNγ^{hi} in cluster #3. Of note, cluster #3 was unique in co-expression of TIGIT^{hi}, TIM-3^{hi}, and PD-1^{hi} and also CD107a^{hi} IFNγ^{hi} which decreased in frequency during cART. To further understand HIV-1-specific single-cell data and highlight TIGIT^{hi} as the main possible IR involved in Tex after long-term durable cART suppression, we analyzed by classical gating strategy the combinations of CD107a, IFNγ, IL-2 in CM CD8+ T cells expressing single TIGIT+ (**Figure 30A**). We found that the combination of one functional marker in CM CD8+ T cells was significantly decreased in cART (Ei vs. S1 and S2; p<0.05) (**Figure 30A**). Notably, the deconvolution of immune profiles revealed CD107a+ as the only functional marker that decreases in TIGIT+ CM CD8+ T cells after long-term suppressive cART (Ei vs. S2; p=0.05) (**Figure 30B**). These data support the depletion observed in the differential cluster #3 with CD107a^{hi} TIGIT^{hi} memory HIV-1-specific CD8+ T cells after long-term cART.

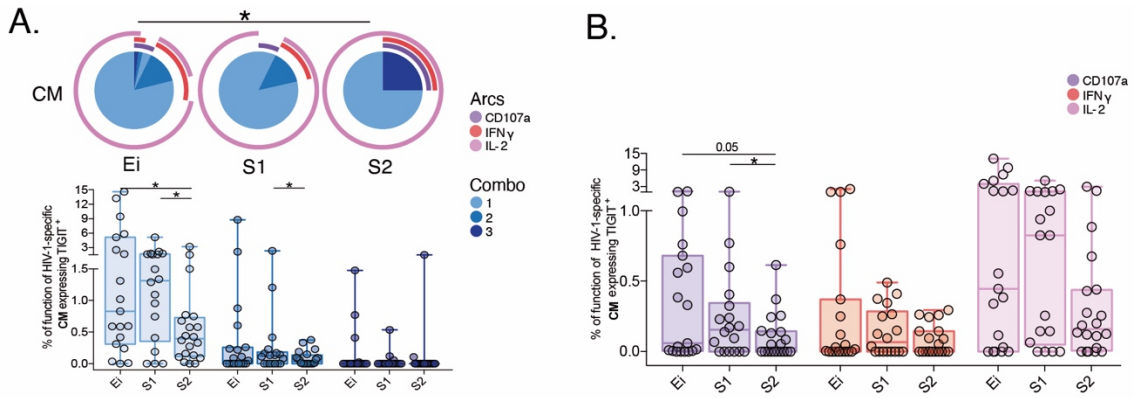

Figure 30. Combinatorial profiling of functional markers of CM CD8+ T cells expressing single TIGIT+. A. Pie chart forms (top panel) and scatter plots (bottom panel) representing the median of the combinations from one, two, or three functional markers. We used permutation tests in pie chart forms for statistical analysis between groups. **B.** Scatter plots showing the median and interquartile ranges for single CD107a+ (purple), IFNγ (red), and IL-2 (pink) from HIV-1-specific TIGIT+ CM CD8+ T cells. We used the Mann-Whitney test in unpaired samples and the Wilcoxon test in paired samples for statistical analysis. P-values: =0.05 and *<0.05.

TIGIT blockade restores degranulation capacity of HIV-1-specific CD8+ T cells during cART

Given the prominent role of TIGIT in our analyses, we next investigated the blockade of TIGIT in HIV-1-specific CD8+ T-cells of suppressed cART individuals. Indeed, we evaluated αTIGIT alone or in combination with αTIM-3 in samples from S1 and S2. As shown in **Figure 31A** and based on the selection of HIV-1-specific CD8+ T cells producing at least CD107a, IFNγ, or IL-2, we concatenated 65,445 events for single-cell data analyses. We observe a reduction of TIGIT expression in the net-SNE flow cytometry projections after the incubation with αTIGIT or αTIGIT+αTIM-3 blockade

antibodies (**Figure 31A**). By classical gating strategy, we identified an increase of CD107a in total HIV-1-specific CD8+ T cells in αTIGIT (0.9% in isotype vs. 1.4% in αTIGIT; p<0.05) and αTIGIT+αTIM-3 (0.6% in isotype vs. 1.2% in αTIGIT+αTIM-3; p<0.05) (**Figure 31B**). No changes in IFNγ and IL-2 production were observed in blockades (**Figure 31A, data not shown**).

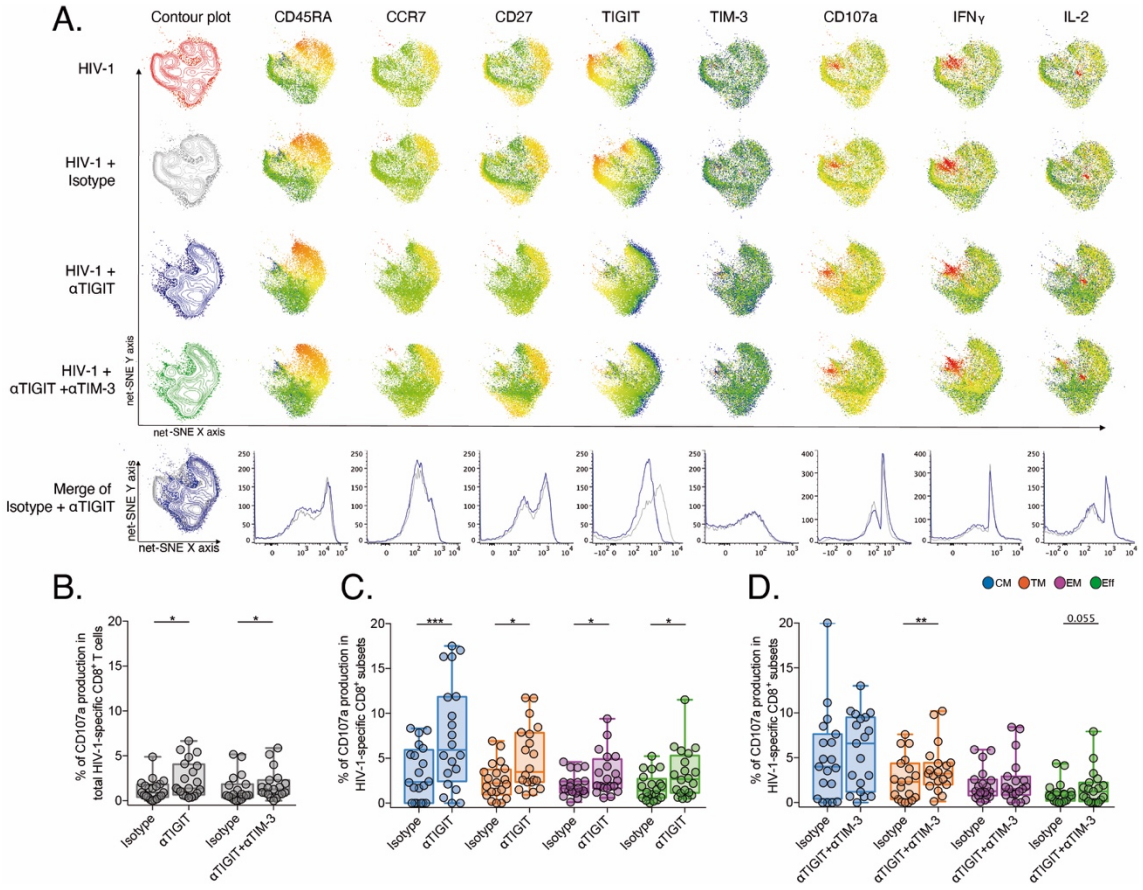

Figure 31. TIGIT blockade restores degranulation in HIV-1-specific CD8+ T cells. A. Representative net-SNE flow cytometry projections with surface and functional markers. 65,445 acquired HIV-1-specific CD8+ T cells from S1 and S2 were concatenated and merged according to the condition; HIV-1 stimulation (red), HIV-1 with isotype (grey), HIV-1 with αTIGIT (blue), and HIV-1 with αTIGIT+αTIM-3 (green). αTIGIT and its respective isotype are merged as histograms to highlight differences between conditions. **B.** Scatter plots showing the median and interquartile ranges of the frequency of CD107a in total HIV-1-specific CD8+ T cells after αTIGIT and αTIGIT+αTIM-3. **C.** Frequency of CD107a in HIV-1-specific CD8+ T-cell subsets after αTIGIT, and **D.** after αTIGIT+αTIM-3. **(B-D)** Statistical differences were calculated by the Wilcoxon matched-pairs signed ranked test. P-values: =0.05, *<0.05, **<0.005 and ***<0.0005.

Moreover, the increase in CD107a was consistent in αTIGIT across HIV-1-specific CD8+ T-cell subsets (isotype vs. αTIGIT; p<0.05). There was a median increase from 2.4% to 5.9% in CM, from 2.2% to 3.5% in TM, from 1.6% to 2.2% in EM, and from 1.3 to 2.7% in Eff CD8+ T cells (**Figure 31C**). Although αTIGIT+αTIM-3 showed an increase of CD107a in total HIV-1-specific CD8+ T cells, this effect was only maintained in the case of TM CD8+ T-cell subset from 2.3% to 3.2% (isotype vs. αTIGIT+αTIM-3; p<0.05).

(**Figure 31D**). The effect of αTIM-3 alone in the functional markers was not significant (data not shown). Collectively, these results demonstrated restoration of degranulation capacity in total and HIV-1-specific CD8+ T-cell subsets by TIGIT blockade during cART.

Discussion

CD8+ T-cell exhaustion or Tex displays a range of functional defects during HIV-1 infection [113], [289]–[291]. The expression of IRs is considered as a hallmark of Tex [95], [97], [98]. Tex and CD8+ T-cell activation share common features in the face of antigen exposure. Therefore, the identification of specific immune signatures of Tex continues to be a challenge. In this sense, while co-expression of IRs is related to cancer severity [117], [137], [138] and progression of disease in HIV-1 infection [100], [123], [126], [293], [298], complementary data on biomarkers, transcriptomics, and functional *in vitro* experiments are required to delineate with precision the role of IRs in Tex. Here, we performed a comparative study between healthy, early HIV-1-infected, and HIV-1-infected cART individuals suppressed over a decade. We combined multiple IRs, functional, and lineage markers in an unsupervised single-cell analysis to gain precision in the immunological parameters across the landscape of Tex in CD8+ T cells of cART individuals. In this way, we overcome some of the previous study limitations, including the focus on single expression of IRs, analysis of total CD8+ T cells, and also cross-sectional data [95], [98], [100], [126], [298].

In agreement with previous studies, our data reported a marked increase of TIGIT expression in memory CD8+ T cells during HIV-1 infection [100], which persisted overtime in cART [99]. We further extended these data by demonstrating that the increase of TIGIT is at single level (TIGIT+) in CM and TM CD8+ T cells and co-expressed with TIM-3 (TIGIT+TIM-3+) in CM and Eff CD8+ T cells. Moreover, single TIGIT+ expression in CD8+ T cells negatively correlated with CD4+ T-cell counts after long-term cART but not in early HIV-1 infection. This observation indicates an increase in the frequency of single TIGIT+ expression overtime despite long-term cART, and its association with immune recovery. TIGIT expression may reflect ongoing immune activation despite fully suppressive durable cART [99], [100], [123], [305]. Previous studies have shown that IL-2, IL-7, IL-15, and IL-21 can upregulate the expression of IRs during viral suppression [134], [147], suggesting that the production of cytokines

during cART may contribute to expand TIGIT+ or TIGIT+TIM-3+ in CD8+ T cells despite low antigenic stimulation.

Our single-cell phenotyping confirmed previously reported IR co-expression of PD-1+LAG-3+ and PD-1+TIGIT+ [96], [123], [125], [289], [306], and further identified a wide diversity and complexity of IR co-expression among the study groups. We characterized cellular homeostatic changes in total CD8+ T cells during HIV-1 infection and after durable cART. We found a contraction of low differentiated CD8+ T cells with a low degree of IR expression concomitant with an expansion of Eff-like CD8+ T cells with high co-expression of IRs. In this sense, we delineated how IR co-expression emerges in early HIV-1 infection and persists over a decade of cART, suggesting that expansion of total CD8+ T cells with IRs co-expression becomes fixed in HIV-1 infection and irreversible despite cART. Previous studies in HCV infection demonstrated that epigenetic scars of HCV-specific and exhausted CD8+ T cells persist after the cure of infection [307].

Moreover, TCR-activated CD8+ T cells through polyclonal stimulation revealed a similar homeostatic contraction and expansion than total CD8+ T cells after long-term cART. While polyclonal memory clusters with IR co-expression showed a contraction, polyclonal Eff-like clusters co-expressing IRs increased the frequency in cART. These data indicate that polyclonal phenotypes are linked to IR co-expression and these IR combinations may comprise the functional status of CD8+ T cells. In this sense, IRs in memory phenotypes may involve Tex through cell frequency reduction or activation through the expansion of Eff clusters after long-term cART. Previous studies showed exhausted CD8+ T cells lack self-renewal and can enter into apoptosis [308], [309], indicating that IR co-expression may have impacted the survival of CD8+ T cells leading to final stages of Tex.

Our immune profiling to HIV-1-specific CD8+ T cells revealed four cellular clusters with a reduction in frequency during cART. Indeed, these clusters showed common features of memory-like phenotypes, TIGIT^{hi}, and co-expression with other IRs^{hi}. In addition, we identified that CM CD8+ T cells expressing TIGIT+ have a low CD107a profile that can be restored after short-term TIGIT blockade in samples from cART individuals. To our knowledge, this is the first time that recovery of degranulation in HIV-1 specific CD8+ T cells by TIGIT blockade has been reported. Previous data support an increase in IFNγ

and proliferation by TIGIT blockade. However, differences in time on cART in the study groups should be considered [100]. Low CD107a expression has been related to terminal T-bet^{dim}Eomes^{hi} exhausted phenotype [99], [290], and Taurantin *et al*. demonstrated that TIGIT^{hi} HIV-1-specific CD8+ T cells can degranulate, but most degranulating cells do not express granzyme B [99]. In addition, the molecular pathway behind TIGIT and CD107a in CD8+ T cells during HIV-1 infection is unknown. In our study, TIGIT and TIM-3 combinational blockade did not demonstrate an additive or synergistic effect but rather the opposite by reducing the recovery of degranulation of TIGIT blockade. This effect might be related to the complexity of TIM-3's biology and the promiscuity of the receptor to engage multiple ligands such as Gal-9, CEACAM-1, PtdSer, and HMGB-1 [129] [310], suggesting that TIM-3 blockade may not be enough to disrupt all the interactions with the different ligands. In this context, we cannot exclude the impact of TIGIT and TIM-3 combinational blockade in other cell types [136]–[138]. Additional experiments could bring new information in other cell types targeted by TIGIT and TIM-3 in combination.

Our study has some limitations in the size of the study groups and the limited sampling to peripheral blood that may underestimate IR alterations on lymphoid tissues and the total contribution of TIGIT expression to Tex. Moreover, complementary studies on transcriptomic, epigenetic, and metabolic profiles of TIGIT expressing CD8+ T cells could bring additional information on the role of this marker in Tex of HIV-1-specific CD8+ T cells.

In summary, our study characterizes highly and complex profiling of IRs and functional markers across the landscape of CD8+ T cells at the single-cell level. We identified TIGIT as a potential biomarker of Tex. We proposed the targeting of TIGIT to recover degranulation functional activity of antigen-specific CD8+ T cells in chronic HIV-1 infection and long-term durable cART.

Acknowledgements, funding, and conflict of interest

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All other authors: none to declare.

Supplemental Material

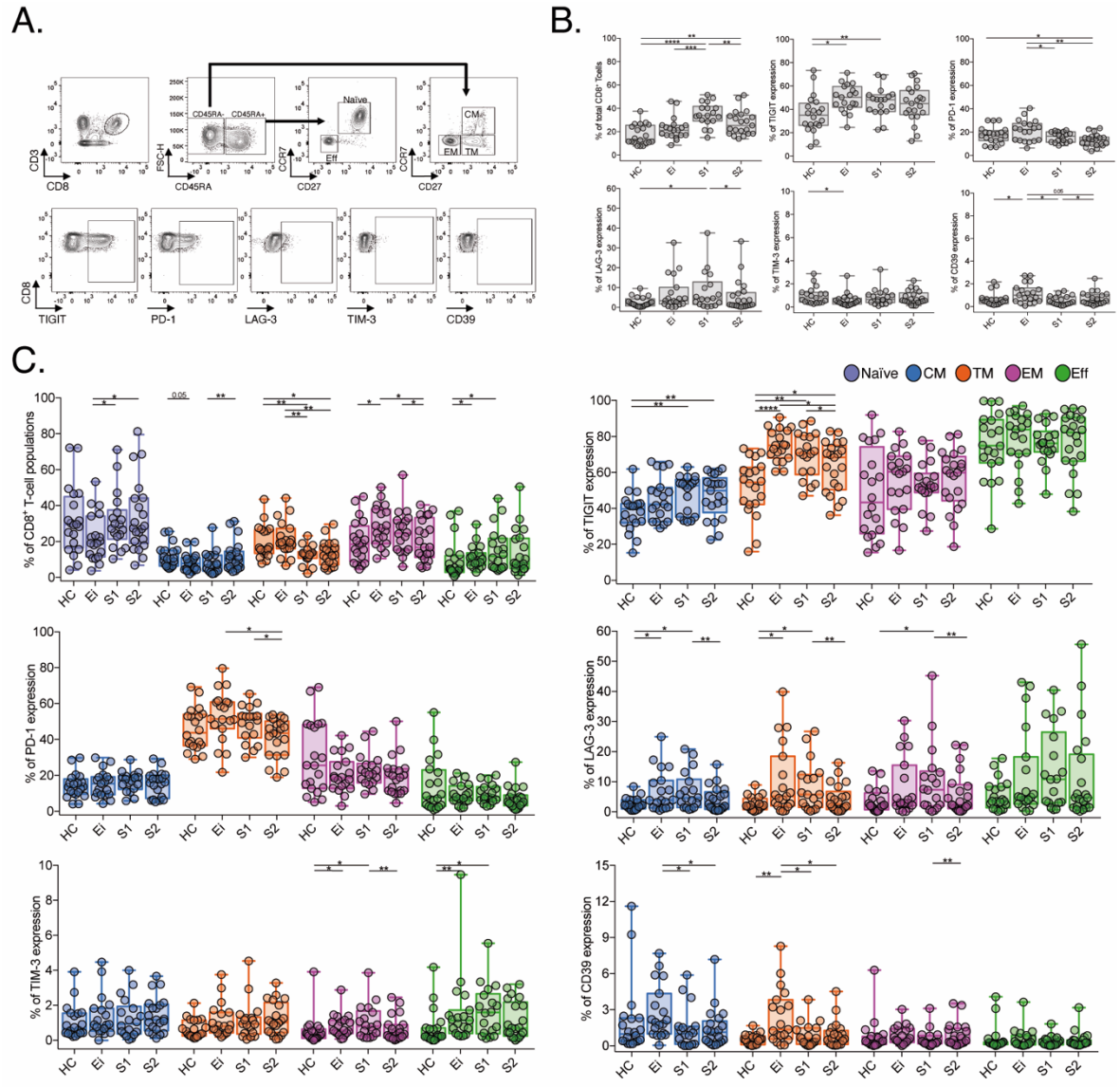

Figure supplementary 1. Changes of IR expression using the classical manual gating strategy. A. Flow cytometric gating strategy to analyze the frequency of TIGIT, PD-1, LAG-3, TIM-3, and CD39 in total and CD8+ T-cell subsets. **B.** Scatter plots with the median and interquartile ranges of frequency in population and IR expression for total CD8+ T cells between HC, Ei, S1, and S2 groups, and **C.** for CD8+ T-cell subsets. Due the low expression of IRs on naïve, data are not shown. We used the Mann-Whitney test for unpaired samples and the Wilcoxon test for paired samples for statistical analysis. P-values: *<0.05, **<0.005, ***<0.0005, ****<0.00005.

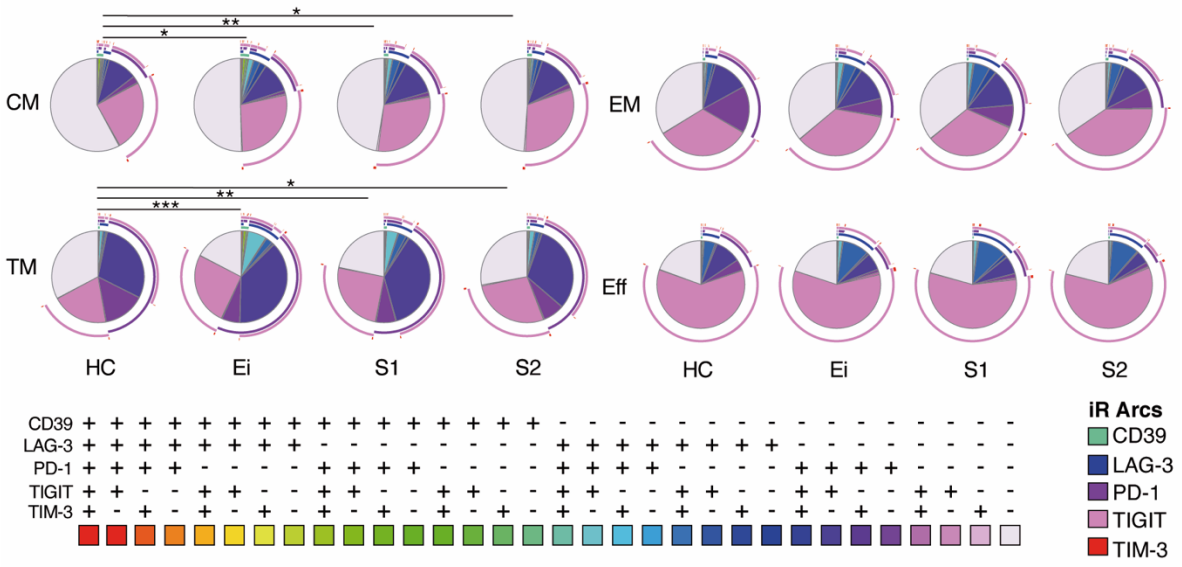

Figure supplementary 2. Changes of IR poly-expression using SPICE. Poly-expression patterns for the 32 possible combinations for TIGIT, PD-1, LAG-3, TIM-3, and CD39 in CD8+ T-cell subsets. Each pie chart form and arcs are color-coded according to the combinations of IRs. We used permutation tests of SPICE software for statistical analysis. P-values: *<0.05, **<0.005, ***<0.0005, ****<0.00005.

Table Supplementary 1. Epidemiological and baseline characteristics of the study groups.

Study Groups	Healthy control	HIV-1 early infection	HIV-1 with cART		ƥ
	HC	Ei	S1	S2	
Number	24	24	24		
Age (years) [median (range)]	43.1 (38.1 - 52.5)	43.3 (41.8 - 46.2)	40.6 (35.1 - 44.7)	50.0 (43.7 - 55.1)	0.059
Male gender [n (%)]	16 (67%)	23 (95.8%)	18 (75%)		0.091
Female gender [n (%)]	8 (33%)	1 (4%)	6 (25%)		0.091
Nadir CD4+ T-cell counts per mm^3 [median (IQR)]	-	-	275 (241 - 366.8)		
CD4+ T-cell counts per mm^3 [median (IQR)]	-	630 (557 - 757.3)	573 (464 - 992)	910 (799.8 - 1111)	**0.0001**
Weeks since seroconversion [median (IQR)]	-	1.3 (0.77 - 17.8)	-	-	
Years on cART [median (IQR)]	-	-	7.1 (3.8 - 11.4)	17.6 (11.7 - 20.9)	
Years undetectable VL [median (IQR)]	-	-	2.2 (1.8 - 2.8)	10.1 (7.4 - 12.9)	
VL (log copies/ml)	-	4.5 (4.3 - 5.1)	undetectable	undetectable	**<0.0001**

Abbreviations: IQR, interquartile range; P-values were calculated using Kruskal-Wallis test (Dunn´s correction) or X^2-test.

CHAPTER III. Enhancement of antiviral CD8+ T-cell responses and complete remission of metastatic melanoma in an HIV-1-infected subject treated with pembrolizumab

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Autor's contributions: The author of this thesis contributed by measuring the immune phenotype of total CD8+ T cells and also the functional responses of HIV-1-specific CD8+ T cells. The author also contributed designing the study, analyzing and interpreting the results, and writing the manuscript.

Abstract

Pembrolizumab is an ICI against PD-1 approved for therapy in metastatic melanoma. PD-1 expression is associated with a diminished functionality in HIV-1-specific CD8+ T cells. It is thought that PD-1 blockade could contribute to reinvigorate antiviral immunity and reduce the HIV-1 reservoir. Upon metastatic melanoma diagnosis, an HIV-1-infected individual on stable suppressive antiretroviral regimen was treated with pembrolizumab. A PET-CT was performed before and one year after pembrolizumab initiation. We monitored changes in the immunophenotype and HIV-1-specific CD8+ T-cell responses during 36 weeks of treatment. Furthermore, we assessed changes in the viral reservoir by total HIV-1 DNA, cell-associated HIV-1 RNA, and ultrasensitive plasma viral load. Complete metabolic response was achieved after pembrolizumab treatment of metastatic melanoma. Activated CD8+ T cells expressing HLA-DR+/CD38+ transiently increased over the first nine weeks of treatment. Concomitantly, there was an augmented response of HIV-1-specific CD8+ T cells with TNF production and poly-functionality, transitioning from TNF to an IL-2 profile. Furthermore, a transient reduction of 24% and 32% in total HIV-1 DNA was observed at weeks 3 and 27, respectively, without changes in other markers of viral persistence. These data demonstrate that pembrolizumab may enhance the HIV-1-specific CD8+ T-cell response, marginally affecting the HIV-1 reservoir. A transient increase of CD8+ T-cell activation, TNF production, and poly-functionality resulted from PD-1 blockade. However, the lack of sustained changes in the viral reservoir suggests that viral reactivation is needed concomitantly with HIV-1-specific immune enhancement.

Introduction

As mentioned in the section of introduction, IRs play a key role in the regulation of persistent immune activation during cancer and chronic infection to avoid self-damage. ICIs are monoclonal antibodies that target IRs to reverse Tex due to continuous antigen stimulation. Pembrolizumab (Keytruda®, MSD) is an ICI directed against PD-1, which blocks the interaction with its ligands PD-L1 and PD-L2. These ligands are overexpressed on activated APCs as well as cancer cells, and their blockade promotes T-cell activation against tumor cells [311]. Currently, pembrolizumab, approved by FDA, has become a first-line treatment against metastatic or unresectable melanoma providing a five-year survival rate between 34% and 41% [312].

Expression of PD-1 has also been associated with dysfunctional HIV-1-specific CD8+ T-cell responses and disease progression. In this context, *in vitro* PD-1 blockade has demonstrated a recovery of HIV-1-specific CD8+ T-cell functionality [101], [116]. On the other hand, IR-expressing CD4+ T cells have been associated with cell-based measures of viral persistence in HIV-1-infected patients on cART [119], [313], and *ex vivo* experiments have shown an enhancement of viral production by CD4+ T cells after PD-1 blockade [190], [314]. Therefore, ICIs could impact on the HIV-1 reservoir through the so-called 'shock and kill' mechanism; i.e., reactivating latent HIV-1 provirus from infected CD4+ T cells and reversing exhausted HIV-1-specific CD8+ T cells against HIV-1 producing cells, altogether resulting in a potential reduction of the reservoir that ultimately might help viral remission [179].

Different case reports, and a couple of case series, suggest that ICI therapy appears to be safe and efficacious in HIV-1-infected individuals with advanced stage cancer [315], although ongoing prospective trials of ICI need to confirm these findings [316]–[318]. However, the effectiveness of ICI therapy to help eliminate the viral reservoir in cART-treated individuals is still controversial and only based on limited case reports. These cases range from showing no major changes in either HIV-1-specific CD8+ T-cell responses or HIV-1 persistence [232], transient enhancement of HIV-1-specific CD8+ T cells with no variation in viral persistence [233]–[235], transient increase in viral transcription without changes in viral reservoirs [236], or cases of depletion of the HIV-1 reservoir [190], [236]. Thus, as the immunological and virological outcome of ICIs in HIV-1-infected patients remains unclear, additional data are needed to determine the use of ICIs in HIV-1 cure strategies.

Here, we report a case of an HIV-1-infected individual on cART that received pembrolizumab treatment for metastatic melanoma. We combine the description of clinical response to melanoma after pembrolizumab treatment with a detailed characterization of functional T-cell responses as well 81 as the therapeutic impact on viral persistence.

Case report

A 46-year-old man was diagnosed with HIV-1 infection in 2008, immediately receiving suppressive cART (**Figure 32**). In 2011, his cART was simplified to ritonavir-boosted darunavir. In June 2016, he was diagnosed with an amelanotic melanoma on the right hemithorax, with palpable right axillary lymphadenopathy.

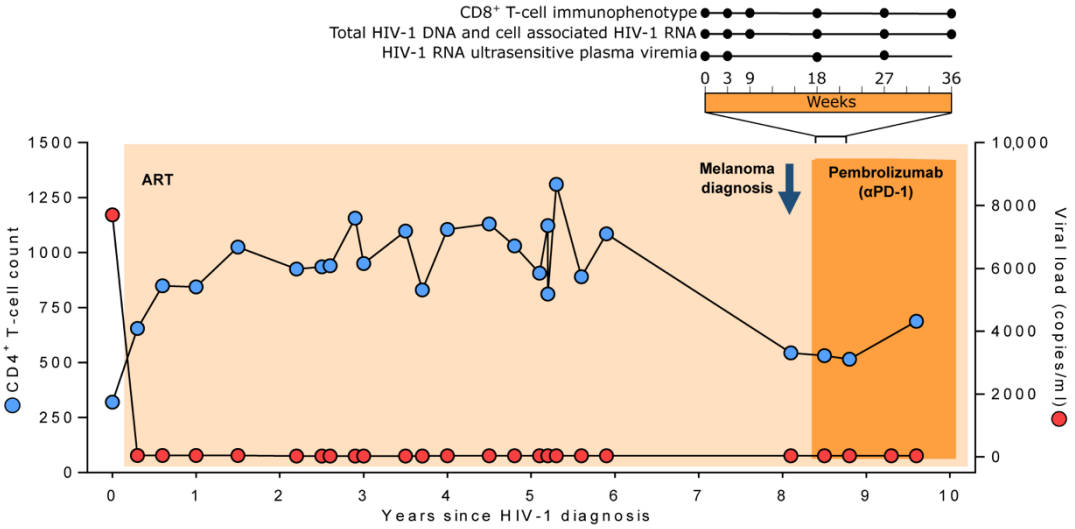

Figure 32. Clinical follow-up of pembrolizumab administration in an HIV-1-infected individual on active cART who developed metastatic melanoma. Longitudinal analysis in years of CD4+ T-cell count (blue circles) and HIV-1 viral load (red circles) since HIV-1 diagnosis. Light orange area indicates the time on cART and dark orange area depicts the time of pembrolizumab administration under cART. Blue arrow indicates melanoma diagnosis. Top bar represents the cycles of pembrolizumab administrations and the biological samples that were analyzed at 0, 3, 9, 18, 27, and 36 weeks. Black lines indicate measures in CD8+ T-cell immunophenotype, total HIV-1 DNA, cell-associated HIV-1 RNA and, ultrasensitive viral load in plasma (usVL).

Staging positron emission tomography-computed tomography (PET-CT) and neurologic magnetic resonance image (MRI) showed no evidence of distant metastatic disease. In September 2016, an axillary lymphadenectomy was performed with involvement of 3/22 lymph nodes by melanoma. There were no mutations in the codon V600 of the BRAF gene, NRAS in exon 2 or 3, or in the KIT gene (PCR Sanger sequencing, Therascreen BRAF Pyro Kit, Qiagen). Repeated PET-CT showed disease progression with hypermetabolic focal lesions in the right axillary, right pleura, and D4 vertebral body (**Figure 33 A1 to A3**). In October 2016, the subject initiated

pembrolizumab (2mg/kg every three weeks). At the onset of melanoma, the cART was switched to RAL 400mg twice daily and truvada (tenofovir fumarate (TDF) and emtricitabine (FTC)) to avoid drug interactions. His CD4+ T-cell count was 544 cells/µl and plasma HIV-1 RNA was suppressed (<40 copies/ml). The subject had never presented any AIDS-associated illness.

A new PET-CT after twelve cycles of pembrolizumab treatment showed complete disappearance of the right axillary, right pleura, and D4 vertebral body lesions (**Figure 33 A4 and 5**). The infusions were well tolerated, without requiring systemic steroids for signs of local inflammation to pembrolizumab. PBMCs and plasma samples were collected before each pembrolizumab administration at weeks (w) 0, 3, 9, 18, 27, and 36 (**Figure 32**). Additionally, we evaluated the short-term effect of pembrolizumab in a sample one day after pembrolizumab administration at w18 (w18+1). The subject provided informed consent and the institutional review board approved the investigational protocol (ref PI-18-229).

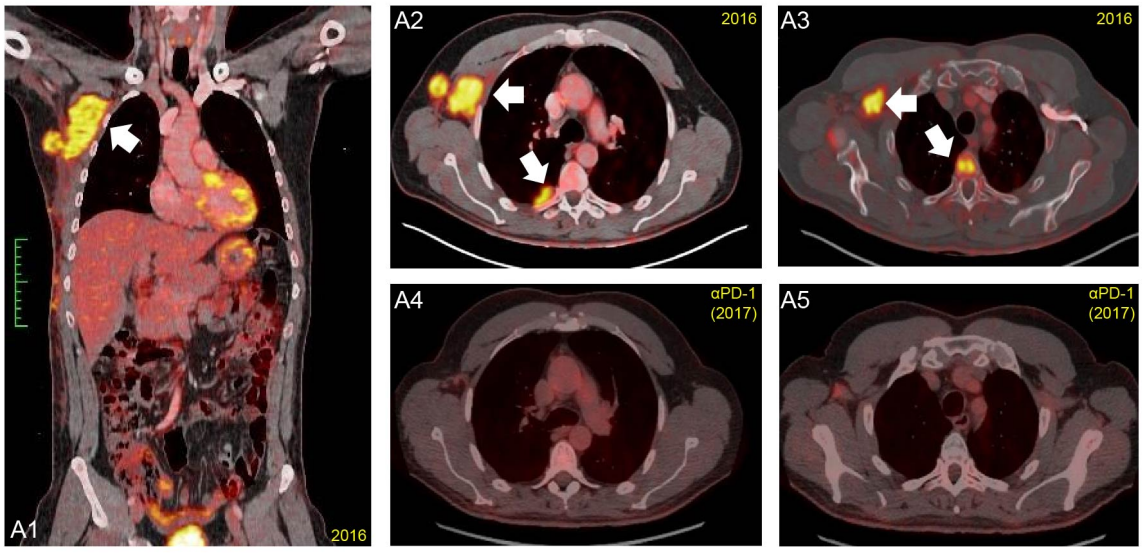

Figure 33. Coronal (left panel) and axial (right panel) images of positron emission tomography with 2-deoxy-2-[fluorine-18] fluoro-D-glucose integrated with computed tomography (18F-FDG PET/CT). A1, A2 and, A3 images depict the lesions before pembrolizumab administration (27/9/2016) by the increased uptake of 18F-FDG in the right axillary lymphoid node and local soft tissue invasion in pleura and osteoblastic spine metastasis. A4 and A5 panels show serial imaging after one year of pembrolizumab administration (27/9/2017) of axial baseline and indicating sustained complete metabolic response and the resolution of the lesions. For A1 to A5, white arrows indicate the localization of the metastatic lesions of melanoma.

Materials and Methods

Immune phenotype of total CD8+ and HIV-1-specific CD8+ T cells

To monitor changes in CD8+ T cells and HIV-1-specific CD8+ T-cell responses after pembrolizumab initiation (αPD-1), PBMCs were stimulated with HIV-1-Gag peptide pool (2µg/peptide/ml, EzBiolab), SEB (1µg/ml, Sigma-Aldrich) or no stimuli, in the presence of CD28/49d co-stimulatory molecules (1µl/ml, BD), Monensin A (1µl/ml, BD Golgi STOP), and anti-CD107a (PE-Cy5, clone eBioH4A3, Thermo Fisher Scientific) for 6 hours at 37ºC in a 5% CO_2 incubator and rested overnight at 4ºC. After incubation, cells were washed with PBS 1X and stained with a viability dye (APC-Cy7, Thermo Fisher Scientific) for 30 minutes at RT. Cells were surface stained for 30 minutes at RT with anti-human antibodies for CD3 (A700, clone UCHT1, BD), CD4 (APC-Cy7, clone SK3, BD), CD8 (V500, clone RPA-T8, BD), CD45RA (BV786, clone HI100, BD), CCR7 (PE-CF594, clone 150503, BD), CD27 (BV605, clone L128, BD), PD-1 (BV421, clone EH12.1, BD), CD38 (PE, clone HB-7, BD), and HLA-DR (A647, clone L243, BioLegend). Afterwards, cells were fixed with Fix/Perm Buffer A (Thermo Fisher Scientific) for 15 minutes at RT and intracellular stained with Fix/Perm Buffer B and antibodies for TNF (PE-Cy7, clone MAb11, BioLegend), IFNγ (BV711, clone B27, BD), and IL-2 (BV650, clone MQ1-17H12, BD) for 20 minutes at RT. Finally, cells were resuspended and fixed in formaldehyde 1% and acquired on LSR Fortessa cytometer using FACSDiVa software (BD). Data were analyzed with FlowJo software v10. We excluded dump and CD4+ T cells, and gates were defined using FMO controls. Surface markers were measured in total CD8+ T cells and CD8+ T-cell subsets including Naïve, CM, TM, EM, and Eff, as previously described [95]. The frequency of each surface marker was expressed as the mean of two independent replicates. For Gag HIV-1-specific CD8+ T-cell cytokine production, only positive replicates were included. GraphPad Prism v4.0 software was used for graph plotting. Pestle and SPICE software [272] was applied to visualize poly-functionality of HIV-1-specific CD8+ T-cell responses for the evaluation of functional pattern distribution.

Total HIV-1 DNA, cell-associated HIV-1 RNA and ultrasensitive viral load

To evaluate the size of the proviral reservoir, total HIV-1 DNA was measured in purified peripheral CD4+ T-cell lysates using droplet digital PCR (ddPCR), as previously described [163]. Briefly, 5'-LTR region was amplified in duplicate, and the RPP30 housekeeping gene was quantified in parallel to normalize sample input. Raw ddPCR

data were analyzed using the QX100™ Droplet Reader and the QuantaSoft v.1.6 software (Bio-Rad).

Also, viral transcription was evaluated by quantification of cell-associated HIV-1 RNA (caHIV-1 RNA) in purified CD4+ T cells, using one-step reverse-transcription ddPCR22. The 5'-LTR gene and the housekeeping gene TATA-binding protein (TBP) were measured in parallel. Residual low-level viremia was determined through ultrasensitive viral load (usVL) by ultracentrifugation of 5 to 6 ml of plasma and quantified using the Abbott Real-Time HIV-1 assay (Abbott Molecular Inc.), as previously described [319], [320].

Results

Pembrolizumab administration induces a marked and transient increase of HLA-DR+/CD38+ expression 2w in EM and Eff CD8+ T cells

Immunotherapies are supposed to affect the distribution of immune cell populations and their activation status. We measured changes in the frequency of total CD8+ T cells and stable counts were observed despite a transient decrease at w9 (**Figure 34A**). The frequency of total CD8+ T cells was 68.5% at baseline and was contracted to 62.5% at w9, returning to baseline levels beyond w18.

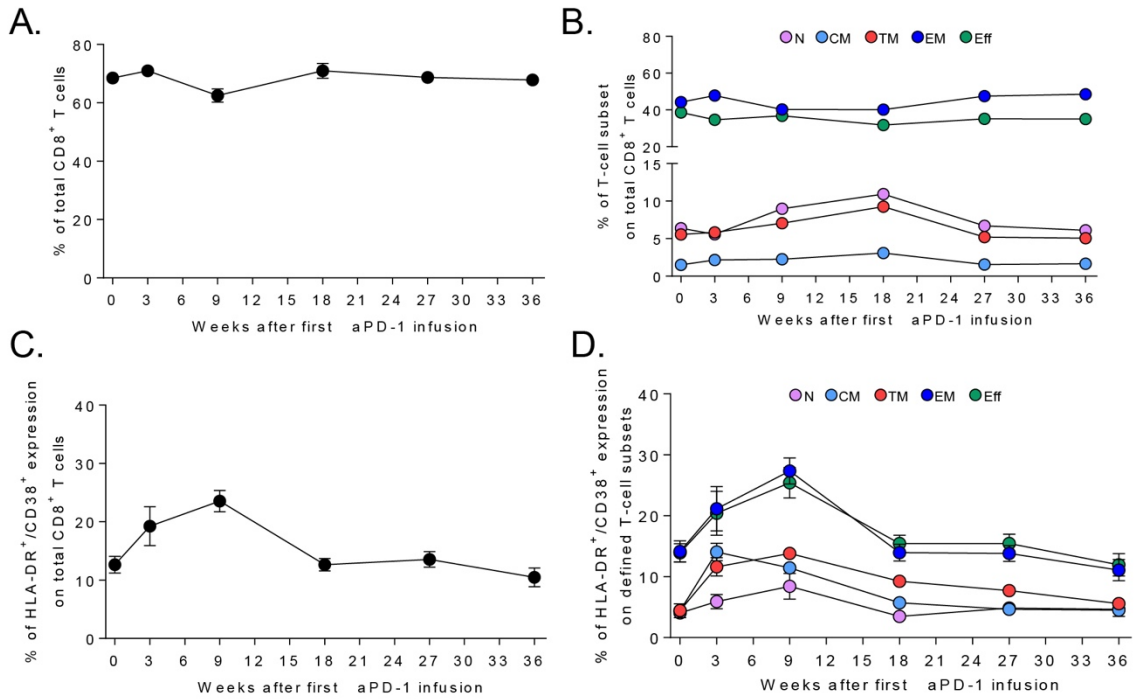

Figure 34. Impact of pembrolizumab administration (αPD-1) in total CD8+ T cells and subpopulations. **A.** Changes in total CD8+ T cells after the first pembrolizumab administration in weeks. **B.** Changes in CD8+ T-cell subsets. **C.** Analysis of total CD8+ T cells expressing HLA-DR+/CD38+ and, **D.** CD8+ T-cell subsets expressing HLA-DR+/CD38+. Results are expressed as the mean of two measurements.

Regarding CD8+ T-cell subpopulations, no major changes were observed in the distribution over the analyzed period (**Figure 34B**). Only the expression of Naïve and TM CD8+ T cells showed a transient increase between w9 and w18. When we evaluated the short-term effect at w18+1, we identified a slight decrease in total CD8+ T cells (70.9% to 67.4%) that was concomitant in Naïve, CM, and TM subpopulations. However, Eff and EM CD8+ T cells raised 7.3% and 7.2%, respectively, after 24 hours of pembrolizumab infusion (**Figure supplementary 1A**).

Concerning the activation status, total CD8+ T cells showed increased expression of the activation markers HLA-DR and CD38 after the first pembrolizumab infusion [321], [322] (**Figure 34C**). Activated CD8+ T cells raised progressively from w0 to w9 (12.7% to 23.6%) and returned to baseline levels at w18 (12.7%). This transient increase of HLA-DR+/CD38+ expressing cells was also observed in all the CD8+ T-cell subpopulations. The percentage of activated Naïve, TM, EM, and Eff CD8+ T cells increased gradually after the first infusion and peaked at w9 (**Figure 34D**). Particularly, the expression of HLA-DR+/CD38+ in EM and Eff CD8+ T cells doubled from 14.2% to 27.4% and from 13.9% to 25.4%, respectively. Nevertheless, activated CM CD8+ T cells peaked at w3 and their levels decreased progressively overtime. Regarding the short-term effect at w18+1, we observed a small increase in the activation status of total CD8+ T cells (12.7% to 13.9%) but no changes in CD8+ T-cell subsets (**Figure supplementary 1B**). Altogether, these data show that pembrolizumab transiently activates peripheral CD8+ T cells, more specifically Eff CD8+ T cells, within the first few infusions in line with its immune therapeutic mechanism.

Enhancement of TNF production in HIV-1-specific CD8+ T-cell responses after pembrolizumab initiation

In addition to the evaluation of changes in CD8+ T-cell populations and their activation status, we wanted to measure the magnitude and functional profile of HIV-1-specific immune responses. Thus, we performed a detailed characterization of HIV-1-specific CD8+ T cells by a combination of surface lineage markers, degranulation and intracellular cytokine detection in response to HIV-1 Gag. We found an increase in HIV-1-specific CD8+ T-cells defined by an augment of TNF production between w0 and w9 (0.3% to 0.5%) after the first infusion of pembrolizumab (**Figure 35A**). The response decreased progressively to undetectable levels at w36. Interestingly, concomitant to TNF reduction, we observed a slight increase of CD107a, IFNγ and IL-2 production by

HIV-1-specific CD8+ T cells at w18, which stayed that way until the end of the follow-up. Additionally, at w18+1 we observed an increase from 0.2% to 0.4% on TNF production by HIV-1-specific CD8+ T cells. However, no changes were observed in CD107a, IFNγ, and IL-2 (**Figure supplementary 1C**). Also, we observed a high frequency of TNF, IFNγ, and IL-2 cytokine-producing cells and degranulation in response to CD8+ T-cell polyclonal activation with the SEB stimuli that transiently peaked at w3 (**Figure 35B**).

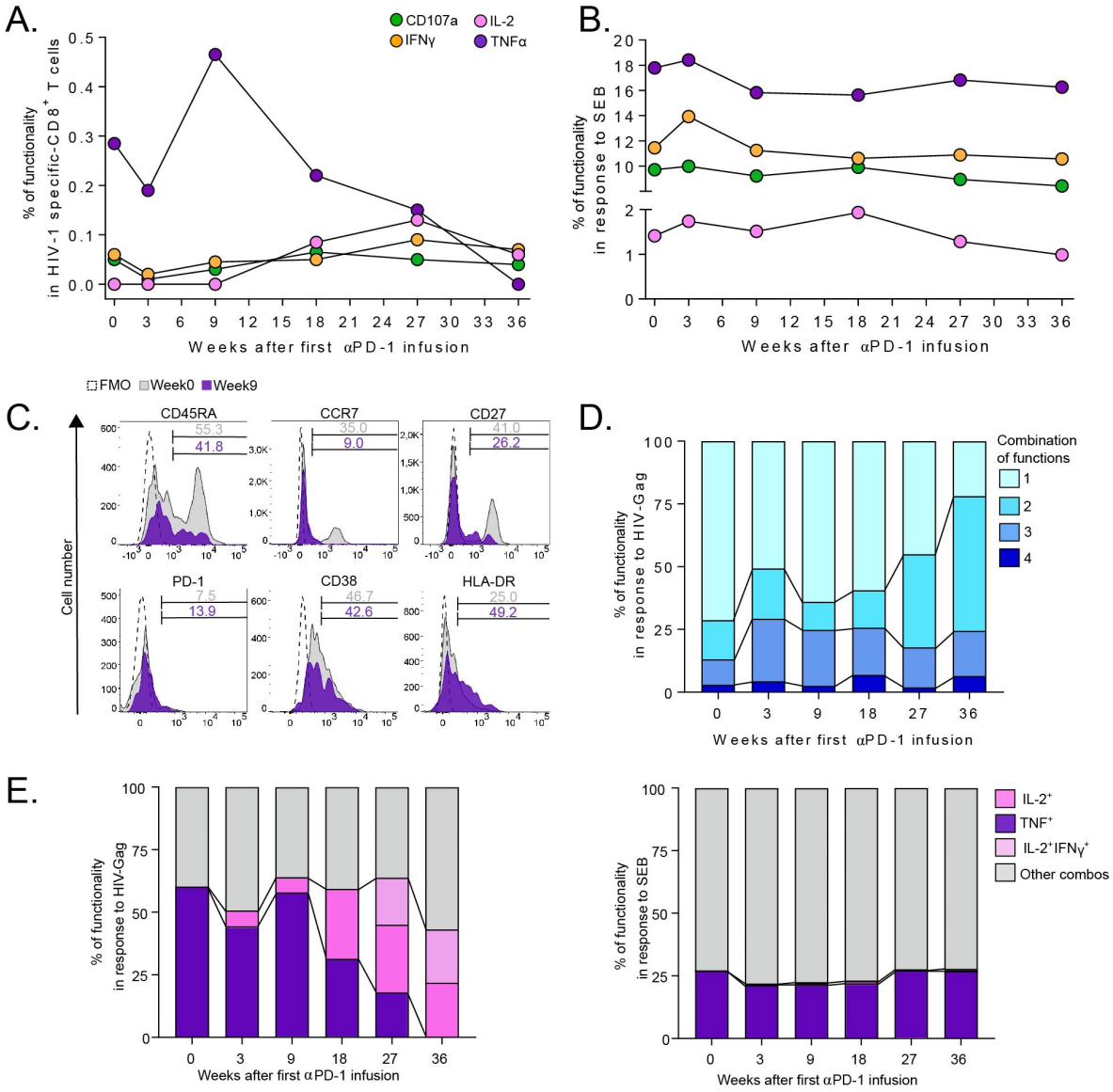

Figure 35. Immunophenotype of the HIV-1-specific CD8+ T-cell response during pembrolizumab administration (αPD-1). A. The graph represents the changes of degranulation and TNF, IFNγ, and IL-2 production in Gag HIV-1-specific CD8+ T-cells responses. **B.** Functional changes in response to SEB. **C.** Histograms depict the expression of CD45RA, CCR7, CD27, PD-1, CD38, and HLA-DR on TNF HIV-1-specific CD8+ T cells at week 0 (grey) and week 9 (purple). FMO controls for each marker are included. **D.** Poly-functionality expressed as the combination of one, two, three, or four functions in HIV-1-specific CD8+ T cells. **E.** Detailed poly-functional profiles of HIV-1-specific CD8+ T cells (right) and under SEB stimulation (left). The bars in purple represent the evolution of TNF^+ frequency overtime in single combination and pink represents the changes of $IL\text{-}2^+$ and $IL\text{-}2^+IFN\gamma^+$ over the week 18.

By using lineage expression markers, we tried to decipher the phenotype that underlined the peak of TNF production by HIV-1-specific CD8+ T cells. We observed a decrease in CCR7, CD27, and an increase in HLA-DR and PD-1 markers between w0 and w9 (**Figure 35C**). These data indicate an effector profile of HIV-1-specific CD8+ T cells responsible of TNF production at the peak of response to pembrolizumab.

Functional switch from TNF to IL-2 profile in HIV-1-specific CD8+ T-cell responses after pembrolizumab follow up

By using a detailed functional profile of cytokine production based on TNF, IFNγ and IL-2 as well as CD107a degranulation, we analyzed the evolution of HIV-1-specific CD8+ T-cell responses overtime (**Figure 35D and E**). Pembrolizumab raised the cell's polyfunctionality, expressed as any combination of two or more functional markers, from 28.7% at w0 to 78.2% at w36 (**Figure 35D**). Regarding combinations of three functional markers, although we observed peaks at w3 (24.9%) and w9 (22.3%), these combinations remained high until the end of the study. Indeed, detailed analysis showed a transient increase driven by HIV-1-specific CD8+ T-cells expressing TNF (TNF^+) during the first nine weeks of pembrolizumab treatment. Nonetheless, the TNF dominated profile switched to an IL-2 dominant profile ($IL\text{-}2^+$ and $IL\text{-}2^+IFN\gamma^+$) since w9 (**Figure 35E**). In the short-term effect, we found an augment of TNF expressing cells (TNF^+) at w18+1, concomitant with a reduction in $IL\text{-}2^+$ production (**Figure supplementary 1D and E**). However, the combination $IL\text{-}2^+IFN\gamma^+$ seems to start to raise at this timepoint. These data contrast with those from polyclonal stimuli where no changes were observed. Thus, the initial release of TNF by HIV-1-specific CD8+ T cells is switched to an IL-2 release profile in the long-term antiviral response.

Transient decays of total HIV-1 DNA

To monitor the perturbation in the HIV-1-infected CD4+ T-cell reservoir induced by pembrolizumab, we measured viral persistence by HIV-1 DNA, caHIV-1 RNA, and usVL. For all the analyzed samples, we detected total HIV-1 DNA with a median IQR of 1,008 (862–1,141) copies/million CD4+ T cells. We detected transient decays in total HIV-1 DNA from 1,080 to 820 copies per million CD4+ T cells (24%) at w3, and 727 copies per million CD4+ T cells (32%) at w27 (**Figure 36A**). However, after each reduction, the levels of total HIV-1 DNA recovered to values similar to those of the baseline. Moreover, the expression of cell-associated unspliced HIV-1 RNA in CD4+ T cells was stable over

time in all samples with a median level of 40 (36-47) ratio (HIV/TBP) x 1,000 (**Figure 36A**). In order to determine the effect of pembrolizumab on HIV-1 reactivation, we measured HIV-1 RNA by ultrasensitive viral load. We detected HIV-1 RNA in 50% of analyzed plasma samples. Interestingly, rather than seeing the production of new virions, we observed a reduction of viremia below the limit of detection (0.8 copies/ml) at w3 and w27 (**Figure 36B**).

This decrease in HIV-1 RNA was concomitant to the transient reduction of total HIV-1 DNA observed. When we evaluated the short-term effect of pembrolizumab at w18+1, we found levels of total HIV-1 DNA and caHIV-1 RNA within the same magnitude than at w18 (**Figure supplementary 1F**). Thus, we found transient decreases of total proviral HIV-1 DNA and ultrasensitive plasma HIV-1 RNA at w3 and w27 after pembrolizumab treatment without affecting cell-associated unspliced HIV-1 RNA in CD4+ T cells.

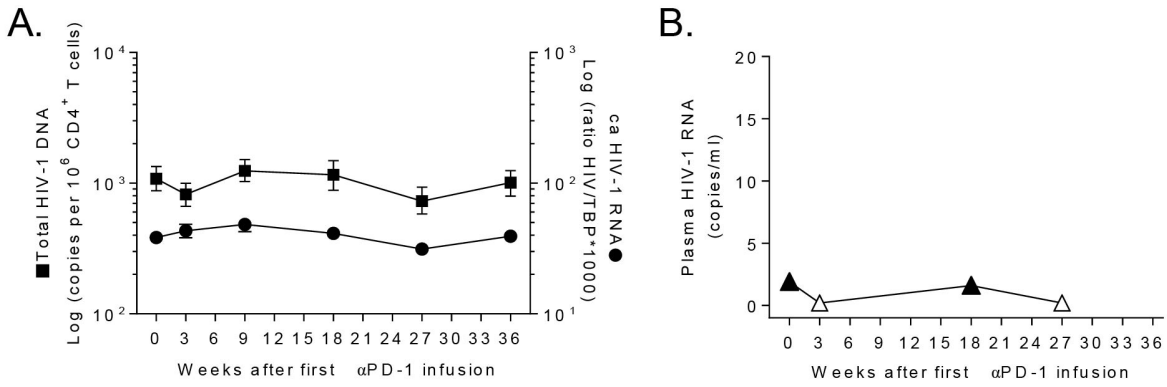

Figure 36. Longitudinal analysis of HIV-1 reservoir during pembrolizumab administration (αPD-1). A. Total HIV-1 DNA (squares) and cell-associated (CA) HIV-1 RNA (circles) in CD4+ T cells, measured by ddPCR. **B.** Ultrasensitive viral load in plasma (triangles). Open symbols represent determinations below the limit of quantification.

Discussion

Previous studies have hypothesized that ICIs might be possible candidates to the shock and kill curative strategies by the reactivation of the HIV-1 reservoir [190], [314], [323] and the concomitant enhancement of antiviral immune responses [101], [116], [324], leading to the clearance of reactivated cells and consequently reducing the HIV-1 reservoir size. However, the effect of PD-1 blockade in HIV-1-infected individuals is incompletely understood, and the results are controversial, in part due to limited experience in this clinical setting. Here, we not only show a case of complete remission of metastatic melanoma after treatment with pembrolizumab in a HIV-1-infected

individual, but also an enhancement of T-cell activation and the HIV-1-specific CD8+ T-cell responses. However, this T-cell invigoration was associated only with a transient perturbation on the HIV-1 reservoir size.

Pembrolizumab had a good safety profile and a sustained antitumoral response in this HIV-1-infected subject. Although the experience of pembrolizumab, as well as other ICIs, in people with HIV-1 is limited, these results are in line with other preliminary studies assessing the safety of ICI therapies in HIV-1-infected individuals with advanced-stage cancer [315], [316]. However, only a fraction of these patients with advanced melanoma benefit from ICI blockade [315], [316], which highlights this case of complete remission of this metastatic melanoma. Immunologically, over the first nine weeks, we found an increase of HLA-DR+/CD38+ expressing cells, especially in EM and Eff CD8+ T-cell subsets, which had also been reported previously [236]. While previous studies only found IFNγ production by HIV-1-specific CD8+ T cells [233], [234], [236], we describe for the first time the production of a peak of TNF production by HIV-1-specific CD8+ T cells at w9 as a result of the PD-1/PD-L1 pathway disruption by pembrolizumab, in agreement with the initiation of an inflammatory response. TNF is a pleiotropic cytokine, mainly produced by activated cells, regulating the immune response, promoting inflammation, and inhibiting tumorigenesis [325].

After the peak of TNF, we observed a transition from TNF to an IL-2 cytokine profile consistent with the reduction of T-cell activation through the antigen receptor [326]. IL-2 plays a crucial role in the expansion of Eff CD8+ T cells that get activated upon antigen encounter, and regulates proliferation and homeostasis of T, B, and NK cell responses [325], [327]. IL-2 helps to resolve the inflammatory response caused by pembrolizumab in the first weeks of treatment, contributing to the maintenance of HIV-1-specific CD8+ T cells and generating a long-term memory responses [327]. However, we cannot exclude an impact of pembrolizumab on other cell types including NKs. Indeed, NK responses can have crucial immunotherapeutic effects upon PD-1/PD-L1 blockade in tumors with low levels of MHC-I expression [328]. Additional studies profiling functional immune cell responses during treatment with ICIs could bring new insights on the mechanism of these new therapies in the remission of cancer and viral diseases.

Despite observing two punctual drops on proviral DNA during the study, we were unable to detect a consistent decrease in the viral reservoir. This observation has been previously reported by other studies using ICIs in combination with cART [232]–[234], [244]. These results differ from a single case report that shows a decrease from 369 to 30 copies HIV-1 DNA per million cells at day 120 after nivolumab administration [236].

Also, it has recently been reported that pembrolizumab led to transiently short-term small increase in unspliced cell-associated HIV-1 RNA in CD4+ T cells *in vivo* in individuals on cART [231]; however, due to differences in sampling times, we cannot corroborate such observations.

The absence of sustained reduction on proviral DNA in our study case might be due to the lack of expression of cell-associated HIV-1 RNA and the subsequent production of viral antigen needed for the targeting of infected cells by HIV-1-specific Eff CD8+ T cells [323]. Although there was an enhancement on HIV-1-specific CD8+ T-cell responses, we did not observe a sustained reduction of viral reservoir in peripheral CD4+ T cells, suggesting that pembrolizumab was unable to effectively reverse viral latency. Alternatively, any sustained reduction in the viral reservoir might have been unnoticed due to homeostatic proliferation of latently HIV-1-infected CD4+ T cells expressing PD-1, which might mask the detection of any effective "shock and kill" events under pembrolizumab treatment. Furthermore, the limitation of our analyses to peripheral blood samples underestimate the effects on lymphoid tissues where follicular helper CD4+ T cells expressing PD-1 play an important role as HIV-1 reservoirs [329]. Furthermore, data from *ex vivo* measurements on CD4+ T cells from cART-suppressed individuals suggest that pembrolizumab enhances the induction of HIV-1 production when combined with the latency reversing agent Bryo, without providing data in reservoir clearance [190].

This study is restricted to peripheral blood samples from a single patient with complete control of viral replication under suppressive cART, which might have masked any beneficial effect of αPD-1 treatment on plasma HIV-1 viral load. These limitations should be carefully considered, in view of interindividual variability, especially in cancer patients. Ongoing clinical trials administrating ICI alone [231], [330] or in combination with LRAs to HIV-1-infected individuals on cART would help to clarify the potential contribution of ICIs in curative strategies.

Overall, our study shows that pembrolizumab treatment resulted in a successful remission of metastatic melanoma in an HIV-1-infected individual, and concomitantly enhanced the HIV-1-specific CD8+ T-cell responses in terms of activation and function. Nevertheless, this was not sufficient to consistently reduce the size of the viral reservoir, questioning the stand-alone effect of ICIs in terms of "shock and kill" HIV-1 curative strategies.

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Supplemental Material

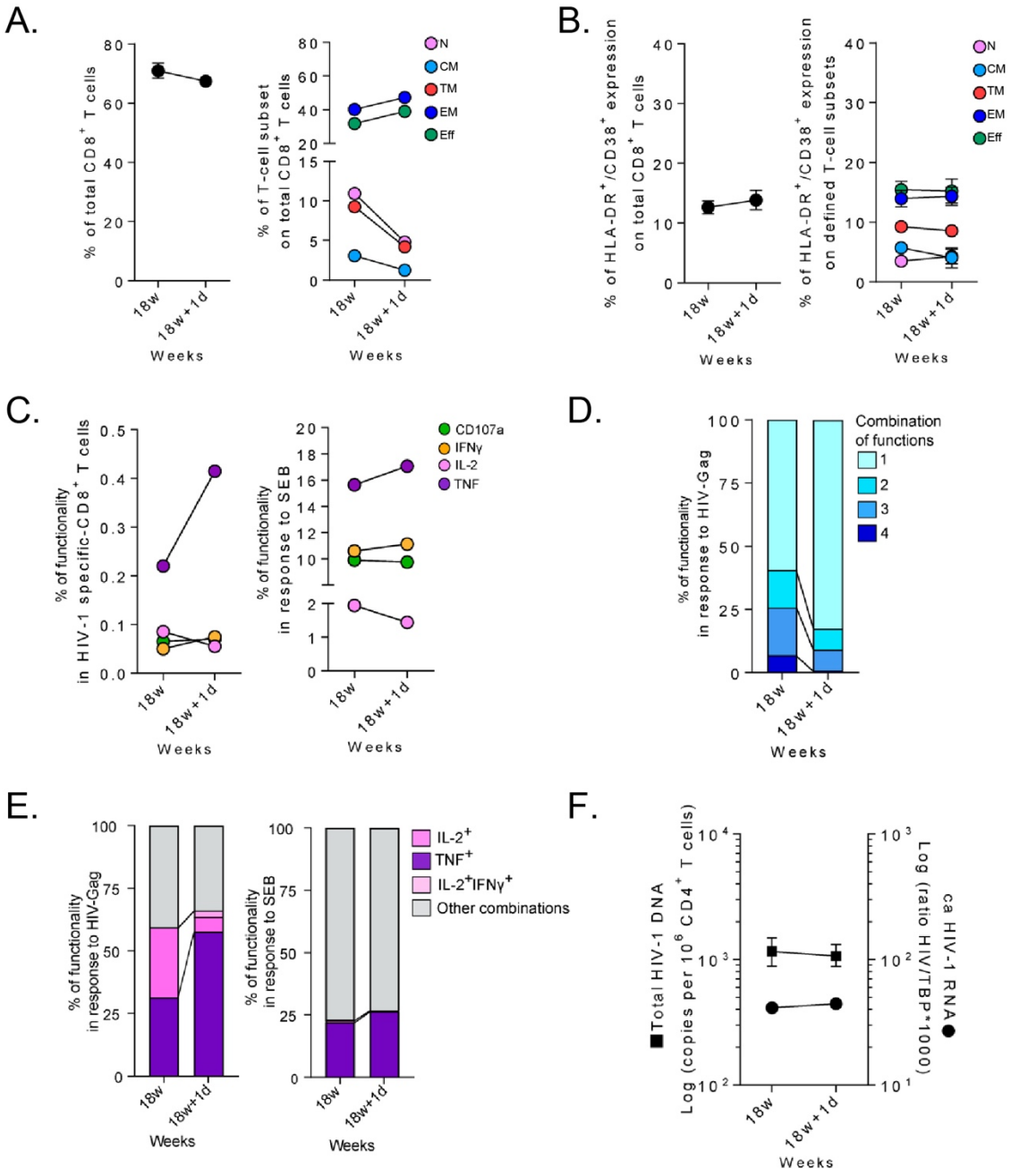

Figure supplementary 1. Immunophenotype of the HIV-1-specific CD8+ T-cell responses after 24 hours of pembrolizumab administration from week 18 (w18+1). A. Changes in total CD8+ T-cell population (left graph) and, in CD8+ T-cell subsets (right graph) after 24 hours from the administration at week 18. **B.** The graphs represent the changes in total CD8+ T cells expressing HLA-DR+/CD38+ (left graph) and, in CD8+ T-cell subsets (right graph). **C.** Changes of CD107a degranulation and TNF, IFNγ and, IL-2 production for HIV-1-specific CD8+ T cells in response to HIV-1 Gag (left graph) and in response to SEB (right graph). **D.** Frequency of TNF, IFNγ, CD107a and, IL-2 combinations expressing as 1, 2, 3 or 4 functions in total HIV-1-specific CD8+ T cells. **E.** Detailed poly-functional profiles of HIV-1-specific CD8+ T cells. The bars in purple represent the changes on TNF frequency and in pink it is represented the changes of IL-2^{+} and IL-2^{+}IFNγ^{+}. **F.** Total HIV-1 DNA (squares) and cell-associated HIV-1 RNA (circles) in CD4+ T cells at week 18+1.

DISCUSSION

Understanding Viro-immunological limitations for HIV-1 cure therapeutics

The natural course of HIV-1 infection is characterized by an exponential rise in the viral load, progressive decline in CD4+ T cells, and chronic immune activation (**Figure 37A**). Following the first wave of innate immunity, HIV-1-specific CD8+ T cells are primed and activated to further control viral replication and plasma viremia is reduced to the viral set point [68]–[74]. However, as HIV-1 remains replicating at the level of reservoirs, the persistent antigen exposure and sustained immune activation lead to the exhaustion of HIV-1-specific CD8+ T cells driven by the expression of IRs among other factors (**Figure 37A**) [103]–[106].

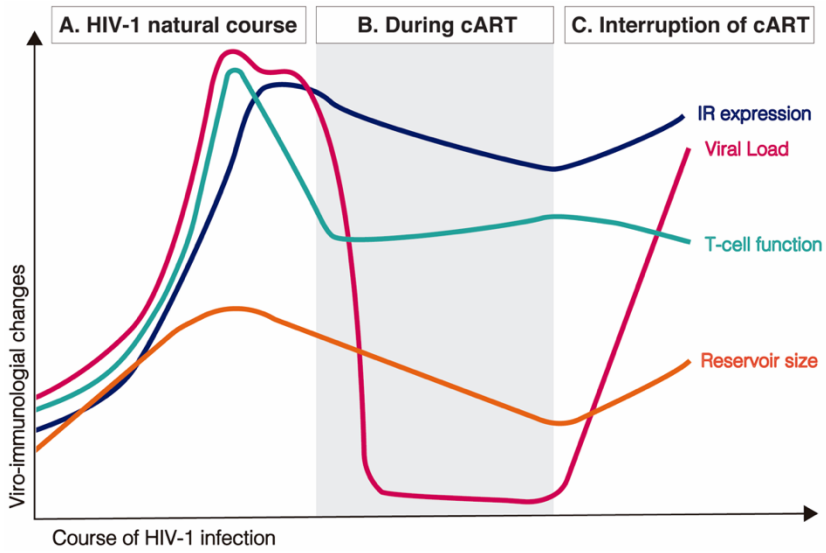

Figure 37. Viro-immunological changes in the course of HIV-1 infection. A. Natural course of HIV-1 infection. **B.** During cART and **C.** After cART-interruption. Viral load (VL), reservoir size, the functionality of T cells, and IR expression are represented as changes in the course of HIV-1 infection in A, B, and C. cART is represented with a grey shadow. Image created with Biorender and Adobe Illustrator CC 2018.

The most important advance in the clinical management of HIV-1 infection has been the development of antiretroviral drugs capable of suppressing the systemic viral load. Indeed, cART has been effective in decreasing global AIDS-related deaths by 50% between 2005 and 2021 [10]. Although cART effectively suppresses viral replication and partially contributes to immune recovery, HIV-1-specific CD8+ T cells remain dysfunctional (**Figure 37B**). In addition, HIV-1 infection remains incurable as cART is unable to remove latently infected cells from the body. Treatment is thus needed throughout a lifetime, raising concerns about the long-term healthcare of HIV-1-infected individuals. Indeed, as soon as cART is interrupted, the virus rebounds within weeks in nearly all individuals to pre-cART levels (**Figure 37C**) [30], [39]–[42]. These findings highlight the need to cure HIV-1 infection by developing novel therapeutic strategies.

Both antigen expression by latently infected CD4+ T cells and the dysfunctional phenotype of CD8+ T cells need to be overcome to find effective HIV-1 curative strategies.

Crossing the barrier of antigen expression and CD8+ T-cell exhaustion for effective HIV-1 shock and kill strategies

An encouraging amount of work has been performed in the field of the HIV-1 cure during the last years looking for LRAs to induce HIV-1-antigen expression on latently infected CD4+ T cells [180]–[185]. Over 160 LRAs have been identified to date [331], but none of these candidates has yet led to a promising *in vivo* functional cure [180], [181], [191]. Furthermore, basic and clinical research have highlighted the complexity and highly heterogeneous nature of the HIV-1 reservoirs [185], [331]–[333], shedding doubts about the feasibility of the shock and kill approach.

In the first chapter of this thesis, we aimed to identify potential limitations of HIV-1 shock and kill strategies (**Figure 38**).

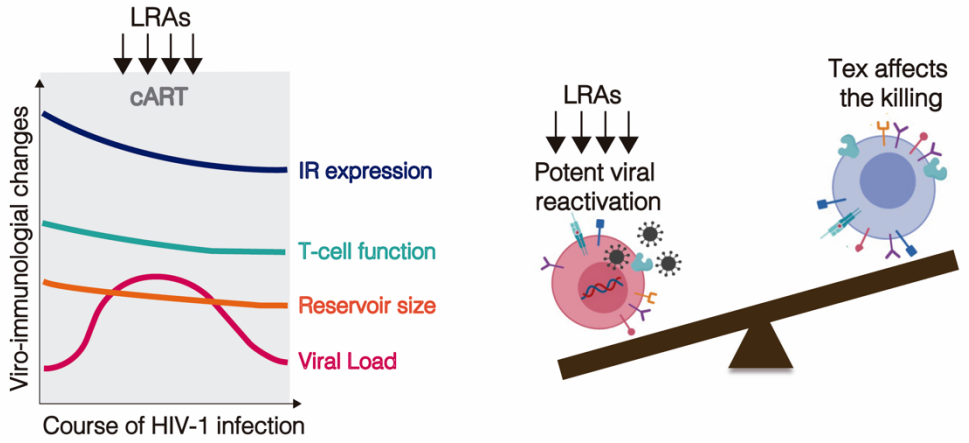

Figure 38. Viro-immunological parameters in shock and kill strategy under cART. On the left, a hypothesis of what happens in viral load, reservoir size, T-cell function, and IR expression after LRA administration under cART is represented. Only viral load changes are observed after LRA treatment. On the right, the imbalance of the figure indicates that the weight of the shock and kill strategy is focused on the reactivation part without taking into account the importance of the functional profile of CD8+ T cells that kill the LRA-reactivated cells. cART is represented with a grey shadow. Image created with Biorender and Adobe Illustrator CC 2018.

Our results showed that potent LRAs are essential to ensure the presentation of viral antigens on the surface of reactivated cells for rapid recognition and killing by HIV-1-specific CD8+ T cells (**Figure 38**). These limitations in the potency of LRAs indicate the need to develop novel molecules with higher potency and specificity. We believe that novel LRAs should be able to reactivate the latent reservoir targeting specifically HIV-1-chromosomic locations such as active genes or gene-rich chromosomes [39], [334]. Of

note, Chen *et al*. concluded that the efficacy of each LRA depends on the integration site of HIV-1 [334], suggesting that the reactivation of the whole latent viral reservoir should be achieved using a cocktail of LRAs to reactivate any provirus regardless of its integration site. Thus, LRAs should not focus on global changes on the transcriptome on their own, but rather on the use of LRA combinations to cover the reactivation of the complete HIV-1 genome. Moreover, novel LRAs should ensure adequate blood and tissue biodistribution. The latent reservoirs are composed mainly of memory CD4+ T cells that reside in cellular compartments or sanctuaries including but not limited to gut-associated lymphoid tissues (GALT), genital tract, lymph nodes, and central nervous system in which cART penetration is limited [185], [332]. These sites may be preferentially responsible for the viral rebound after cART interruption. Thus, given the characteristics of the latent viral reservoir, in terms of location, size, composition as well as mechanisms of maintenance, it will be challenging to develop LRAs able to completely reactivate the HIV-1 reservoir at once in the entire body. In this sense, only highly invasive strategies such as bone marrow transplants with CCR5-Δ32 donors have been successful in the elimination of the reservoir from the body leading to HIV-1 cure [171]–[173].

Moreover, we described by immune profiling a correlation between the lack of IR expression in HIV-1-specific CD8+ T cells and the magnitude of the killing of the LRA-reactivated cells in chapter I (**Figure 38**). We observed that upon continuous exposure to HIV-1 antigens, a contraction in the frequency of CTLs without IR expression concomitant with an expansion of CTLs co-expressing at least three IRs. These data reinforce the idea that Tex in HIV-1-specific CD8+ T cells may be associated with high levels of IRs such as PD-1, TIM-3, LAG-3, and TIGIT [101], [116], [265]. Additional efforts are required to clarify the role of IRs in the context of Tex in HIV-1-infected and cART individuals. Therefore, immunotherapy based on targeting IRs may have a key role in overcoming the exhaustion of HIV-1-specific CD8+ T cells and favor the killing of HIV-1-infected cells in the context of LRAs with a broad and potent spectrum of reactivation.

The interest of IRs as biomarkers of CD8+ T-cell exhaustion and targets for HIV-1 cure

Tex arises in the face of persistent immune activation by continuous antigen expression in cancer and chronic infections [103]–[106]. Tex's surface markers and transcription factors are shared with activated and functional T cells. Although both exhausted and functional CD8+ T cells express genes related to the T-cell cycle, homing, and function,

Tex results in dysfunctional HIV-1-specific CD8+ T cells. As a result, the identification of specific biomarkers of Tex is required to specifically target dysfunctional T cells for HIV-1 immunotherapies [106], [110], [292]. Despite considering IRs as hallmarks of Tex in HIV-1-specific CD8+ T cells [95], [97], [98], novel evidence using functional markers, blockade assays, and transcriptomics are required to demonstrate that IRs are directly associated with Tex.

In the second chapter of this thesis, we delineated the expression of IRs across the landscape of CD8+ T cells in HIV-1 infection and long-term cART. We identified that TIGIT expression was increased in HIV-1 infection and after long-term cART. Also, the frequency of TIGIT in CD8+ T cells was inversely correlated with CD4+ T-cell counts. Previous studies have suggested a role for TIGIT as a predictor of immune status and disease pathogenesis in HIV-1 chronic infection [99], [100]. Our single-cell data analyses revealed wide heterogeneity in IR expression with homeostatic changes in CD8+ T-cell clusters in the course of HIV-1 infection and long-term suppressive cART. Indeed, we identified the selective depletion of a cluster of memory-like HIV-1-specific CD8+ T cells with CD107a^{hi} TIGIT^{hi} during cART. In this context, low CD107a and TIGIT expression have been associated with the T-bet^{dim}Eomes^{hi} phenotype of terminal exhausted cells in chronic HIV-1 infection [100], [126], [290]. Nevertheless, no study has shown whether the expression of TIGIT^{hi} can directly suppress CD107a. In the last years, several biomarkers including Nr4a and TOX transcription factors have been associated with Tex in the microenvironment of tumors [292]. TOX distinguishes exhausted from memory CD8+ T cells in various mouse models being required for the epigenetic remodeling and survival of exhausted CD8+ T cells [289]. Meanwhile, our work identifies TIGIT as a potential Tex biomarker, additional data are needed on epigenetics and survival profiles to complement our findings. To date, new pathways and molecules including LAG-3, TIM-3, CD73, NKG2A, and CEACAM-1 are also being explored as biomarkers of Tex in chronic viral infections and cancer [335].

In chapter II, we proposed the targeting of TIGIT to reverse Tex and particularly the loss of degranulation capacity. We evaluated the impact of αTIGIT antibodies in terms of changes in CD8+ T-cell function in HIV-1 infection and cART (**Figure 39**). Through short-term TIGIT blockade experiments, we identified a recovery of degranulation profile in HIV-1-specific CD8+ T cells from HIV-1-infected individuals on cART. To our knowledge, this is the first study reporting the recovery of degranulation profiles in HIV-

1-specific CD8+ T cells by TIGIT blockade. Additional experiments will be needed to unravel the molecular pathway behind it. Our study did not evaluate the impact of αTIGIT on other cell types expressing TIGIT, such as NKs and CD4+ T cells [136]–[138]. However, TIGIT blockade has been shown to protect against various solid and hematological cancers through NK responses [222]. Thus, new studies measuring the impact of αTIGIT in other cell types could bring complementary information to characterize the full effect of αTIGIT in immune function. Furthermore, the blockade of TIGIT can act as an LRA (**Figure 39**). It has been reported that TIGIT-expressing CD4+ T cells harbor latent HIV-1 provirus, and therefore, targeting TIGIT may contribute to viral reactivation [114]. To date, no reports are available about the potential of TIGIT as an LRA [216]. Therefore, further investigations of αTIGIT as LRA will be essential to bring new insights into the cure of HIV-1.

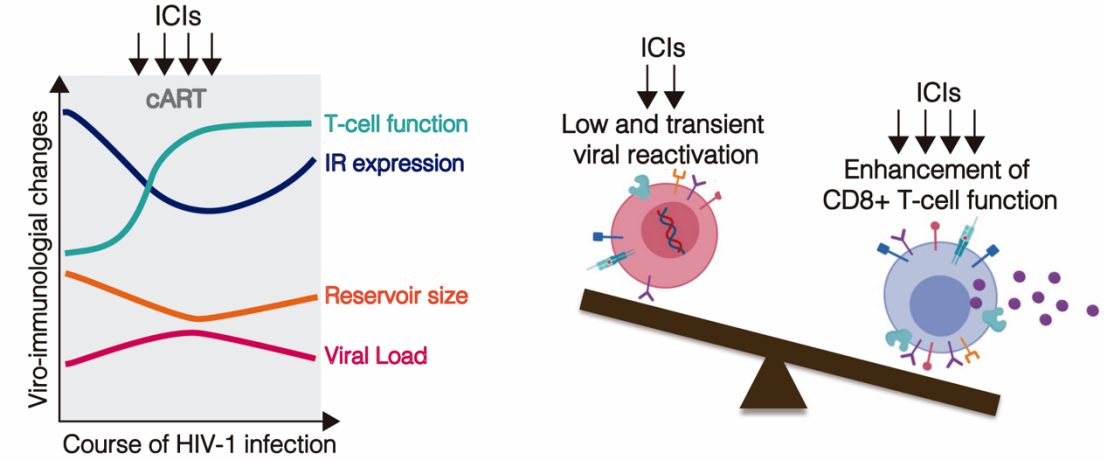

Figure 39. Viro-immunological parameters on ICI treatment under cART. Both αTIGIT and αPD-1 effectively restored the functional profiles of HIV-1-specific CD8+ Tex cells. In addition, the effect of the αPD-1 was not sufficient to consistently reduce the size of the viral reservoir. The left represents the possible changes in viral load, reservoir size, T-cell function, and IR expression after ICI administration under cART. After the IR blockade, an increase in T-cell function can be observed concomitant with a reduction of IR expression. In addition, viral load and reservoir size are transitionally affected. cART is represented with a grey shadow. On the right, the imbalance indicates the stand-alone effect of ICIs in terms of shock and kill curative strategies with low viral reactivation and enhancement of CD8+ T-cell function. Image created with Biorender and Adobe Illustrator CC 2018.

Clinical use of antibodies targeting IRs for HIV-1 cure

Given the potential benefits of ICIs in enhancing immunity and leading tumor remission [223], [224], the blockade of IRs is an attractive candidate to move forward into clinical evaluation in HIV-1-infected individuals. Although several studies suggest the safety and efficacy of the use of ICIs in HIV-1-infected individuals [315], [316], their effectiveness in the context of the cure is not fully understood and only based on limited case reports [232]–[234], [236], [244]. These studies range from no significant changes [232] to a

transient enhancement of functional CD8+ T cells without affecting viral persistence [233]–[235], transient increase of viral transcription but no changes in viral reservoirs [236], as well as some cases with reduction of the viral reservoir [190], [236].

In chapter III, we analyzed the impact of the use of pembrolizumab (αPD-1) in a cART suppressed HIV-1-infected patient with metastatic melanoma. Our results showed a case of complete remission of metastatic melanoma in response to treatment and an enhancement of T-cell activation and the frequency of HIV-1-specific CD8+ T-cell responses during the ICI treatment (**Figure 39**). We observed a peak of TNF production and a transition from TNF to an IL-2 cytokine profile in HIV-1-specific CD8+ T cells over time. Nevertheless, we cannot exclude the impact of pembrolizumab on other cell types expressing PD-1 such as NKs, DCs, monocytes, and B cells. PD-1 blockade has shown crucial immunotherapeutic effects in NK and DC responses [328] and increased B-cell activation, proliferation, and production of inflammatory cytokines [336]. Thus, future studies of ICIs measuring multiple cell types are essential to bringing new insights into the remission of cancer and HIV-1.

In addition, we assessed virological changes in the reservoir during pembrolizumab treatment. By measuring total HIV-1 DNA, cell-associated HIV-1 RNA, and ultrasensitive plasma viral load, we could not observe a consistent decrease in the total HIV-1 DNA or the plasma RNA, showing only two transient drops on proviral DNA during the study (**Figure 39**). These data reinforce the idea that ICIs alone *in vivo* is not enough to induce HIV-1 remission and are in line with previous studies that also found ICIs unable to detect a consistent decrease in the viral reservoir [232]–[234], [244]. Thus, clinical trials in HIV-1-infected individuals administrating ICIs with additional interventions such as vaccines or LRAs may be required to clarify the potential contribution of ICIs to the cure of HIV-1. In this context, it has been shown that combining ICIs and therapeutic cancer vaccines has the potential to improve the clinical outcomes modulating the tumor microenvironment better on their own [337].

Combinatorial therapeutic approaches to achieve the HIV-1 cure

Since each therapeutic approach presented here has its specific shortcomings, the combination of therapeutic approaches may increase the efficacy of each treatment alone. In this context, therapeutic vaccines in combination with LRAs have been tested in clinical trials RISVAC03, BCN02, REDUC, and RIVER [182], [187]–[189]. Although MVA.HIVconsv vaccine and romidepsin in BCN02 trial resulted in robust and broad HIVconsv-specific CD8+ T cells with significant reduction of the viral reservoir

[187], RISVAC03, RIVER, and REDUC did not show any impact on the latent reservoir or the rebound of plasma viral load after interruption of cART [182], [188], [189]. Thus, further optimization of combinatorial strategies may be required to achieve a sizeable effect on the latent reservoir that will translate into clinical benefits for people living with HIV-1.

Moreover, few clinical trials are ongoing using alternative approaches. Recent data by Fromentin *et al.* demonstrated *ex vivo* that latently HIV-1-infected cells showed synergically more reactivation after PD-1 blockade plus bryostatin than αPD-1 alone [190]. Additionally, a study by Borducchi *et al.* demonstrated that the combination of the agonist of TLR7 together with the bNAb PGT121 was able to delay SIV rebound for more than six months [338]. The use of different bNAbs in combination, such as 3BNC117 and 10–1074 in clinical trials, has shown suppression of viral rebound for a median of 21 weeks [339]. In addition, our group is exploring *ex vivo* the effect of therapeutic vaccination in combination with PD-1 blockade to boost vaccine-induced antiviral responses. We have observed an enhancement of proliferative capacity, IFNγ production, and HLA-DR+/CD38+ activation of HIV-1-specific CD8+ T cells after PD-1 treatment (Marin *et al.*, manuscript in preparation).

Thus, combinational approaches such as ICIs, therapeutic vaccines, LRAs, or bNAbs have been tested preclinically as well as in animal models, showing improved efficacy. However, these therapies raise concerns about undesirable irAEs. Reducing adverse events will be essential for its implementation in people living with HIV-1 and cART with a near-normal life expectancy and good quality of life.

Future perspectives

Although multiple advances have been made over the last years in designing and testing novel therapeutic strategies to attempt the cure of HIV-1, only allo-HSCT has eradicated the virus, proved by the lack of viral rebound after cART interruption [171]–[173]. However, the complexity of allo-HSCT implementation and safety concerns warrant further investigation to improve therapeutics for the HIV-1 cure. In addition, many scientific questions need to be addressed, including how to completely reactivate the latent viral reservoir, boost the immune responses, and the hurdles to reach viral sanctuaries in tissues.

Firstly, cure therapeutics will need to ensure a systemic reactivation of the replication-competent virus in blood and tissues from latently HIV-1-infected cells. Secondly, ICIs able to restore Tex in HIV-1 infection are essential. Moreover, thirdly, an effective HIV-

1 vaccine consisting of T-cell epitopes and selected B-cell epitopes should be explored to induce highly specific T-cell responses, bNAbs, NAbs, and antibody-dependent cellular cytotoxicity (ADCC), and antibody-dependent cellular viral inhibition (ADCVI) [340]. Of note, several studies are in progress in which T-cell-based conserved vaccines have been shown to enhance T-cell responses [341].

The optimal scenario would be the presence of low or undetectable viral reservoirs and potent HIV-1-specific immune responses that would prevent the viral rebound once cART is interrupted (**Figure 40**).

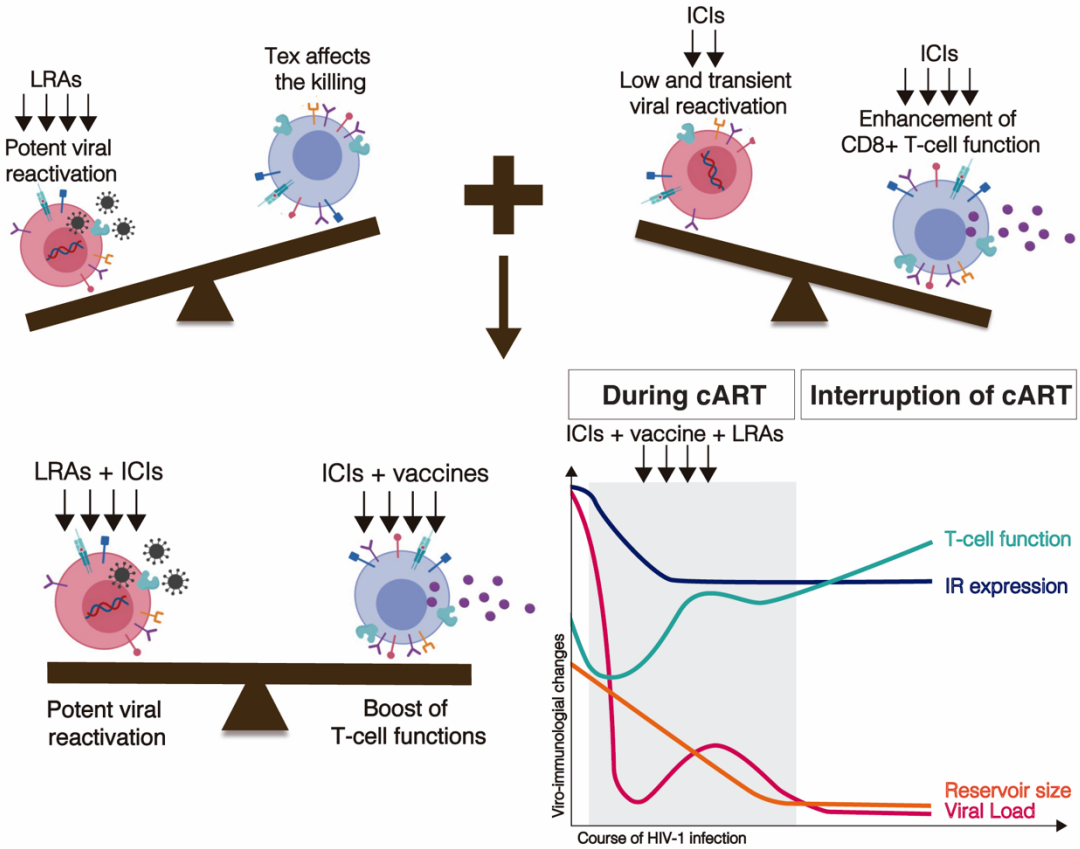

Figure 40. Optimal scenario to achieve a cure of HIV-1. Following the information provided in the chapters of this thesis (balances of the top), we need to balance viral reactivation and immunity to clear the HIV-1 reservoir. Viral load, reservoir size, the functionality of T cells, and IR expression are represented as changes after immunotherapy with LRAs, ICIs, and vaccines under cART to achieve the desired viro-immunological parameters to control and eliminate the virus from the body once cART is interrupted. cART is represented with a grey shadow. Image created with Biorender and Adobe Illustrator CC 2018.

The clue to establish a successful cure for HIV-1 will likely be established when all these viro-immunological components are combined in specific ways to release the brakes on exhausted immune cells and concurrently help force HIV-1 out of its hiding places. The goal for a cure of HIV-1 would be to attain the similar phenotype of elite controllers (ECs), which are a small fraction of HIV-1-infected individuals with the ability to remain clinically and immunologically stable without progression of AIDS for extended periods

and with undetectable viral loads [75], [162], [342]. Understanding HIV-1-controllers may provide a blueprint for developing CD8+ T cell-mediated therapeutics to obviate the lifelong daily cART and end the HIV pandemic.

Overall, this thesis provides an overview of Viro-immunological HIV-1 cure strategies tested in the last years. The thesis starts by identifying the limitations of the current shock and kill curative strategies, continuing a better understanding of IR expression in CD8+ T cells during HIV-1 infection to identify candidates for immune restoration, and finishing with the evaluation of current IR blockades *in vivo* as potential strategies for a cure of HIV-1.

In summary, the key for a potential cure of HIV-1 may rely on finding a way to effectively awaken the viral reservoir and potentially enhance cellular and humoral immune responses to clear the body from latently HIV-1-infected cells completely.

CONCLUSIONS

Objective I: To identify the immunological barriers that prevent "shock and kill" strategies from successfully clearing the viral reservoir

i. Development of a RELI-model to simultaneously evaluate HIV-1 reactivation by LRAs and killing of reactivated cells by HIV-1-specific CD8+ T-cell lines.

ii. The potency of LRAs is crucial to modulate the speed of HIV-1 antigen recognition and the magnitude of killing by HIV-1-specific CD8+ T cells.

iii. The increase of LRA dosage and the frequency of CD8+ T cells optimized the immune clearance of HIV-1-reactivated target cells.

iv. The expression of IRs in HIV-1-specific CD8+ T cells can limit the magnitude of the killing of LRA-reactivated cells.

Objective II: To delineate the expression of IRs across the landscape of CD8+ T cells and evaluate their impact in terms of functionality in HIV-1 infection and cART

i. The increase of TIGIT+ and TIGIT+TIM-3+ expression in CD8+ T-cell subsets persists throughout HIV-1 infection and long-term cART.

ii. Single TIGIT+ expression in CD8+ T cells inversely correlates with CD4+ T-cell counts.

iii. Highly complex profiles of IR expression across three levels in the landscape of CD8+ T cells are present in HIV-1 infection and long-term cART.

iv. Homeostatic changes of contraction and expansion in specific CD8+ T-cell clusters occur througout HIV-1 infection and long-term cART.

v. There is a selective depletion in a differential cellular cluster of memory-like HIV-1-specific CD8+ T cells with $CD107a^{hi}$ $TIGIT^{hi}$ characteristics despite long-term cART.

vi. TIGIT blockade effectively restores degranulation profile of total and HIV-1-specific CD8+ T-cell subsets.

Objective III: To determine the stand-alone effect of ICIs *in vivo* as possible candidates of shock and kill strategy in HIV-1 infection.

i. Complete remission of metastatic melanoma after treatment with pembrolizumab in a HIV-1-infected individual with cART.

ii. Pembrolizumab induced a transient increase of activation markers in EM and Eff CD8+ T cells.

iii. Pembrolizumab transiently enhanced the TNF production and switched to IL-2 profile in HIV-1-specific CD8+ T-cell responses.

iv. HIV-1-specific CD8+ T-cell reinvigoration was associated with a transient perturbation on the size of HIV-1 reservoir size.

LIST OF PUBLICATIONS

1. Athina Kilpeläinen, Esther Jimenez-Moyano, **Oscar Blanch-Lombarte**, Dan Ouchi, Ruth Peña, Bibiana Quirant-Sanchez, Anna Chamorro, Ignacio Blanco, Eva Martínez-Caceres, Roger Paredes, Lourdes Mateu, Jorge Carrillo, Julià Blanco, Christian Brander, Marta Massanella, Bonaventura Clotet, Julia G. Prado. "*Skewed cellular distribution and functional T-cell responses in SARS-CoV-2 Non-Seroconvertors*". Under review.

2. Ana Moyano, **Oscar Blanch-Lombarte**, Laura Tarancón, Nuria Pedreño, Miguel Arenas, Tamara Alvaro, Concepción Casado, Isabel Olivares, Mar Vera, Carmen Rodriguez, Jorge del Romero, Cecilio López-Galíndez, Ezequiel Ruiz-Mateos, Julia G. Prado, María Pernas. "*Loss of twenty-seven years of HIV-1 control in HLA B*14:02 LTNP driven by Env-EL9 CD8+ T-cell response*". Under review.

3. Antonio Astorga-Gamaza, Michele Vitali, Mireya L Borrajo, Rosa Suárez-López, Carlos Jaime, Neus Bastus, Carla Serra-Peinado, Laura Luque-Ballesteros, **Oscar Blanch-Lombarte**, Julia G Prado, Juan Lorente, Felix Pumarola, Marc Pellicer, Vicenç Falcó, Meritxell Genescà, Víctor Puntes and Maria J Buzon. "*Antibody cooperative adsorption onto AuNPs and its exploitation to force natural killer cells to kill HIV-1-infected T cells*". Nano Today. 36 (2021) 101056. DOI: 10.1016/j.nantod.2020.101056.

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6. Diego Sánchez-Martínez, Matteo L Baroni, Francisco Gutierrez-Agüera, Heleia Roca-Ho, **Oscar Blanch-Lombarte**, Sara González-García, Montserrat Torrebadell, Jordi Junca, Manuel Ramírez-Orellana, Talía Velasco-Hernández, Clara Bueno, José Luís Fuster, Julia G Prado, Julien Calvo, Benjamin Uzan, Jan Cools, Mireia Camos, Françoise Pflumio, María Luisa Toribio, Pablo Menéndez. "*Fratricide-resistant CD1a-specific CAR T cells for the treatment of cortical T-cell acute lymphoblastic leukemia*". Blood 2019 May 23; 133(21): 2291-2304. Epub 2019 Feb 22. PMID: 3079602. PMCID: PMC6554538. DOI: 10.1182/blood-2018-10-882944.

7. Alba Ruiz, **Oscar Blanch-Lombarte**, Esther Jiménez-Moyano, Dan Ouchi, Beatriz Mothe, Ruth Peña, Cristina Galvez, Meritxell Genescà, Javier Martinez-Picado, Philip Goulder, Richard Barnard, Bonnie Howell, Bonaventura Clotet and Julia G Prado. "*Antigen Production after Latency Reversal and Expression of Inhibitory Receptors in CD8+ T cells Limit the Killing of HIV-1 Reactivated cells*". Frontiers in Immunology. 2019 Jan 22; 9:3162. eCollection 2018. PMID: 30723480. PMCID: PMC6349966. DOI: 10.3389/fimmu.2018.03162.

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Skewed cellular distribution and functional T-cell responses in SARS-CoV-2 Non-Seroconvertors

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ABSTRACT

The role of T cells in the control of SARS-CoV-2 infection has been underestimated in favor of neutralizing antibodies. However, cellular immunity is essential for long-term viral control and protection from disease severity. To understand T-cell immunity in the absence of antibody generation we focused on a group of SARS-CoV-2 Non-Seroconvertors (NSC) recovered from infection. We performed an immune comparative analysis of SARS-CoV-2 infected individuals stratified by the absence or presence of seroconversion and disease severity. We report high levels of total naïve and low effector CD8+ T cells in NSC. Moreover, polyfunctional Nucleocapsid (NP)-specific CD8+ T-cell responses, as well as reduced levels of T-cell activation monitored by PD-1 and activation-induced markers were observed. Longitudinal data indicate the stability of the NSC phenotype over three months of follow-up after SARS-CoV-2 infection. Together, these data highlight distinctive immunological traits in NSC. These traits include skewed cellular distribution and functional SARS-CoV-2 T-cell responses as potential contributors to protection from disease severity in the absence of seroconversion.

Loss of twenty-seven years of HIV-1 control in HLA B*14:02 LTNP driven by Env-EL9 CD8+ T-cell response

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ABSTRACT

In the presence of protective HLA-B alleles, control of HIV-1 infection has been associated with strong and effective CD8+ T-cell responses against HIV-1 Gag epitopes. However, HIV-1 control by HLA-B*14:02 allele is associated with Env-specific CD8+ T-cell response. The association between HIV-1 CD8+ T-cell escape variant selection, and disease progression depends on immunogenetics and viral fitness. Here, we performed an in-depth virologic and immunologic longitudinal analysis in an HLA-B*14:02 viraemic long-term non-progressor (LTNP) that lost HIV-1 control (LVC) 27 years after diagnosis. We monitored the quasispecies evolution and searched for amino acid alterations in HLA restricted epitopes. We characterized the magnitude and functionality of longitudinal CD8+ T-cell responses restricted by Gag-DA9 and Env-EL9 HLA-B*14:02 epitopes, and identified Env-EL9 escape variants. Additionally, we evaluated the impact of Env-EL9 escape mutants on viral replicative capacity (RC), and potential Env protein structural changes. We found an immunodominant HLA-B*14:02 restricted response directed towards Env-EL9. We also identified positive selection in Env-EL9, including selection for K588R and L592R escape mutations. We identified double mutants in Env-EL9 with an increase of viral RC, Env protein stability, and loss of virological control after 27 years. These findings indicate the contribution of Env-EL9 immunodominant HLA-B*14:02 restricted CD8+ T-cell responses during long-term HIV-1 control, although the potency of these responses should be further characterized, especially in LTNPs lacking protective alleles. These findings support that Env epitopes constitute relevant CD8+ T-cell immunogens.

Key words: Long-term non-progressor (LTNP), loss of viral control (LVC), EL9 escape HLA-B*14:02, CD8+ T-cells

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Antibody cooperative adsorption onto AuNPs and its exploitation to force natural killer cells to kill HIV-infected T cells

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ABSTRACT

HIV represents a persistent infection which negatively alters the immune system. New tools to reinvigorate different immune cell populations to impact HIV are needed. Herein, a novel nanotool for the specific enhancement of the natural killer (NK) immune response towards HIV-infected T-cells has been developed. Bispecific Au nanoparticles (BiAb-AuNPs), dually conjugated with IgG anti-HIVgp120 and IgG anti-human CD16 antibodies, were generated by a new controlled, linker-free and cooperative conjugation method promoting the ordered distribution and segregation of antibodies in domains. The cooperatively-adsorbed antibodies fully retained the capabilities to recognize their cognate antigen and were able to significantly enhance cell-to-cell contact between HIV-expressing cells and NK cells. As a consequence, the BiAb-AuNPs triggered a potent cytotoxic response against HIV-infected cells in blood and human tonsil explants. Remarkably, the BiAb-AuNPs were able to significantly reduce latent HIV infection after viral reactivation in a primary cell model of HIV latency. This novel molecularly-targeted strategy using a bispecific nanotool to enhance the immune system represents a new approximation with potential applications beyond HIV.

Introduction

The augmentation of the effector immune responses towards specific targets, such as virally-infected or cancerous cells, is positioning itself as a new promising therapeutic intervention [1]. Indeed, an efficient immunological synapse between immune effectors and aberrant cells is a key factor to promote an effective immune-mediated killing of such undesired cells. In this context, cell-to-cell contact is required for natural killer (NK) cells, lymphocytes of the innate immune system that eliminate abnormal cells through several sophisticated biological mechanisms, including antibody-dependent cellular cytotoxicity (ADCC), to accomplish their mission [2–4]. Unfortunately, in the context of a persistent viral infection, like the one established by the human immunodeficiency virus (HIV), the virus integrates into the genome of host cells and is able to escape from the immune response, therefore perpetuating the infection [5]. In turn, chronic infection leads to a persistent immune activation that promotes the appearance of dysfunctional NK cells with diminished capacity to kill infected cells [6], further impairing immune defences. Importantly, this functionality is not completely restored with the current antiretroviral therapy, and discontinuation of the treatment leads to a rapid viral rebound [7]. Thus, new targeted interventions are needed to help the immune system of HIV-infected patients to control the virus. In this regard, novel therapeutic strategies focused on the re-direction, potentiation and/or promotion of an efficient immunological synapse between NK and

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HIV-1 Gag mutations alone are sufficient to reduce darunavir susceptibility during virological failure to boosted PI therapy

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Background: Virological failure (VF) to boosted PIs with a high genetic barrier is not usually linked to the development of resistance-associated mutations in the protease gene.

Methods: From a cohort of 520 HIV-infected subjects treated with lopinavir/ritonavir or darunavir/ritonavir monotherapy, we retrospectively identified nine patients with VF. We sequenced the HIV-1 Gag-protease region and generated clonal virus from plasma samples. We characterized phenotypically clonal variants in terms of replicative capacity and susceptibility to PIs. Also, we used VESPA to identify signature mutations and 3D molecular modelling information to detect conformational changes in the Gag region.

Results: All subjects analysed harboured Gag-associated polymorphisms in the absence of resistance mutations in the protease gene. Most Gag changes occurred outside Gag cleavage sites. VESPA analyses identified K95R and R286K ($P < 0.01$) as signature mutations in Gag present at VF. In one out of four patients with clonal analysis available, we identified clonal variants with high replicative capacity and 8- to 13-fold reduction in darunavir susceptibility. These clonal variants harboured K95R, R286K and additional mutations in Gag. Low susceptibility to darunavir was dependent on the Gag sequence context. All other clonal variants analysed preserved drug susceptibility and virus replicative capacity.

Conclusions: Gag mutations may reduce darunavir susceptibility in the absence of protease mutations while preserving viral fitness. This effect is Gag-sequence context dependent and may occur during boosted PI failure.

Introduction

The introduction of active combined ART (cART) has led to the effective control of viral replication in HIV-1-infected individuals. Although integrase inhibitor-based cART treatments are currently the most widely used first-line regimens, darunavir/ritonavir and lopinavir/ritonavir are used in some simplification strategies and low-income settings.[1–5]

Despite the high genetic barrier of PIs to resistance development, the emergence of mutations at the active site of the protease leads to HIV-1 drug resistance and virological failure (VF).[6–9] The main pathways of drug resistance to PIs are well defined. They initiate with mutational changes in the active site of the viral protease,[10] followed by a step-wise accumulation of mutations surrounding the active site,[10–12] at cleavage sites[13–15] and at non-cleavage sites of the Gag-pol polyprotein,[15–17] which compensate for replication defects and increase phenotypic resistance. The emergence of protease-associated mutations is usually observed in patients who have experienced VF to unboosted or first-generation boosted PIs.[18,19] On the other hand, this rarely occurs in patients experiencing VF to cART based on the most recently developed boosted PIs as first-line regimens or in simplification strategies.[6,8,9] This observation raises questions about the mechanism leading to VF to boosted PIs. Although the answer remains elusive, increasing evidence points to the role played by the presence of mutations outside of the protease and other unexplored mechanisms of resistance.

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Enhancement of Antiviral CD8+ T-Cell Responses and Complete Remission of Metastatic Melanoma in an HIV-1-Infected Subject Treated with Pembrolizumab

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Abstract: Background: Pembrolizumab is an immune checkpoint inhibitor against programmed cell death protein-1 (PD-1) approved for therapy in metastatic melanoma. PD-1 expression is associated with a diminished functionality in HIV-1 specific-CD8+ T cells. It is thought that PD-1 blockade could contribute to reinvigorate antiviral immunity and reduce the HIV-1 reservoir. Methods: Upon metastatic melanoma diagnosis, an HIV-1-infected individual on stable suppressive antiretroviral regimen was treated with pembrolizumab. A PET-CT was performed before and one year after pembrolizumab initiation. We monitored changes in the immunophenotype and HIV-1 specific-CD8+ T-cell responses during 36 weeks of treatment. Furthermore, we assessed changes in the viral reservoir by total HIV-1 DNA, cell-associated HIV-1 RNA, and ultrasensitive plasma viral load. Results: Complete metabolic response was achieved after pembrolizumab treatment of metastatic melanoma. Activated CD8+ T-cells expressing HLA-DR+/CD38+ transiently increased over the first nine weeks of treatment. Concomitantly, there was an augmented response of HIV-1 specific-CD8+ T cells with TNF production and poly-functionality, transitioning from TNF to an IL-2 profile. Furthermore, a transient reduction of 24% and 32% in total HIV-1 DNA was observed at weeks 3 and 27, respectively, without changes in other markers of viral persistence. Conclusions: These data demonstrate that pembrolizumab may enhance the HIV-1 specific-CD8+ T-cell response, marginally affecting the HIV-1 reservoir. A transient increase of CD8+ T-cell activation, TNF production, and poly-functionality resulted from PD-1 blockade. However, the lack of sustained changes in the viral reservoir suggests that viral reactivation is needed concomitantly with HIV-1-specific immune enhancement.

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IMMUNOBIOLOGY AND IMMUNOTHERAPY

Fratricide-resistant CD1a-specific CAR T cells for the treatment of cortical T-cell acute lymphoblastic leukemia

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KEY POINTS

- **CD1a CARTs exhibit specific and robust cytotoxicity in vitro and in vivo using both T-ALL cell lines and primary coT-ALL cells.**
- **CD1a CARTs are fratricide resistant and exhibit long-term persistence in vivo with antileukemic activity in rechallenge experiments.**

Relapsed/refractory T-cell acute lymphoblastic leukemia (T-ALL) has a dismal outcome, and no effective targeted immunotherapies for T-ALL exist. The extension of chimeric antigen receptor (CAR) T cells (CARTs) to T-ALL remains challenging because the shared expression of target antigens between CARTs and T-ALL blasts leads to CART fratricide. CD1a is exclusively expressed in cortical T-ALL (coT-ALL), a major subset of T-ALL, and retained at relapse. This article reports that the expression of CD1a is mainly restricted to developing cortical thymocytes, and neither CD34$^+$ progenitors nor T cells express CD1a during ontogeny, confining the risk of on-target/off-tumor toxicity. We thus developed and preclinically validated a CD1a-specific CAR with robust and specific cytotoxicity in vitro and antileukemic activity in vivo in xenograft models of coT-ALL, using both cell lines and coT-ALL patient–derived primary blasts. CD1a-CARTs are fratricide resistant, persist long term in vivo (retaining antileukemic activity in re-challenge experiments), and respond to viral antigens. Our data support the therapeutic and safe use of fratricide-resistant CD1a-CARTs for relapsed/refractory coT-ALL. (*Blood.* 2019;133(21):2291-2304)

Introduction

T-cell lineage acute lymphoblastic leukemia (T-ALL) is a malignant disorder resulting from leukemic transformation of thymic T-cell precursors.[1] T-ALL is phenotypically and genetically heterogeneous and is commonly associated with genetic alterations/mutations in transcription factors involved in hematopoietic stem and progenitor cell (HSPC) homeostasis and in master regulators of T-cell development.[2] T-ALL comprises 10% to 15% and 20% to 25% of all acute leukemias diagnosed in children and adults, respectively,[3,4] with a median diagnostic age of 9 years.[5-7] Intensive chemotherapy regimens have led to the improved survival of patients with T-ALL; however, the event-free and overall (OS) survival remains <70%, and relapsed/refractory (R/R) T-ALL has a particularly poor outcome. There are currently no potential curative options beyond hematopoietic cell transplantation and conventional chemotherapy, which is linked to large trade-offs in toxicities,[4,8] reinforcing the need for novel targeted therapies.

Immunotherapy has generated unprecedented expectations in cancer treatment and relies on the immune system as a powerful weapon against cancer. In recent years, adoptive cellular immunotherapy based on chimeric antigen receptors (CARs) has shown great potential. CAR therapy redirects genetically modified T cells to specifically recognize and eliminate specific antigen-expressing tumor cells in a major histocompatibility complex–independent fashion.[9,10] The success of CAR T cells (CARTs) redirected against CD19 or CD22 is now indisputable for B-cell malignancies (mainly B-ALL).[11-14] However, strategies targeting T-cell malignancies using CARTs remain challenging because of the shared expression of target antigens between CARTs and T-lineage tumoral cells. In this regard, CARTs against pan T-cell antigens have

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Antigen Production After Latency Reversal and Expression of Inhibitory Receptors in CD8+ T Cells Limit the Killing of HIV-1 Reactivated Cells

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The so-called shock and kill therapies aim to combine HIV-1 reactivation by latency-reversing agents (LRA) with immune clearance to purge the HIV-1 reservoir. The clinical use of LRA has demonstrated detectable perturbations in the HIV-1 reservoir without measurable reductions to date. Consequently, fundamental questions concerning the limitations of the recognition and killing of LRA-reactivated cells by effector cells such as CD8+ T cells remain to be answered. Here, we developed a novel experimental framework where we combine the use of cytotoxic CD8+ T-cell lines and *ex vivo* CD8+ T cells from HIV-1-infected individuals with functional assays of LRA-inducible reactivation to delineate immune barriers to clear the reservoir. Our results demonstrate the potential for early recognition and killing of reactivated cells by CD8+ T cells. However, the potency of LRAs when crossing the barrier for antigen presentation in target cells, together with the lack of expression of inhibitory receptors in CD8+ T cells, are critical events to maximize the speed of recognition and the magnitude of the killing of LRA-inducible provirus. Taken together, our findings highlight direct limitations in LRA potency and CD8+ T cell functional status to succeed in the cure of HIV-1 infection.

Keywords: human immunodeficiency virus, HIV-1 reservoir, HIV-1 immunogen, shock and kill, CTL (Cytotoxic T lymphocyte), inhibitory receptors

INTRODUCTION

The introduction of antiretroviral treatment (ART) has increased life expectancy and improved the health of people living with HIV-1 infection. However, ART does not cure HIV-1 infection, and treatment is needed for decades, thus raising the alarm about long-term health and sustainability of treatment and care of HIV-1-infected individuals (1). The current limitations of ART highlight the need for therapeutic strategies to eradicate HIV-1 from the body. A major obstacle to eradication is

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Gag-protease coevolution analyses define novel structural surfaces in the HIV-1 matrix and capsid involved in resistance to Protease Inhibitors

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Despite the major role of Gag in establishing resistance of HIV-1 to protease inhibitors (PIs), very limited data are available on the total contribution of Gag residues to resistance to PIs. To identify in detail Gag residues and structural interfaces associated with the development of HIV-1 resistance to PIs, we traced viral evolution under the pressure of PIs using Gag-protease single genome sequencing and coevolution analysis of protein sequences in 4 patients treated with PIs over a 9-year period. We identified a total of 38 Gag residues correlated with the protease, 32 of which were outside Gag cleavage sites. These residues were distributed in 23 Gag-protease groups of coevolution, with the viral matrix and the capsid represented in 87% and 52% of the groups. In addition, we uncovered the distribution of Gag correlated residues in specific protein surfaces of the inner face of the viral matrix and at the Cyclophilin A binding loop of the capsid. In summary, our findings suggest a tight interdependency between Gag structural proteins and the protease during the development of resistance of HIV-1 to PIs.

The introduction of protease inhibitors (PIs) as part of the highly active antiretroviral therapy (HAART) have led to a dramatic reduction in morbidity and mortality rates in HIV-1–infected patients[1]. PIs have high intrinsic antiviral activity and are among the most potent antiretroviral drugs (ART) available in clinical practice to date. In fact, only simplification strategies with boosted PIs have proven to be as efficacious as triple ART in maintaining continuous virological suppression[2,3].

PIs target the active site of the HIV-1 protease (PR). Protease activity is essential for the generation of full infectious viral particles through the cleavage of Gag and Gag-pol polyproteins. Despite the high genetic barrier of the PI, the emergence of mutations at the protease active site leads to drug resistance. Mutations in HIV-1 causing resistance to PIs reduce the affinity of the drug for the active site. These mutations are generally followed by a stepwise accumulation of additional mutations in protease that partially rescue its activity[4]. Moreover, mutations in the Gag polyprotein at protease cleavage sites have generally been shown to contribute to resistance to PIs by restoring the interaction with the cleavage sites and compensating for defects in viral replicative capacity[5,6].

However, the above-mentioned "traditional" PI resistance pathways have been challenged by studies that evidence virological failure of PI-treated patients in the absence of PI resistance mutations[7]. Various studies have demonstrated the direct contribution of Gag mutations to drug susceptibility. Thus, mutations at Gag cleavage site positions A431V, K436E and I437V/T conferred resistance to PIs in the absence of drug resistance mutations at

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¡1000 millones de gracias a todos ☺ ☺ ☺!

Óscar


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HIV-1 GAG MUTATIONS REDUCE PI-SUSCEPTIBILITY IN THE ABSENCE OF PROTEASE RESISTANCE MUTATIONS
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