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Anti-PD-1 mediated depletion of cellular reservoir from HIV infected donor blood mononuclear cells: A proof of concept study

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UNIL | Université de Lausanne
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Anti-PD-1 mediated depletion of cellular reservoir from HIV infected donor blood mononuclear cells: A proof of concept study

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by

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Summary:

Background: The advances of HIV research and especially the advent of antiretroviral therapy (ART) led to strong and long-lasting viral suppression in peripheral blood of most treated individuals. Despite the efficacy of ART, treatment interruption results in vial rebound and disease progression due to the persistence of HIV in reservoirs that cannot be reached by ART. Recent studies have shown that PD-1$^+$CD4 T cells serve as a major cellular reservoir for HIV-1 replication and production, thus providing a strong rationale for developing therapeutic strategies targeting the elimination of PD-1$^+$CD4 T cells.

Aim: The current project aims at killing HIV infected cells based on PD-1 as a marker for HIV cellular reservoir using the antibody-dependent cellular cytotoxic activity (ADCC) of humanized IgG1 antibodies or antibody-drug conjugates (ADC).

Methods: Humanized anti-human PD-1 antibodies (*h*PD-1 IgG1/IgG4) were developed to evaluate ADCC activity. In parallel, mouse or humanized anti-PD-1 antibodies (*m*PD-1 or *h*PD-1 Ab) were conjugated with an anthracycline toxin to produce an antibody-drug conjugate (ADC). ADCC and ADCs were evaluated on blood mononuclear cells and isolated CD4$^+$ T cells, respectively, isolated from viremic untreated or ART suppressed HIV infected donors in 5-day assays. Cells undergoing apoptosis and cell death were assessed by flow cytometry and culture supernatants were analyzed for viral p24 (ECL COBAS HIV Ag) or HIV RNA (COBAS AmpliPrep/TaqMan HIV-1) production. Cells from ART treated donors were tested in a quantitative viral outgrowth assay (QVOA) to evaluate the frequency of cells harboring replication competent and infectious viruses following Ab or ADC treatment.

Results: In HIV viremic donors, *h*PD-1 IgG1 lead to the targeted depletion of PD-1$^+$CD4 T cells, which was associated with 59% and 43% reduction of HIV RNA and p24 production from infected cells, respectively (n=4). Similarly, *m*/*h*PD-1 ADCs efficiently depleted PD-1$^+$CD4 T cells as indicated by the increased frequencies of apoptotic and dead cells compared to unconjugated *m/h*PD-1 Ab (n=7 and n=5, respectively). Targeted cytotoxicity was associated with 80% and 83% reduced viral p24 production in the culture supernatants compared to unconjugated *m/h*PD-1 Ab, respectively. In ART suppressed individuals, increased frequencies of apoptotic cells and cell death after incubation with *h*PD-1 IgG1 treatment reduced the frequency of infected cells harboring replication competent viruses by 66% (n=4). Similarly, *m*PD-1 ADC was associated with 88% reduction of infected cells harboring replication competent viruses and cells harboring infectious viruses were undetectable (n=5; n=4).

Discussion: These results indicate that the depletion of PD-1$^+$CD4 T cells by *h*PD-1 IgG1 or *m*/*h*PD-1 ADC strongly reduces HIV-1 infected cells capable to produce infectious viruses. In these proof of concept studies, *h*PD-1 IgG1 or anti-PD-1 ADC represent novel interventions that could contribute to a functional cure for HIV-1 infected individuals.

Résumé

Contexte : Les avancées effectuées en recherche sur le VIH et tout particulièrement le développement des antirétroviraux (ARV) ont conduit à une suppression forte et durable du virus dans le sang de la plupart des individus infectés. Toutefois le virus resurgit chez la plupart des patients infectés si le traitement est interrompu, impliquant la persistance du VIH dans des réservoirs cellulaires qui ne sont pas ciblés par les antirétroviraux. Des études récentes ont démontré que les cellules T PD-1^{+}CD4^{+} constituent un réservoir important pour la réplication et la production du VIH, justifiant le développement de stratégies thérapeutiques visant l'élimination de ces cellules.

But : Ce projet vise l'élimination des cellules infectées par le VIH en se basant sur l'expression de PD-1 comme marqueur du réservoir cellulaire. Les cellules T PD-1^{+}CD4^{+} sont ciblées grâce à l'activité cytotoxique cellulaire dépendante des anticorps (ADCC) d'un anticorps anti-PD-1 IgG1 humanisé ou à l'aide d'anticorps anti-PD-1 couplés à une toxine.

Méthode : Des isoformes IgG1 and IgG4 ont été développées pour l'anticorps humanisé anti-PD-1 humain (*h*PD-1), afin d'évaluer l'activité ADCC. Pour créer le conjugué médicament-anticorps (ADC), les anticorps souris ou humanisés anti-PD-1 humain (*m*PD-1 ou *h*PD-1) ont été conjugués à une toxine de la classe des anthracyclines. En parallèle, l'activité ADCC et l'ADC ont été évalués au moyen d'un test de cinq jours respectivement sur des cellules sanguines mononucléaires et sur des cellules T CD4^{+} isolées provenant de donneurs infectés virémiques et non-traîtés, ou de donneurs avirémiques sous ARV. Les cellules apoptotiques ainsi que la mort cellulaire ont été évaluées par cytométrie en flux. La présence d'antigène p24 (ECL COBAS HIV Ag) ainsi que d'ARN viral (COBAS AmpliPrep/TaqMan HIV-1) a été déterminée dans les surnageants de culture. Finalement, les cellules de donneurs traités par ARV ont été récoltées, et les fréquences des cellules abritant des virus capables de réplication et infectieux déterminées par un test d'amplification viral (QVOA).

Résulats : Sur les donneurs virémiques, *h*PD-1 IgG1 a efficacement tué les cellules T PD-1^{+}CD4^{+}, comme l'indique l'augmentation des fréquences de cellules apoptotiques et mortes comparé au traitement *h*PD-1 IgG4. La cytotoxicité est associée à une réduction de 44% de p24 viral produit dans les surnageants comparé à *h*PD-1 IgG4 (n=4). Similairement, la mort ciblée des cellules T PD-1^{+}CD4^{+} par les ADCs *m*PD-1 et *h*PD-1 (respectivement n=7 et n=5) est associée à une réduction de respectivement 80% et 83% de p24 viral produit dans les surnageants comparé aux anticorps non-conjugués. Sur les donneurs avirémiques sous ARV, le traitement *h*PD-1 IgG1 a causé 66% de réduction de cellules infectées abritant des virus capables de réplication (n=4). Similairement, le traitement *m*PD-1 ADC est associé à 88% de réduction de cellules infectées abritant des virus capables de réplication, et aucune cellule abritant des virus infectieux n'a été détectée (respectivement n=5 et n=4).

Discussion : Ces résultats démontrent que la déplétion ciblée des cellules T PD-1^{+}CD4^{+} causée par *h*PD-1 IgG1 ou *m*/*h*PD-1 ADC induit une forte réduction du nombre de cellules infectées par le VIH capables de produire du virus infectieux. Dans ces études de démonstration de faisabilité, *h*PD-1 IgG1

et *m/h*PD-1 ADCs représentent de nouvelles approches interventionnelles qui pourraient contribuer à l'élaboration d'un traitement curatif pour les patients infectés par le VIH.

List of abbreviations

Ab	antibody		IL	interleukin
ADC	antibody-drug conjugate		IN	integrase
ADCC	antibody-dependent cell cytotoxicity		INI	integrase inhibitor
ADCP	antibody-dependent cell phagocytosis		IRF	interferon regulatory factor
AIDS	acquired immunodeficiency syndrome		LN	lymph node
APOBEC	apolipoprotein B mRNA editing enzyme		LRA	latency reversing agents
ART	antiretroviral therapy		LTNP	long-term non-progressor
ATI	analytical treatment interruption		LTR	long terminal repeats
AZT	azidothymidine		MA	matrix protein
BiTE	bispecific T cell engager		MDM	monocyte derived macrophage
bNAb	broadly neutralizing antibody		MFI	mean fluorescence intensity
BsAb	bispecific antibody		MHC	major histocompatibility complex
BST-2	bone marrow stromal cell antigen 2		moDC	monocyte derived dendritic cell
CA	capsid		MSM	men having sex with men
CAR	chimeric antigen receptor		NC	nucleocapsid
CDC	complement dependent cytotoxicity		NHEJ	non-homologous end joining
cGAS	cyclic GMP-AMP synthase		NK	natural killer cell
CRISPR/Cas9	clustered regularly interspaced short palindromic repeats associated protein 9		nNAb	non-neutralizing antibody
CTL	cytotoxic T lymphocyte		NNRTi	non-nucleosid reverse transcriptase inhibitor
DART	dual-affinity retargeting antibody		NRTi	nucleosid analog reverse transcriptase inhibitor
DC	dendritic cell		PAMP	pathogen associated molecular pattern
dNTP	desoxynucleotide tri-phosphate		PD-1	programmed cell death 1
Env	enveloppe		pDC	plasmacytoid dendritic cell
ESCRT	endosomal sorting complexes required for transport		PI	protease inhibitor
Fab	antigen-binding fragment		PIC	pre-integration complex
Fc	crystallizable fragment		PKC	phosphokinase C
FCγR	crystallizable fragment gamma receptor		PR	protease
FDA	food and drug administration (US)		PRR	pathogen recognition receptor
FDC	follicular dendritic cell		QVOA	quantitative viral outgrowth assay
GALT	gut associated lymphoid tissues		RIG-1	retinoic acid-inducible gene I
GPI	glycosylphosphatidylinositol		RRE	Rev-response element
gRNA	guide RNA		RT	reverse transcriptase
HDACi	histone deacetylase inhibitor		RTC	reverse transcriptase complex
HIV /SIV	human/simian immunodeficiency virus		scFv	single chain variable fragment
HLA	human leukocyte antigen		TALEN	transcription activator-like effector nuclease
HPSC	hematopoietic progenitor stem cell		TCR	T cell receptor
i/aNKR	inhibitory/activatory natural killer receptor		T_{FH}	follicular helper T cell
ICI	immune checkpoint inhibitor		TNF	tumor necrosis factor
IFI16	gamma-interferon inducible protein 16		TRIM5α	tripartite motif-containing protein 5
IFITM	interferon induced transmembrane protein		ZFN	zinc finger nuclease
IFN	interferon			

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1 Introduction

1.1 General introduction on human immunodeficiency virus

1.1.1 HIV viruses

The human immunodeficiency virus (HIV) belongs to the Lentivirus genus of the Retroviridae family and has been shown to be the causative agent for acquired immunodeficiency syndrome (AIDS)[1]. Based on genetic characteristics and viral antigens, HIV is classified into type 1 and 2 (HIV-1 and HIV-2)[2,3]. Epidemiologic and phylogenetic studies suggest that HIV viruses originate from cross-species transmissions of simian immunodeficiency viruses (SIV) infecting African monkeys and were transmitted to human around 1920 and 1940.

1.1.2 HIV-1

1.1.2.1 Genome and particle structures

HIV is a spherical particle of about 100 nm diameter, with an outer lipid bilayer membrane as envelope (**Figure 1A-B**)[4]. The envelope displays 72 knobs composed of trimers of Env glycoproteins. Gp120 trimers are anchored to the envelope membrane by the trimers of gp41[5]. Envelope membrane covers the symmetrical outer core membrane and the inner capsid protein (CA), which forms a conical shape that encapsulates two identical molecules of single stranded RNA as well as several viral enzymes[6-8].

HIV genome is about 9.7 Kb and contains nine genes encoding 15 proteins (**Figure 1C**). The genome of HIV provirus, resulting from reverse transcription of HIV RNA into double-stranded DNA and integration in the host cell genome, is flanked by long terminal repeat sequences (LTRs) at both ends[9]. 5' LTR encodes the promotor for the transcription of viral genes, including *gag*, *pol*, *env*, *tat*, *rev*, *nef*, *vif*, *vpr* and *vpu* (or *vpx* in HIV-2). *gag* encodes the outer core membrane matrix protein (MA, p17), the capsid protein (CA, p24), the nucleocapsid (NC, p7), and a small nucleic acid stabilizing protein; *pol* encodes the enzyme machinery, namely the protease (PR, p10), reverse transcriptase (RT, p66/p51) and the integrase (IN, p32); *env* encodes the surface glycoproteins gp120 and gp41. Beside structural proteins, HIV genome codes for several regulatory proteins: Tat, Rev, Nef, Vif, Vpr and Vpu (or Vpx). While Tat and Rev are mostly involved in the regulation of HIV transcription, Nef, Vif, Vpr and Vpu are dedicated to evasion and modulation of innate and adaptive immune mechanisms[10-12].

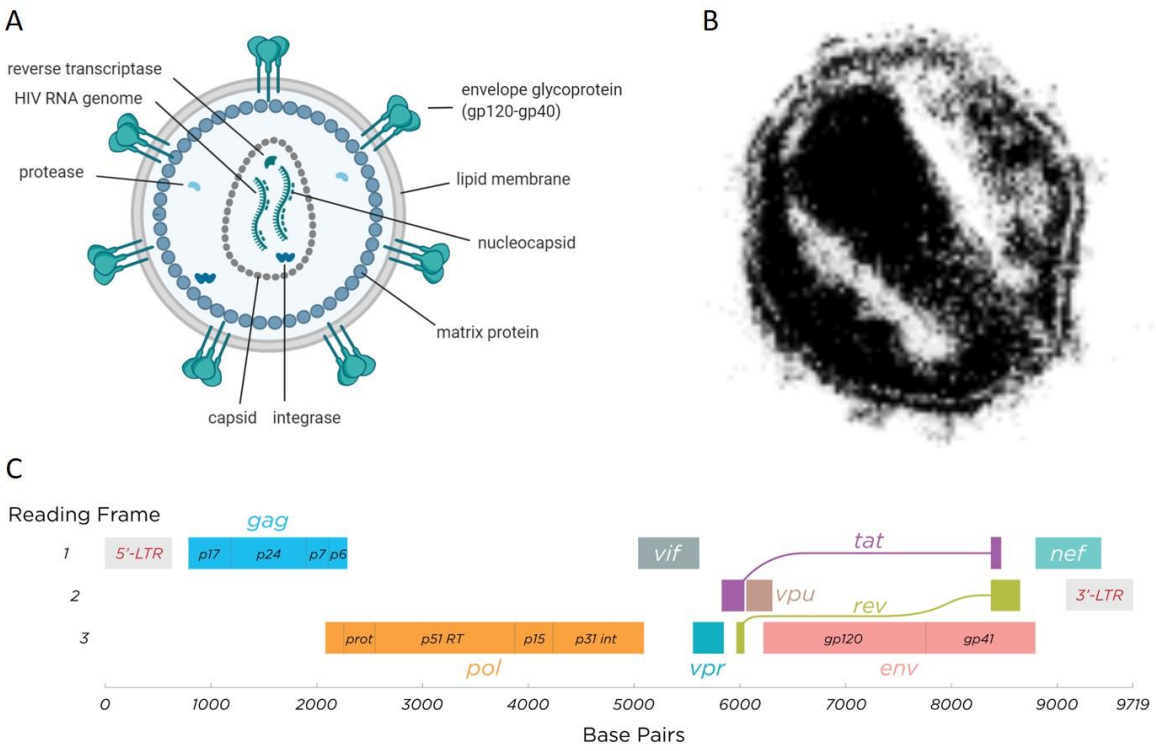


Figure 1: Structure of HIV virion and genome organization. *(A) Schematic representation of HIV structure and (B) figuration by electron microscopy[4]. (C) Organization of the nine genes encoded by HIV genome, gag, pol, env, rev, tat, nef, vif, vpr and vpu.*

1.1.2.2 HIV infection cycle

The infection cycle of HIV is divided in two phases: the early phase describes events from cell binding to the integration of HIV DNA in the cell genome; the late phase describes post-integration events, which starts with the transcription of HIV genes and ends with the release of HIV particles.

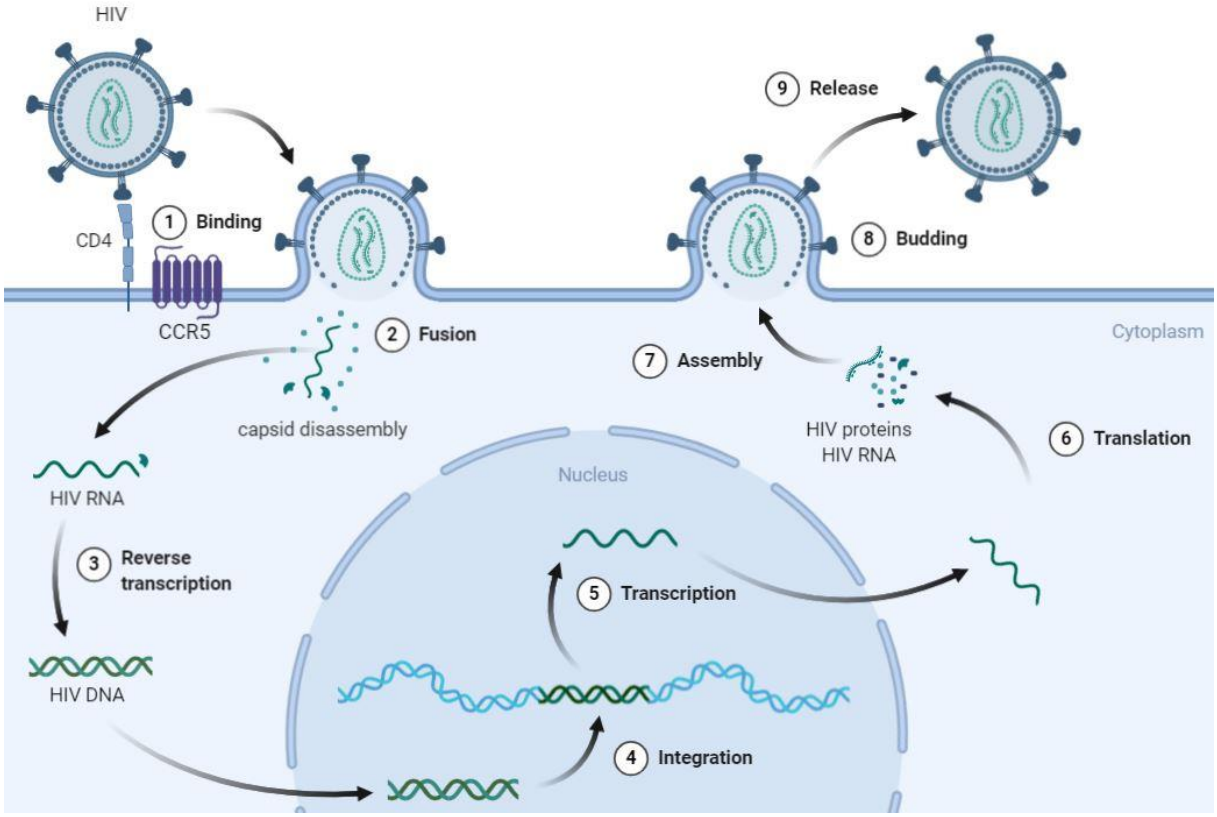


Figure 2: HIV replication cycle. *Binding to CD4 and CCR5 (or CXCR4) co-receptor (1) allows particle fusion with the cell membrane (2) and release of the viral capsid in the cytoplasm. Capsid disassembles and HIV RNA is reverse transcribed (3) to cDNA and then to dsDNA. dsDNA is imported to the nucleus with the pre-integration complex and integrated in the cell genome (4). Viral genome is then transcribed (5) and mRNA translated into viral proteins by the host machinery (6). Viral proteins and genomic RNA are recruited to the cell membrane for the particle assembly into an immature virion (7). The budding virion matures when the protease releases mature viral proteins (8) and new made virion is then released from the cell (9).*

1. Virus binding and entry

The early attachment of virions to the cell surface involves interactions with surface molecules such as heparan sulfate proteoglycan, LFA-1 and nucleolin[13-15]. This allows the virions to concentrate at the cell surface and helps specific receptor engagement. Trimer of gp120 glycoprotein present on mature virions specifically binds to CD4 receptor present on host cell surface. Attachment of CD4 to gp120 trimer induces conformational changes, exposing a site for co-receptor binding to CCR5 or CXCR4 depending upon the tropism of the

virus[16,17]. Binding to CD4 and co-receptor leads to additional conformational changes, allowing N-termini of gp41 to insert in the host cell membrane and subsequent fusion of both viral and host cell membranes and release of the viral capsid into the cytoplasm[18].

2. Uncoating and reverse transcription

The release of the viral core in the cytoplasm is associated with the gradual disassembly of capsid molecules (CA) (uncoating) and the generation of the reverse-transcription complexes (RTCs) and pre-integration complexes (PICs). Though the exact timing and mechanism of those events are still poorly understood, recent articles suggest that uncoating may succeed HIV RNA transcription[19-21].

Mature reverse transcriptase (RT) has two enzymatic sites: a polymerase and a HIV RNase H[19]. To carry out the transcription, HIV RNA bound to host cell $tRNA^{Lys3}$ is associated to the viral proteins reverse transcriptase (RT), integrase (IN) and nucleocapsid in a complex called the reverse-transcription complex (RTC). RT transcribes HIV RNA into cDNA. While HIV RNA is degraded by the RNase H, HIV cDNA is converted into double-stranded DNA (proviral DNA) by the DNA-dependent polymerase activity of the RT.

After completion of the reverse transcription, the RTC/PIC is transported to the nucleus for translocation and subsequent integration of the proviral DNA into the host genome. This process requires trafficking in the host cell and involves interactions with the cytoskeleton actin and microtubules[22].

3. Translocation into the nucleus and integration in the host genome

The proviral DNA is transported to the nucleus as the pre-integration complex (PIC), formed by the integrase (IN), proviral DNA, viral protein R (Vpr), matrix protein (MA) and several host co-factors. Due to the large size of PIC relative to the nuclear pore (56 vs 25 nm diameter, respectively) and the ability of HIV to infect non-dividing cells, the complex must be actively transported through nuclear pore complexes[23,24]. Though the mechanism of nuclear entry remains unclear, several studies have demonstrated the implication of CA protein for active nuclear transport via Nup153 binding[25-28].

Once in the nucleus, the integration of proviral DNA in the host cell genome finalizes the infection and provides persistence to HIV. To insert in the host cell genome, integrase (IN) catalyzes first 3' processing step where the 3'GT dinucleotide is removed from the U5 and U3 ends. Then, IN introduces a staggered cut unto the host chromatin and catalyzes the strand transfer reaction[29,30].

Integration in the host genome is not random and is assisted by cell host factors[31,32]. Sequencing studies revealed that HIV DNA integration favored transcriptionally active

genes[33,34]. Important factors are LEDGF/p75 and CPSF6. LEDGF/p75 has been shown to greatly enhance IN activity *in vitro* and to have a crucial role for HIV DNA integration *in vivo*[35,36]. Recent studies suggested that CPSF6 was involved in directing integration in transcriptionally active genes and LEDGF in directing the position in the gene[37].

4. Expression of viral genes and proteins

HIV transcription efficiency depends on chromatin environment but also on cell activation status[38,39]. HIV transcription is mediated by the 5' LTR promoter, which has binding sites for many host transcription factors such as NFkB, NFAT, AP-1 and SP-1[40-44]. Successful HIV transcription requires host RNA polymerase II (RNAP II) as well as many host and viral co-factors[45,46].

HIV RNA has many splicing sites so that it can yield 40 different transcripts that code for nine different proteins[47]. The early transcription products result in fully spliced mRNA that is quickly delivered to the cytosol for translation into regulatory proteins Nef, Tat and Rev. Those regulatory proteins are required for efficient transcription and can alter the viral transcription output. While Nef has been shown to positively affect the activity of transcription factors, Tat and Rev are responsible for the conversion to late gene expression[48-53].

Late gene expression results in single-spliced and unspliced transcripts, coding for Vpu, Vpr, Vif and Env, and Gag-Pol polyprotein, respectively. The nuclear export of intron-containing transcripts is allowed by the interaction of Rev with a secondary structure called the Rev Responsive Element (RRE), present in within 3' intronic region[52,53].

5. HIV particle assembly, budding and maturation

HIV Env protein is a trans-membrane protein and therefore migrates to the cell surface using the cellular vesicular transport. Env is inserted co-translationally into the ER where it is glycosylated, assembled in trimeric complexes and processed into gp41 (transmembrane domain) and gp120 (surface domain) before it is delivered to the plasma membrane.

Virion assembly occurs at the plasma membrane in specialized microdomains and is mostly mediated by HIV structural polyproteins Gag and Gag-Pol[54]. Gag inserts in the plasma membrane via MA (amino-terminal domain) and helps the recruiting and concentrating of viral Env proteins. The central domain of Gag (CA) mediates protein-protein interactions that are required for viral assembly. Gag NC domain binds the packing sequence (Ψ) present in HIV gRNA. Finally Gag carboxy terminal domain p6 binds several proteins such as HIV accessory protein Vpr. Although Gag mediates viral assembly, HIV requires the host ESCRT machinery to terminate Gag polymerization and catalyze the release of the virion.

The viral proteins first assemble in spherical immature particles. While the immature virion buds, HIV protease is activated and cleaves Gag polyproteins into its constituents (MA, CA, NC and p6).

To summarize, attachment of HIV virion to the cell membrane requires 30 minutes to 2 hours, transcription of genomic RNA to proviral DNA up to 6 hours, and integration process 6 hours. Twelve more hours are required to detect the first viral particles after provirus integration, meaning that a fully successful viral replication cycle requires an average of 24 hours.

1.1.2.3 Groups and subtypes

HIV-1 is divided in groups M, N, O and P, resulting from independent cross-species transmission events. Based on phylogenetic analysis, groups M and N directly originate from strains present in chimpanzees (SIVcpz*ptt*). HIV-1 group P may come from a strain circulating in gorilla (SIVgor), whereas no direct common ancestor was found for HIV-1 group O (SIVgor or SIVcpz*ptt*)[2].

The main strain responsible for HIV-1 pandemic is the group M. Group M is subdivided into subtypes corresponding to distinct lineages or clades, A-D, F-H, J and K[55]. Subtypes originate from HIV-1 M dissemination that lead to several founder events resulting in the predominance of one or the other subtype in different geographic areas[2].

1.1.3 HIV-2

The reservoir for HIV-2 ancestors was identified as being the sooty mangabey (SIVsmm)[56,57]. Similarly to HIV-1, HIV-2 is classified in groups, namely A to H, though groups A and B only have spread considerably within humans[2].

HIV-2 has been shown to be generally less pathogenic than HIV-1. Patients infected with HIV-2 usually have lower viral loads and rare progression to AIDS[58,59].

1.2 HIV infection / AIDS

1.2.1 Epidemiology

The first documented case of AIDS was published in 1981 in the US. In 1982, further cases were reported in US metropolitan areas, mostly among men having sex with men (MSM) and intravenous drug users[60]. AIDS was then described as a disease associated with defects in cellular immunity and the occurrence of opportunistic infections or malignant neoplasms. The agent responsible for AIDS, HIV-1, was first isolated in 1983 from AIDS patients and described as a T cell-tropic retrovirus[61]. HIV-1 was confirmed as the etiological agent for AIDS in 1985[62].

Starting from 1983, several reports point the prevalence of AIDS in African patients who had emigrated in Europe, and in Africa itself, suggesting that HIV-1 infection was not localized but in fact a pandemic[63,64]. In addition, studies performed in Africa observed an even distribution of cases between men and women, pointing toward a heterosexual transmission as well. AIDS in children suggests a possible mother-to-child transmission of the disease.

Retrospective analyses of infected blood and tissue samples from Kinshasa residents in 1959 and 1960 showed that HIV-1 infection was present and had already diversified at that time[65]. Phylogenetic and statistical analyses dated the last common ancestor for HIV-1 group M from 1920's[66,67]. Similarly, group P may have appeared around 1940 and group N around 1960. HIV-1 M was probably exported to Haiti in 1966 and then to North America. Since then, HIV-1 M subtypes have been spreading to a global pandemic, with predominance of different subtypes in different geographic area resulting from a number of bottleneck-founder events (**Figure 3**)[68].

In contrast, HIV-2 appeared through zoonotic transmission of SIVsmm (sooty mangabey) around 1940[2,69]. It remained mostly restricted to West Africa due to its significantly lower infectivity. HIV-2 was exported to Portugal and France probably in the mid-60's, leading to wide spread in Europe, South America and Asia, though with low prevalence[70].

Globally, 37.9 million people were living with HIV-1 and 1.7 million people were newly infected in 2018 (UNAIDS Data 2019). More than 80% of HIV-infected individuals live in developing countries, with the highest prevalence in the Sub-Saharan Africa. The distribution of new HIV infections varies significantly between countries. While HIV is predominantly transmitted within the general population in eastern/southern Africa, there is a marked higher prevalence among key populations in developed countries, including MSM, drug users, transgenders and prostitutes.

The access to antiretrovirals and education of the population has been a key event in the progress made to control HIV infection, though huge disparities are still observed between

countries. Despite noticeable progresses, 28% reduction of new infections compared to 2010 (16% reduction worldwide), eastern/southern African countries still remain the most affected by the epidemic.

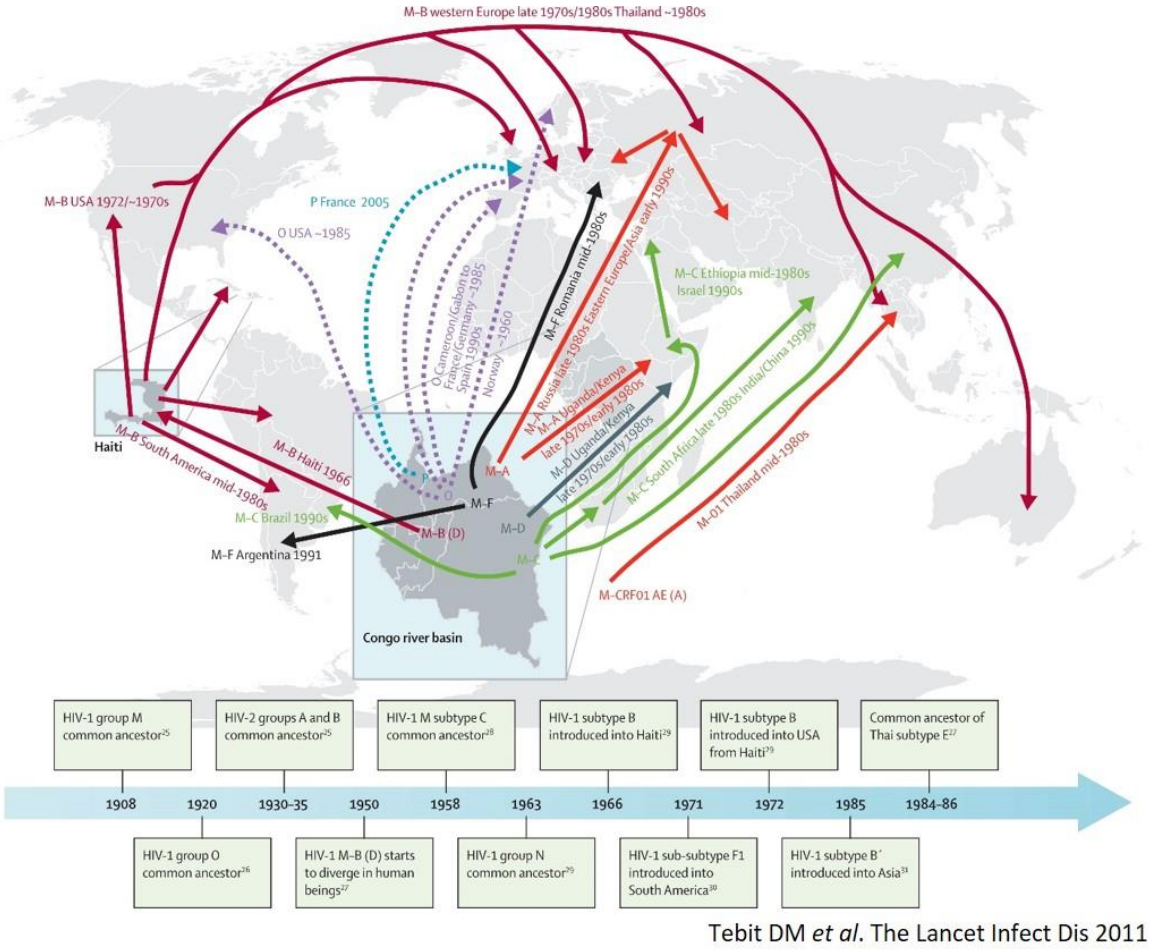


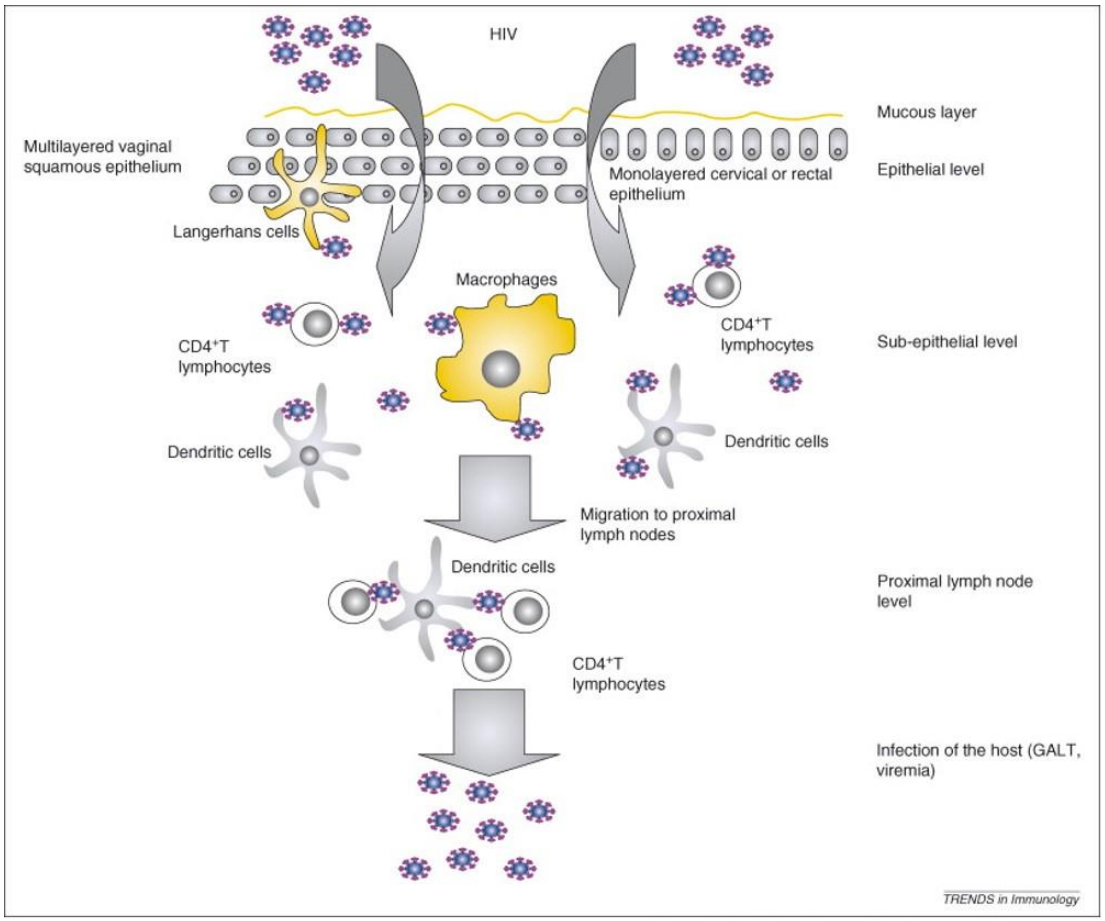
Figure 3: Global spread of HIV. *Timeline indicates key events in the spread of HIV-1 groups M, N and O, and of HIV-2.*

1.2.2 HIV transmission and pathogenesis

1.2.2.1 Acute phase

HIV enters the body via intact mucosa, eczematous or injured skin/mucosa and by parenteral inoculation. During sexual transmission, which is the most common route for HIV infection, HIV virus enters through vaginal or gastro-intestinal mucosa by interacting with dendritic cells (Langerhan's cells) (**Figure 4**)[71]. Though the virus can infect all cells expressing CD4 and CCR5 including macrophages/monocytes and dendritic cells, it preferentially replicates in resident memory $CD4^{+}CCR5^{+}$ T cells[71-75]. The propagation of the virus may be enhanced by the activation of the innate immune cells, which recruit cells that are potentially new targets for HIV infection[76].

One or two days after local infection, infected cells and virions are drained toward gut associated lymphoid tissues (GALT), where the abundance of activated $CD4^+CCR5^+$ T cells supports active replication, resulting in a massive depletion of target and bystander cells and dissemination of the virus[77]. HIV is detectable in lymph nodes (LN) five to six days after transmission. Assisted by surrounding dendritic cells and B cells, secondary lymphoid organs support amplification and propagation of the virus to the whole body[78,79].



Piguet V. *et al*. Trends in Immunology 2007

Figure 4: HIV mucosal transmission. *HIV crosses the mucosal barrier through interaction with DCs. Once in the sub-epithelial level, HIV can infect macrophages, other DCs and mucosal $CD4^+$ T cells. The migration of infected cells to proximal lymph nodes and then to GALT contributes to the wide spreading of HIV infection.*

The onset of humoral response starts after 3-6 weeks of infection and is associated with unspecific clinical symptoms such as fever, LN enlargement, fatigue, malaise, skin rash and gastro-intestinal symptoms[80]. The initial absence of immunity results in aggressive HIV replication and dissemination throughout the body, associated with a strong depletion of peripheral blood $CD4^+$ T cells. With the onset of the first humoral immune responses, peripheral $CD4^+$ T cells rebound and viral load decreases.

Since acute HIV infection poorly targets naïve and resting central memory T cells, this pool provides possible regeneration of $CD4^+$ T cells[81]. In fact, the chronic phase of HIV infection is associated with reduction of viral load and an almost normal circulatory $CD4^+$ count. However, the increased general immune activation and the rapid $CD4^+$ T cell turnover eventually progresses to AIDS in most infected individuals[82].

HIV is detectable in regional lymphatic tissues after 1-2 days, in lymph nodes after 5-6 days, and in the whole body 10-14 days post-infection[83,84]. The first anti-HIV antibodies are typically detected after 4 to 5 weeks (up to 8 weeks) and are IgM and IgGs directed against p24 and glycoproteins gp120 and gp41[85]. A specific T cell response is induced against a variety of HIV epitopes as well, which is associated with significant reduction of the viral load and clinical latency that can last for years[86].

1.2.2.2 Chronic phase and associated immune dysfunction

During the chronic phase of HIV infection, the balance between immune response and viral evolution leads to a viral setpoint, which can remain stable for ten years in average (**Figure 5**)[87,88]. Two main mechanisms are involved in the spontaneous control of HIV infection. First, the early drop of viral load is attributed to $CD8^+$ T cell response[86,89]. However, mutations arise very quickly in HIV specific epitopes, allowing the virus to escape $CD8^+$ T cell recognition[90]. Rounds of new HIV specific $CD8^+$ T cell generation and viral escape contribute to the partial control of viremia. Second, anti-HIV neutralizing antibodies appear around twelve weeks post-exposure[85]. Again, the small breadth and potency of those antibodies favors HIV escape mutants, which ultimately leads to the persistence of infection[91-93].

The persistence of HIV infection and ongoing viral replication greatly affect the immune system on both tissue and cellular levels. Profound $CD4^+$ T cell depletion in GALT during acute HIV infection persists despite normalization of blood CD4 count, resulting in a loss of intestinal mucosal integrity and translocation of microbial product[77,94]. More importantly, GALT Th17, which are essential to the maintenance of mucosal integrity, are not recovered. The differentiation of cells that repopulate GALT is skewed toward a Th1 phenotype, which creates a more pro-inflammatory environment, and ultimately contributes to the increased immune activation observed during the chronic phase of HIV infection[95]. Moreover, ongoing viral replication has deleterious effects on the structure of peripheral lymphoid tissues. Indeed, germinal centers of lymphoid organs are the main source of viral production during chronic HIV infection, though minimal viral activity is detected in the periphery[96]. The burden of HIV replication ultimately leads to tissue fibrosis and the disruption of germinal center architecture, associated with viral rebound in the periphery[97].

On the cellular level, HIV infection targets and depletes $CD4^+$ T cells, which leads to an increased cell turnover to replenish the cell pool[98]. Though an immune response is mounted, the absence of viral control ultimately results in a loss of an effective cellular and humoral response[99]. Moreover, $CD4^+$ T cells ultimately lose their regenerative potential, resulting in a dramatic decrease of peripheral $CD4^+$ T cell count[81]. Once $CD4^+$ T cell count reaches <300 cells/µl, the immune system is greatly weakened, and opportunistic infections including Pneumocystis pneumonia, candidiasis and reactivation of cytomegalovirus infection, as well as neoplasms such as Kaposi's sarcoma and non-Hodgkin's lymphoma develop.

The course of HIV infection and progression to AIDS may vary a lot between individuals and greatly depends on the rate of $CD4^+$ T cell depletion[81]. The amplitude of initial HIV-specific $CD4^+$ and $CD8^+$ T cell response correlates with disease control[100]. Overall, timing, location and magnitude of the initial immune response along with the genetics of infected individuals that will be discussed below influence the disease outcome.

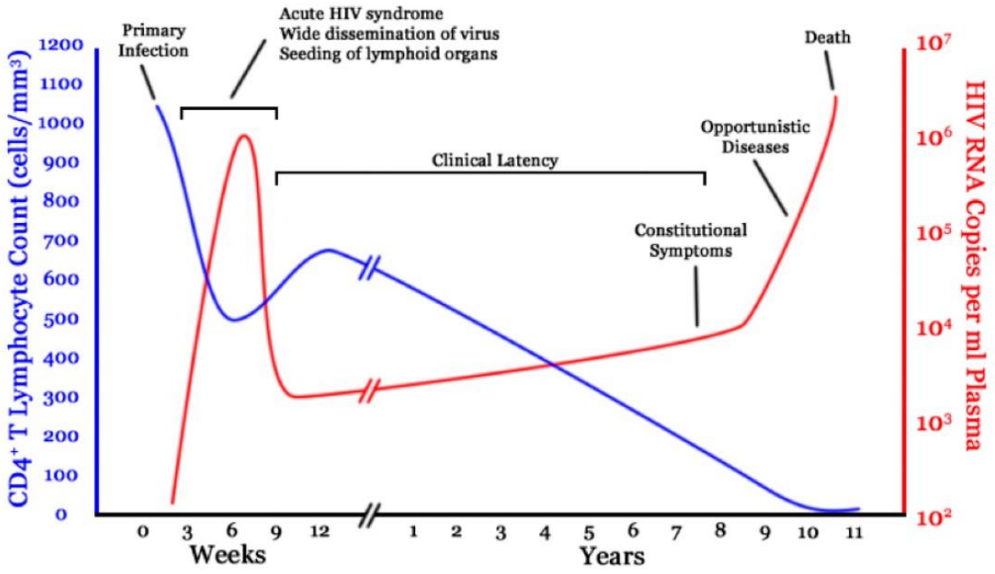


Figure 5: Course of untreated HIV infection. *Three phases are described for HIV infection. 1) The acute HIV syndrome involves wide-spreading of the virus through the body, which is associated with dramatic increased plasma viral loads (red) and a strong depletion of $CD4^+$ T cells (blue). 2) Clinical latency and the maintenance of a viral setpoint results from partial control of the infection. 3) Clinical manifestation of AIDS appears after years of ongoing infection, once $CD4^+$ T cells are profoundly depleted (<300 cells/µl).*

1.2.3 Antiretroviral therapy

Antiretroviral drugs suppress HIV replication by inhibiting key steps of viral replication cycle (**Figure 6**). Many drug classes have been developed, including fusion inhibitors, non-nucleoside reverse transcriptase inhibitors (NNRTI), nucleoside reverse transcriptase inhibitors (NRTI), integrase inhibitors (INI) and protease inhibitors (PI)[101]. The first successful antiretroviral drug for HIV was the NRTi azidothymidine (AZT) and was approved in 1987 by the FDA. Studies have shown that AZT monotherapy delayed the occurrence of opportunistic diseases and improved short-termed survival in AIDS patients, though no improved survival in asymptomatic patients has been observed[102]. Since 1987, about 43 drugs have been approved and the modern combination therapy typically consist in two NRTi and a PI or NNRTi or INI for better virologic suppression and avoid resistance[101,103]. Moreover, a lot of effort has been involved in the development of improved drug formulations such as fixed-dose combinations and long acting formulations, thus increasing patient adherence to the treatment[104,105].

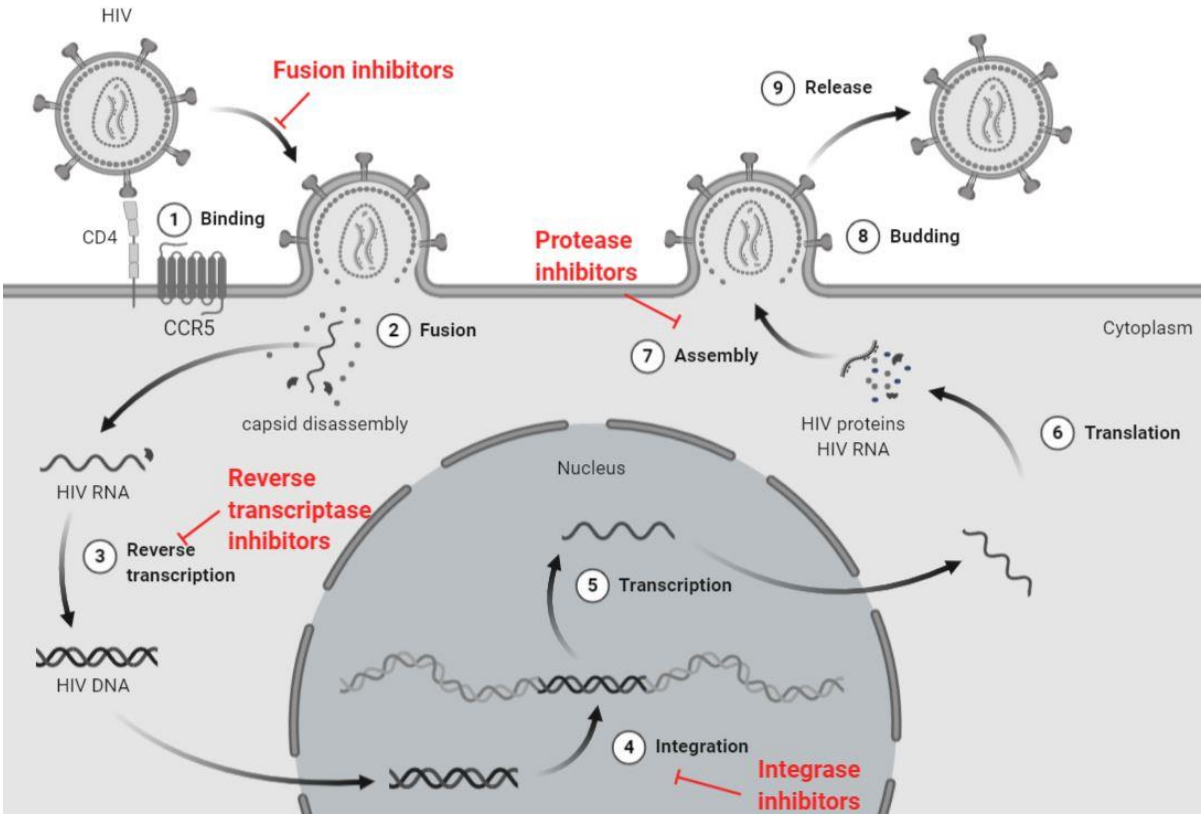


Figure 6: Targets for HIV antiretroviral drugs. *Antiretroviral drugs target steps of the viral replication cycle, including fusion of the virion with plasma membrane, reverse transcription of HIV RNA, proviral integration and protease splicing that release mature viral proteins.*

The modern ART has dramatically improved the life span of HIV infected individuals. The suppression of HIV replication is associated with many benefits for the patient, including the restoration of blood memory $CD4^+$ T cell levels, the partial resolution of chronic immune

activation and the normalization of homeostatic regulation, resulting in significantly lower HIV associated morbidity and mortality[106-111]. Though studies have shown better immune recovery if ART onset early in infection compared to late infection status, patients with low $CD4^+$ T cell count still show immunological benefits from ART[107,112,113]. Moreover, low/undetectable plasma viremia achieved with the modern ART regimen have markedly reduced infectiousness for sexual transmission and improved prevention outcomes[114,115].

Though ART is very efficient at suppressing viral replication, the immune system fails to recover to pre-infection levels, which is associated with increased risks for cardiovascular diseases, malignant, and hepatic diseases compared to HIV uninfected individuals[116,117]. Underlying reason for lower $CD4^+$ T cell reconstitution is a persistent immune activation that may result from low levels of ongoing viral replication in privileged reservoirs, coinfection with other viruses, and microbial translocation due to poor reconstitution of mucosal immune system[118-121]. Besides, HIV persists in a pool of long-lived latently infected cells that is not targeted by ART[122,123]. Though the HIV reservoir size increases with time of infection and correlates with ART initiation, it is established very early in infection and is a barrier to a cure[124,125]. Following this, many studies have focused on the description of HIV reservoir and developed strategies to cure HIV infection.

1.3 Immune response in HIV infection

1.3.1 Innate immunity

Early immune response to virus infection occurs in the infected cell with mechanisms of pathogen sensing and innate immune signaling. The sensing of pathogen-associated molecular patterns (PAMPs) by pathogen-recognition receptors (PRRs) initiates a cascade of events that drives antiviral defenses and virus restriction. The response to PRRs stimulation also leads to the production of type I interferons (IFN), as well as pro-inflammatory cytokines and chemokines that recruit and activate innate immune cells such as macrophages, NK cells, and dendritic cells to promote viral control and priming of the adaptive immunity.

1.3.1.1 Pathogen-recognition receptors (PRRs)

PRRs describe many classes of proteins that recognize PAMPs and are located in various cellular compartments such as plasma membrane, endosomes, cytoplasm and the nucleus. PAMPs associated with HIV are the envelope glycoprotein gp120, nucleic acid products from the reverse transcriptase, single stranded RNA (ssRNA) and the capsid protein. The engagement of PRRs induces a complex cascade of protein interaction leading to the activation of transcription factors from the NF-κB and interferon regulatory factor (IRF) families, which coordinate the expression of type I IFNs, pro-inflammatory cytokines and chemokines[126]. Classes of PRRs involved in HIV PAMPs recognition include Toll-like receptors (TLR), retinoic acid-inducible gene 1 (RIG-1), cyclic GM-AMP synthase (cGAS) and interferon inducible protein 16 (IFI16)[127].

TLRs are expressed on cell surface (TLR2, TLR4) and in endosomes (TLR7/8; TLR3). Surface TLR2 and TLR4 sense HIV gp120[128]. TLR2 and TLR4 are present especially on epithelial mucosal cells and activation leads to the production of pro-inflammatory cytokines and IFN-I. Endosomal TLR7/8 recognize guanosine- and uridine-rich ssRNA and play an important role in phagocytic immune cells such as DC, pDC and macrophages[129,130].

Cytoplasmic PRRs such as RIG-1 recognize secondary structure of HIV ssRNA. Activation of RIG-1 leads to the expression of IFN stimulated genes, thus favoring viral restriction[131]. Reverse transcription results in diverse nucleic acid products such as ssDNA, RNA:DNA hybrid and dsDNA. The presence of dsDNA in the cytoplasm is a danger signal and is recognized by cGAS and IFI16[132-135]. The activation of cGAS and IFI16 induces the expression of IFN-I genes and ISGs.

***Table 1**: PRR detection in target cells. Adapted from Sumner RP et al[127].*

Cell type	PRR	PAMP	Consequence	References
pDCs	TLR7	viral RNA	IFN, pro-inflammatory cytokines	130
Immature DCs	TLR8	ssRNA	NFkB activation, transcription of HIV provirus	129
Monocyte-derived dendritic cells	cGAS	RT products	IFN and ISG induction	132,133
Monocyte-derived macrophages	cGAS	RT products	NFkB and IRF3 activation, IFN and ISG induction	133
	IFI16	RT products	Reduced replication, ISG induction	134,135
Genital epithelial cells	TLR2 TLR4	Gp120	NFkB activation, pro-inflammatory cytokine	128
$CD4^+$ T cells	cGAS	Post-integration step	IFN and ISG induction	136
	TLR7	Viral RNA	Anergy	137

1.3.1.2 Host restriction factors

Host restriction factors are proteins that inhibit viral replication by targeting conserved mechanisms of virus replication cycle shared among a variety of viruses. In HIV-1 infection, restriction factors target steps such as virus entry, uncoating, reverse transcription and budding. Restriction factors against HIV-1 include APOBEC3G, SAMHD1, Thetherin/BST-2, TRIM5α, IFITM, and Schlafen11. Restriction factors are encoded by antiviral genes that are targets of IRF3 as well as interferon stimulated genes (ISGs)[127,138].

IFITMs

IFITMs are small transmembrane proteins that restrict a wide number of RNA viruses[139]. They localize at the plasma membrane and membrane of endocytic vesicles and lysosomes. IFITMs inhibit virus cell fusion process by reducing membrane fluidity[140].

TRIM5α

TRIM5α are cytoplasmic proteins that bind to viral capsid and promote premature disassembly, thus preventing reverse transcription and subsequent integration[141,142]. However, TRIM5α restriction of HIV-1 is cell type and entry pathway specific[143]. HIV-1 capsid is poorly targeted by TRIM5α, meaning that HIV-1 may have evolved to escape TRIM5α targeting.

Additionally, TRIM5α acts as a capsid PRR activating NF-κB and AP-1 transcription factors that leads to the production of pro-inflammatory cytokines[144].

SAMHD1:

SAMHD1 binds cytoplasmic nucleotides and prevents reverse transcription by reducing dNTPs supply for the RT[145]. SAMHD1 restriction occurs in monocyte-derived dendritic cells (moDCs), in monocyte-derived macrophages (MDM) and in resting $CD4^+$ T cells[146]. However,

CDK-mediated phosphorylation inhibits SAMHD-1 restriction in activated $CD4^+$ T cells due to the increased need of dNTPs for cellular activity[147].

APOBEC3G:

APOBEC3G (A3G) restricts HIV-1 by suppressing viral DNA synthesis and inducing mutation in viral DNA[148,149]. A3G interferes with reverse transcription by converting cytosines to uracils in the negative strand of cDNA, and those resulting G to A mutations in the viral genome lead to replication incompetent viruses. A3G is expressed in $CD4^+$ T cells and MDM.

Tetherin/BST-2

BST-2 is a transmembrane protein with a N-terminal transmembrane domain and a C-terminal GPI anchor that can both interact with the membrane of virions and prevent the release of budding virions[150].

Schlafen 11:

Schlafen 11 has been shown to impair HIV translation by counteracting HIV-1 induced change in tRNA composition[151]. The activity of Schlafen 11 exploits the differential codon usage between viral and host proteins, since HIV codons have a high frequency of A/T nucleotide compared to cellular codons.

1.3.1.3 Antagonism of immune mechanisms by HIV accessory proteins

Despite innate immune mechanisms to sense HIV pathogen, HIV is able to evade many of them due to the expression of the accessory proteins Nef, Vif, Vpr and Vpu.

- Nef targets cell surface receptors including CD4, MHC I and TCR for proteasomal degradation[152,153]. The ensuing downregulation of surface receptors helps the virus infected cells to evade immune recognition.
- Vif is mostly known for its antagonistic activity on APOBEC3 proteins[154]. Vif targets APOBEC3 for proteasomal degradation, thus restricting its activity and preventing its packaging in newly made virions.
- Vpr is the less well defined HIV accessory protein but studies have demonstrated its significance for viral pathogenicity *in vivo*[155]. Vpr targets many host proteins for proteasomal degradation, modulating cellular pathways in the favor of the virus. Attributes of Vpr are its ability to delay or arrest cell cycle in the G2 phase and promote cytopathicity, though the exact mechanisms remain unclear.
- Vpu antagonizes tetherin activity, which prevents viral budding and release[150,156].

1.3.1.4 Dendritic cells

As an antigen presenting cell, dendritic cells (DCs) coordinate the host immunity against invading pathogens and are one of the first responders in mucosal infection. DCs are IFN producing cells, display high levels of surface MHC molecules, and therefore play a major role in T cell priming[157].

As previously discussed, the most common route for HIV infection is through genital or gastrointestinal mucosa. Dendritic cells being a very heterogeneous population, the type of mucosa exposed to the virus and therefore the subset of DC encountered will influence the mode of infection and spreading[158]. Two major phases of HIV transfer have been described: 1) uptake of the virus through interaction with the various receptors present on DC cell surface, and 2) the direct infection of DCs through CD4/CCR5 binding[71].

DCs display various receptors on their cell surface that interact with HIV glycoprotein gp120, including DC-SIGN, Siglec-1 and langerins[159-162]. The outcome highly depends on DC subset and maturation status[71,163]. Immature DCs usually express higher levels of receptors than mature DCs, but both can capture virions and store them in intracellular compartments[71,164,165]. This process is totally independent of infection and allows subsequent transfer to $CD4^+$ T cells in a mechanism called *trans*-infection[166,167]. DCs can either directly transmit the virus to tissue resident $CD4^+$ T cells or migrate to draining LN and transfer the virus to $CD4^+$ T cells that reside there. This phase has a rather short duration, since a large amount of viruses are degraded within 24 hours[71].

In regard of the short half-life of the first phase, productive infection of DCs provides a more sustainable mode of spreading for HIV[71,72]. Expressing CD4 and CCR5 on their cell surface, immature DCs and to some extent mature DCs can be readily infected by HIV, though at low frequency[168]. Despite low infection levels, DCs have a high viral transfer capacity to T cells and migration to lymph nodes facilitates HIV infection of resident $CD4^+$ T cells, thus playing a central role in HIV transmission[169].

Besides direct transfer to DCs, the presence of HIV viruses is sensed by the epithelial cells in the mucosa, leading to strong chemokine production and recruitment of plasmacytoid DCs (pDC)[76]. In turn, pDCs produce high levels of chemokines that drive the migration of $CD4^+$ T cells to tissues, enhancing CCR5 expression and therefore facilitating local infection of $CD4^+$ T cells and HIV spread.

Dendritic cells, and more specifically follicular dendritic cells (FDC), also play a major role in HIV pathogenesis. FDCs are found in germinal centers of B cell follicles and are essential to the germinal center development and T cell dependent humoral immune response[170]. FDCs

trap antigens in the form of immune complexes, which can persist for months in an unprocessed form, allowing the development of high affinity antibodies.

Germinal centers have been described as a privileged compartment for HIV production through all stages of the disease[96,171,172]. Large stores of intact HIV particles associated to FDCs as well as close contact with $CD4^+$ T cells result in sustained HIV infection[173]. The abnormal persistence of HIV antigen impairs T_{FH} and B cell response, associated with follicular hyperplasia and suboptimal HIV specific antibody production[174].

Though FDCs are not susceptible to productive infection, studies demonstrated that trapped viral particles remained infectious for months[175]. Moreover, a recent study showed that FDCs isolated from HIV infected donors still carry infectious viruses even after up to 24 years antiretroviral treatment, thus contributing to HIV reservoir[176].

1.3.1.5 Monocytes/Macrophages

Monocytes and macrophages are part of the innate immune system. They sense and clear pathogens, serve as antigen presenting cells for priming the adaptive immunity and are involved in both, pro-inflammatory and anti-inflammatory processes and tissue repair. While monocytes are circulating cells, macrophages can be found in every tissue in the body and are either tissue-resident, or monocyte-derived[177-179].

HIV enters the body mostly through mucosal surface of the genital or gastrointestinal tract. The underlying lamina propria is highly populated with lymphocytes and macrophages, which express CD4 receptor and co-receptors CCR5 and CXCR4, and are therefore susceptible to HIV infection[73,180]. Due to low CD4 expression levels, however, they are not easily infected by transmitted/founder viruses and the virus must acquire macrophage tropism[181].

Macrophage phenotype differs depending on cell location, making them a very heterogeneous population and differently susceptible to infection. For instance, studies have shown that macrophages from vaginal tissues were susceptible to HIV infection, whereas gut resident macrophages were resistant[73,182]. Though mucosal macrophages can be readily infected, their contribution to HIV replication and spread still is controversial.

Viral replication and bacterial translocation associated with HIV infection induces a general increase in inflammation and immune activation, impacting the activation and differentiation of blood monocytes[183-186]. During the differentiation process, monocytes may have increased susceptibility to HIV infection. Monocyte migration to the tissues may thus contribute to the spread of the virus in virtually every tissue of the body[184,187,188]. In addition, monocytes and macrophages have been associated with a lot of HIV related comorbidites such as neurological disorders, atherosclerosis and cardiovascular diseases[186,189-191].

Though monocyte/macrophage contribution to HIV replication and spread is controversial, tissue macrophages are long lived cells (half-life of months to years) resistant to cytopathic effect and resides in privileged anatomic sites, and therefore might represent an important reservoir for HIV persistence.

1.3.1.6 NK cells

NK cells are lymphoid cells and constitute the first line defense against viral infections and tumor cells in absence of adaptive immunity. Two main subsets have been described, characterized by the differential expression of CD56 and CD16[192]: $CD56^{high}/CD16^{-/low}$ NK cells are very important for the production of chemokines and cytokines[193]. In contrast, $CD56^{low}/CD16^{high}$ are responsible for the cytotoxic activity and can mediate killing of target cells by direct recognition of viral peptides, stress ligands, sensing the downregulation MHC-I molecules and mediating antibody-dependent cell cytotoxicity (ADCC). The activation of NK cells are regulated by a number of activating (aNKR) and inhibitory receptors (iNKR)[194].

NK cells are increased during acute HIV infection and contribute to the antiviral innate immune response by secretory and cytolytic mechanisms[195]. NK cells also have a regulatory function on dendritic cells and T cells[196,197]. However, HIV viremia induces phenotypical and functional abnormalities in NK cells[198]. The pathologic redistribution of NK cell subsets includes the expansion of an anergic $CD56^{neg}/CD16^{high}$ population associated with an aberrant expression of NKR repertoire that includes increased expression of inhibitory receptors p58.2/KIR2DL2 and LIR/ILT2, and decreased expression of activatory receptors NKp46 and NKp30[199,200]. As a result, NK effector functions are impaired, leading to defective control of viral replication and disease progression.

Due to the critical role of initial immune events in the course of HIV infection, proper modulation of innate immunity is one of the goals to achieve in vaccination.

1.3.2 Adaptive immunity

Immune failure is a hallmark of HIV infection. Strong $CD4^+$ T cell depletion, mutational escape of HIV variants and immune exhaustion all contribute to the inability to control HIV infection.

1.3.2.1 $CD8^+$ T cell

In a normal immune response, naïve $CD8^+$ T cells are activated by antigen presenting cells, mainly dendritic cells, through MHC-I – peptide recognition[201]. Co-stimulation and cytokine production leads to clonal expansion and differentiation into primary effector $CD8^+$ T cells,

which are very potent at killing antigen-bearing cells through perforin/granzyme production and release of antimicrobial cytokines such as TNFα and IFNγ. Prolonged exposure to antigen drives an efficient effector response and promotes $CD8^+$ T cell differentiation into memory subsets.

$CD8^+$ T cell response is crucial in the acute phase of HIV infection since expansion of HIV-specific $CD8^+$ T cells correlates with the decrease of plasma viral load[86,89]. Moreover, studies in macaques have shown that depletion of $CD8^+$ T cells results in increased SIV replication, emphasizing the importance of $CD8^+$ T cells in mediating viral control[202,203]. However, most of HIV infected individuals inevitably progress to AIDS if left untreated.

Different mechanisms involving $CD8^+$ T cells can contribute to a failure in HIV control. On one hand, the activation of $CD8^+$ T cells depends on TCR interaction with peptide-associated MHC-I. Therefore, $CD8^+$ T cells apply a selective pressure on the virus, favoring viral populations with mutations affecting antigen presentation that can ultimately escape immune recognition[90]. On the other hand, antigen persistence in chronic infections such as HIV can result in severely impaired T cell functions called immune exhaustion[201,204]. $CD8^+$ T cell effector mechanisms including cytolytic activity, IL-2, TNFα and IFNγ production are progressively lost following antigenic stimulation. Exhaustion has been correlated with the upregulation of the co-inhibitory molecule PD-1 and has been shown to predict disease progression[205,206].

However, about one infected patient in 300 control HIV replication and maintain plasma viremia below 2000 RNA copies/ml plasma without ART, the so-called controllers[207,208]. Spontaneous control of HIV infection has been associated with some HLA alleles, including HLAB57, HLAB27, HLAB52 and HLAB14, and with HIV-specific $CD8^+$ T cells displaying increased polyfunctionality and proliferation compared to the progressors[209-212]. Overall, controllers usually have a small HIV reservoir and do not progress toward AIDS, providing a model of functional cure and vaccine strategies.

1.3.2.2 $CD4^+$ T cells

$CD4^+$ T cells are the major target of HIV infection and replication. They express not only high levels of CD4, but also the co-receptors necessary for HIV entry, CCR5 and CXCR4 that have differential expression patterns on $CD4^+$ T cells[213]. While CXCR4 is mostly expressed on circulating peripheral T cells, CCR5 is a tissue homing receptor and is expressed on late differentiated effector and effector memory $CD4^+$ T cells[214]. CCR5 allows the migration of effector cells to extra-lymphoid sites of inflammation. Therefore, $CCR5^+CD4$ T cells are found predominantly in effector sites such as lung and the *lamina propria* of the intestinal mucosa.

Acute HIV infection and progression towards AIDS are associated with a strong depletion of $CD4^+$ T cells, involving different mechanisms. Primary transmitted HIV variants are usually CCR5-tropic, while CXCR4-tropic variants usually develop later in the infection course[215,216]. During acute HIV infection, $CCR5^+CD4$ T cells are rapidly depleted from gastro-intestinal tissues and from the periphery, while naïve and central memory $CD4^+$ T cells are relatively spared[214,217]. $CCR5^+CD4$ T cells are a subset of differentiated cells and can be regenerated from less differentiated $CCR5^-$ precursors, providing new targets that maintain infection over time[81]. Acute infection with CXCR4 tropic variants is possible and is usually associated with a more rapid progression towards AIDS, which is in accordance with the primary targeting and depletion of undifferentiated $CD4^+$ T cells[218]. The regeneration capacity of target cells strongly influences the disease progression[219].

To counter the acute depletion of T effector memory cells (T_{em}), T central and transitional memory cells (T_{cm}/T_{tm}) will proliferate and differentiate to T_{em}, which will then migrate to extra-lymphoid effector sites and partially restore T_{em} compartement[81]. However, the regeneration of T_{em} compartment is a high turnover process, generating shorter lived and less effective cells. Eventually, T_{cm} intrinsic or extrinsic limit for self-renewal or dysregulation of T_{em} production will lead to homeostasis failure and progression towards AIDS.

Though we described the general impact of HIV infection on the depletion of $CD4^+$ T cells, $CD4^+$ T cells are not a homogeneous population and are made of different, functionally distinct subsets, including Th1, Th2, Th17, T_{FH} and T_{reg}. These subsets have different requirement for homeostasis and regeneration and are differently impacted by HIV infection.

Th17

Th17 are important mediators in the host defense against extra-cellular pathogens such as fungi and bacteria and are key regulators in the maintenance of the gut epithelial barrier integrity[220]. Th17 cells are susceptible to HIV infection and, due to their location in the intestinal mucosa, are one of the first target of HIV mediated depletion[95]. The homeostasis of Th17 cells is strongly affected by the massive depletion of memory T cells in acute infection and the regeneration is skewed to a more pro-inflammatory Th1 phenotype, associated with the loss of intestinal mucosa integrity[94]. The increased permeability for pathogens results in chronic immune activation.

T_{FH}

T_{FH} are localized in the germinal centers of secondary lymphoid tissues and interact with antigen-specific B cells to promote antibody production[221]. Located in privileged sites and exposed to high concentrations of viral particles, T_{FH} play a major role in HIV persistence[222]. Indeed, they are enriched in latently infected cells and have been characterized as the main

reservoir for HIV in untreated and ART treated individuals[171,172]. Acute HIV infection results in increased expansion of T_{FH}, associated with increased antibody production, higher frequencies of germinal center B cells and follicular hyperplasia[171,223]. However, increased levels of activation and persistent antigenic stimulation impairs T_{FH} cell-B cell help during HIV infection, leading to inefficient generation of highly functional antibodies.

T_{reg}

T_{reg} cells are a regulatory subset of $CD4^+$ T cells and are important for immune tolerance mechanisms[224]. T_{reg} cells are susceptible to HIV infection and are rapidly depleted from the gut during acute infection. Homeostasis dysregulation during HIV infection results in an imbalance between Th17 and T_{reg} cells[225]. Increased T_{reg} cells are found in GALT and in blood circulation, which may be associated with increased microbial translocation, contributing to the sustained immune activation, and with a delayed or diminished virus specific response[226].

1.3.2.3 B cells and antibody response

Beside cytokines production and antigen presentation, B cells are essential to the development of humoral immune response by secreting antibodies. Proper B cell activation is supervised by T_{FH} cells in LN germinal centers[227]. T_{FH} co-activation results in B cell proliferation, isotype switching and somatic hypermutation. This allow B cells to differentiate into long-lived plasma cells and memory B cells. T_{FH}-B cell interaction is therefore primordial to B cell differentiation and the development of high affinity antibodies.

HIV infection is, however, associated with abnormal B cell responses, including decreased proliferation capacity and immunoglobulin diversity, and increased terminal differentiation[228,229]. Persistent activation, nonspecific polyclonal antibody response and hypergammaglobulinaemia characterize the viremic phase HIV infection, resulting in impaired humoral response to vaccination and other infections[93,223]. The factors causing B cell abnormalities have not been completely defined yet, though the normalization of B cell subpopulations after antiretroviral therapy onset strongly suggests that HIV replication plays an important role in B cell dysfunction[230].

Though anti-HIV antibodies are produced during the first months of HIV infection, they are either non-neutralizing or strain-specific neutralizing antibodies. Studies have shown that overactivation of T_{FH} and B cells prevents the adequate co-stimulation necessary for isotype switching and somatic hypermutation[223,231]. An inefficient antibody response results in successive selection of neutralization-resistant HIV variants and viral persistance[92,232].

However, broadly neutralizing antibodies (bNAbs) eventually develop in about 20% of infected individuals after 2-4 years of infection[233,234]. bNAbs are enriched in somatic

hypermutation, which is associated with functional T_{FH} co-stimulation[235]. The causes for rare and delayed generation of bNAbs are unclear, though several factor may include the glycan shield on Env that protects highly conserved regions of the protein and high level production of mutated, misfolded and non-infectious viral particles. High viral load, duration of infection and genetic diversity of the virus contribute to bNAb generation, factors that are also correlated to B cell abnormality[236].

Many HIV vaccine strategies aim at inducing potent neutralizing antibodies. However, actual knowledge on bNAb generation during HIV infection suggest that this may be difficult to achieve[237].

1.3.3 T cell exhaustion

During acute infection, naive T cells are activated by antigen and differentiate into effector cells, which is characterized by clonal expansion and acquisition of effector function[238]. Following clearance of the pathogen, most effector T cells die, except for a subset that differentiate into memory T cells. Though memory T cells downregulate most of the activation program, they maintain the capacity to reactivate effector function very rapidly following antigen restimulation. In contrast, chronic infections such as HIV are associated with profound T cell dysfunction characterized by strongly reduced effector functions, upregulation and co-expression of inhibitory receptors and homeostatic failure[239]. This altered differentiation state is termed exhaustion.

The control of HIV replication is attributed to an effective $CD8^+$ T cell response, as it is observed during acute infection and in elite controllers[86,89,211]. However, most individuals do not control HIV infection because of the narrow breadth of $CD8^+$ T cell response, which results in viral escape and persistence[90,240]. High viral loads have been associated with $CD8^+$ T cells impaired proliferation capacity, loss of cytotoxic activity and progression of the disease[241]. Though exhaustion has been predominantly described in $CD8^+$ T cells, it has been recognized that $CD4^+$ T cells also suffer from exhaustion, which is characterized by a loss of proliferative capacity and cytokine production[242]. $CD4^+$ T cell exhaustion may impair proper B and $CD8^+$ T cell priming, thus contributing the general immune dysfunction observed in HIV infection[243-245].

Higher and sustained expression of inhibitory receptors is a hallmark of T cell exhaustion. In particular, the upregulation of the inhibitory receptor PD-1 on HIV-specific T cells has been associated with T cell exhaustion, and expression levels were correlated with viral loads and inversely correlated with CD4 count[205]. In normal infection condition, inhibitory receptors are essential to terminate the immune response by attenuating T cell activation, and therefore represent a self-limiting mechanism to preserve self-tolerance (**Figure 7**)[246]. In addition, inhibitory receptors are activation markers and are upregulated upon TCR stimulation.

However, ineffective clearance of the antigen results in chronic activation of TCR and sustained upregulation of inhibitory receptors, thus contributing to the pathogenesis by downregulating T cell function[240].

Importantly, studies have demonstrated that exhaustion is a reversible state[247]. Indeed, blocking PD-1 interaction with its ligands (PDL-1/PDL-2) restored $CD8^+$ T cells function, as assessed by cytokine production. Many immune checkpoint inhibitors (ICIs) have been developed and are approved in the oncology field, showing strong benefit against refractory cancers[248].

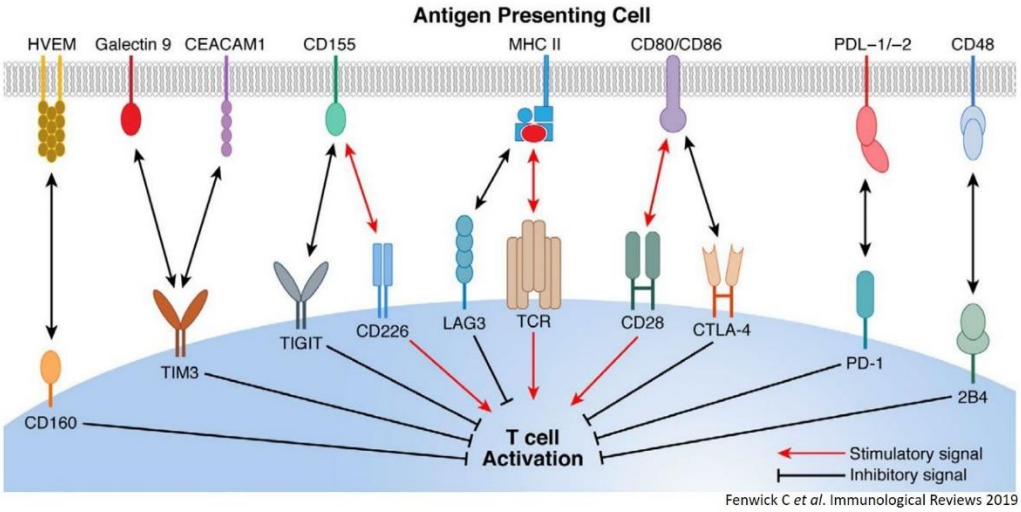


Figure 7: T cell immune checkpoint molecules and their ligands. *MHCII-peptide interaction with T cell receptor delivers the primary signal for T cell activation. Engagement of CD28 and other co-stimulatory receptors enhance this signal, which is essential for proper T cell activation. Co-inhibitory receptors including PD-1, CTLA-4, LAG3, TIGIT, TIM3, CD160 and 2B4 are upregulated following T cell stimulation and engagement of those receptors are required to terminate the immune response.*

The onset of ART results in downregulation of inhibitory receptors on the cell surface, though levels are still higher compared to healthy individuals[206]. Because $CD8^+$ T cells are such an important component in the control of HIV infection, targeting inhibitory receptors such as PD-1 is of particular interest. *In vitro* studies demonstrated that anti-PD-1 could relieve the exhaustion of HIV-specific $CD8^+$ T cells and had a moderate impact on HIV latency in $CD4^+$ T cells[205,206]. *In vivo* studies further revealed that ICI increased the functionality and count of HIV-specific T cells, though the real impact on HIV reservoir remains under debate[249-251].

1.3.4 HIV controllers

Though HIV infection leads to AIDS in absence of treatment for most individuals, some individuals (5-15%) are able to control the infection and maintain low plasma viremia and stable CD4 counts over the years[252,253]. However, most of those individuals, referred as long-term

non-progressors (LTNPs), show signs of disease progression after more than ten years infection[254,255]. In contrast, an even smaller percentage of individuals (<1%) are able to maintain viral loads below the limit of detection (<50 cp/ml), the so-called elite controllers[256]. Consequently, many studies have investigated factors that allow spontaneous control of HIV infection, including cellular restriction factors, viral fitness and host genetics[257].

Studies have shown that some HLA class I haplotypes are associated with better control of HIV infection, either via enhanced $CD8^+$ T cell activity or NK cells[209,258]. Indeed, peptide presentation through HLA I B57 and to some extent B27 has been associated with enhanced $CD8^+$ T cell polyfunctionality, including increased proliferation capacity, cytolytic activity and antiviral cytokine production[210-212,259]. Similarily, some HLA alleles associated to specific NKR demonstrated better control of HIV infection[260-262].

Heterozygosity for CCR5Δ32 has been associated with decreased susceptibility to HIV infection[263]. CCR5 is an important co-receptor for HIV entry into cells and mutation on CCR5 dramatically decrease the infection potential. Corroborating these observations, transplant of allogeneic hematopoietic stem cells (HSCT) from CCR5Δ32 donors resulted in HIV remission in two cancer patients that were infected with HIV[264,265]. However, the low incidence of CCR5Δ32 homozygous genotype in the population (<2%) and the relative toxicity of transplantation associated medical treatment contribute to the limitation of this cure strategy[266].

Overall, this knowledge is encouraging and greatly inspires the development of novel cure strategies, as it will be discussed below.

1.4 HIV reservoirs

As previously discussed, antiretroviral therapy is highly effective at suppressing HIV replication. However, treatment arrest invariably leads to viral rebound even in fully suppressed individuals, meaning that HIV can persist in reservoir that are not targeted by ART. Different non-exclusive mechanisms have been proposed to explain HIV persistence, including a latent HIV cell reservoir, residual HIV replication and privileged anatomical compartments.

Latently infected cells have an integrated HIV provirus but have a deficiency in the ability to produce viral particles, allowing them to escape immune surveillance and to persist despite ART[122]. The best defined reservoir for HIV-1 is a small pool of latently infected resting memory $CD4^+$ T cells[123,267]. Several studies have investigated the contribution of different T cell subsets to the HIV reservoir and have identified T central memory (T_{cm}) and T effector memory (T_{em}) as the major cellular reservoir in blood[268]. Their long *in vivo* half-life as well as the low and/or infrequent viral transcription contribute to making these cells an ideal cellular compartment for HIV persistence.

Kinetic models have estimated a reservoir decay half-life of about 40 months in long-termed treated patients[122,269], but more recently, Riddler and colleagues estimated the decay half-life to be 11.5 years under full antiretroviral suppression in long-term treated patients[270]. Those findings emphasize the reservoir stability and suggest HIV-1 viral eradication is unlikely despite fully suppressive ART.

Different non-exclusive mechanisms have been proposed to explain HIV persistence. On one hand, HIV-1 reservoir cells persist through T cell survival and homeostatic proliferation. The intrinsic stability of latently infected $CD4^+$ T cells supports this hypothesis due to the fact that T_{cm} cells can survive for years and to the subsequent differentiation of T_{cm} toward T_{em} after T cell receptor (TCR) activation inducing proliferation[268]. Moreover, the limited diversity of HIV-1 variants identified after reactivation of latently infected cells provides further evidence that these cells arose from *in vivo* proliferation rather than multiple rounds of infection[271].

On the other hand, the persistence of transcriptionally active cells is thought to play a role in HIV-1 persistence. Low genetic divergence and limited emergence of drug resistance argue against the hypothesis of ongoing low levels of viral replication. However, recent studies show that lymphoid organs act as sanctuary sites in which the virus is able to replicate and evolve[272]. Lower drug concentrations as well as poor accessibility to cytotoxic $CD8^+$ T cells enable the survival of these reservoir cells, even under effective ART[273]. Consequently, persistent viral transcription may be sufficient to replenish latent cellular reservoir.

The existence of sanctuary sites for HIV-1 infection was already described in 1997. In particular, Pantaleo *et al.* demonstrated that the frequency of HIV-1 infected cells is 5 to 10

times higher in lymphoid tissue mononuclear cells compared to peripheral mononuclear cells in early- and middle-stage HIV patients. Undetectable blood viremia and clinical latency were associated with an accumulation of viral particles in the germinal centers, “trapped” in the follicular dendritic cell (FDC) network. In contrast, the disruption of germinal centers as observed in late-stage HIV infected patients is associated with viral particle spillover responsible for increased viral load in the blood compartment and disease progression[96].

Following the importance of germinal centers in supporting viral replication and the identification of T_{cm} and T_{tm} $CD4^+$ T cells as the main cellular compartment enriched in HIV DNA in blood, Perreau *et al*. investigated the role of lymph node memory $CD4^+$ T cell populations isolated from HIV-1 infected patients, before and after the onset of antiretroviral therapy[96,171,268]. Four lymph node $CD4^+$ T cell populations were investigated, based on CXCR5 and PD-1 expression. Of the four populations harboring integrated HIV DNA, the frequency was highest in T follicular helper cells (T_{FH}) ($CXCR5^+/PD\text{-}1^+$), which is in accordance with the observations presented by Chomont *et al*. However, T_{FH} cell subset was only shown to serve as the major $CD4^+$ T cell compartment supporting HIV replication and production in viremic untreated patients. More recently, screening of blood and lymph node $CD4^+$ T cell populations from ART treated HIV-1 infected patients based on the expression of CXCR5 and PD-1 expression identified lymph node $PD\text{-}1^+$ cells (96% T_{FH}) as the major cell compartment to produce replication competent and infectious viruses[172].

Studies have demonstrated that HIV reservoir is generated very early after HIV infection and, despite early diagnosis, ART usually starts after the reservoir establishment. Because of its long half-life and resistance to ART, HIV reservoir remains a major obstacle to HIV eradication.

1.5 HIV cure

Continual suppression of viral loads requires lifelong ART therapy, which is associated with many issues such as side effects, non-compliance leading to the emergence of drug resistance, and high health care related costs. Therefore, new strategies are needed to selectively target and kill cells that serve as HIV-1 reservoir in an effort to allow infected patients to stop or significantly reduce the need for continual drug regimens.

Two approaches are being considered to achieve HIV-1 cure: a) sterilizing cure, which aims to remove any replication competent viruses from the body and b) functional cure, which aims to reduce the burden of HIV infected cells to a level where the virus produced can be controlled by the patient's immune cells in the absence of ART. Many different strategies are being investigated to achieve a functional cure, which appears to be a more achievable outcome. The fact that some infected individual so called elite controller can control HIV infection without ART contribute to the general optimism of finding a cure[274]

1.5.1 Shock and kill strategy

Most popular strategy to achieve HIV cure is the so-called "shock and kill" strategy based on the reactivation of latently infected cells (shock) followed by the immune mediated elimination of those cells (kill). A first trial performed on three HIV patients using anti-CD3/IL-2 showed unspecific stimulation of immune cells, led to severe toxicity and the HIV reservoir of infected cells remained mostly unchanged[275]. Subsequently, compounds that reactivate viral production without global reactivation of the cell, known as latency reversing agents (LRAs), were evaluated.

HIV provirus is inserted in the host genome, meaning that it is subject to the same epigenetic mechanisms that regulate gene expression. Different classes of compounds were evaluated for their ability to reactivate HIV from latency, many of them being anti-cancer agents acting on the chromatin level[276]. Leading classes of LRA's that have been tested in clinical trials are the histone deacetylase inhibitors (HDACi) vorinostat, romidepsin, and panobinostat and the protein kinase C (PKC) activator bryostatin-1[277-284]. The treatment for chronic alcoholism disulfiram, an unclassified LRAs identified in library screening, has a promising therapeutic profile as well[285,286].

LRA's tested on ART-treated HIV patients have shown that treatment was generally safe and well tolerated[287,288]. However, drugs did not reduce the frequency of latently infected cells despite significant increases in HIV transcription (cell associated unspliced HIV RNA) and plasma viremia.

The LRAs' lack of effect on HIV-1 reservoir may be explained by an insufficient immune response against reactivated cells and/or an insufficient degree of reactivation. On one hand, dysfunction in HIV-specific $CD8^+$ T cells as observed in chronically infected individuals may impair their capacity to eliminate reactivated cells[205,206,241,289]. On the other hand, different studies have shown that some LRA's have detrimental effects on $CD8^+$ T cell function[290-293].

To overcome the limitations of the immune system, current strategies to cure HIV infection are based on combined efforts to reactivate the virus from latency and improve immune effector cell function to clear infected cells.

1.5.2 Activation of effector cell function

1.5.2.1 Therapeutic vaccines

Therapeutic vaccines aim at boosting pre-existing immune responses in HIV-infected patients to elicit virologic control in the absence of ART. Therapeutic vaccines are DNA-, RNA vectors or autologous virus peptides that focus on eliciting either a narrow T cell (Gag) or antibody response (Tat) or a broad immune response (Env, Gag, Pol, Nef)[294].

Therapeutic vaccines designed to elicit a narrow immune response are based on observations that have been associated with viral control and slower disease progression, including the induction of gag-specific $CD8^+$ T cells and C5-specific non-neutralizing antibodies[295-297]. However, most trials have shown poor immunogenicity and neither reduction of HIV reservoir, nor control of viral rebound after analytical treatment interruption (ATI) compared to the control arm. Since the host immune response is directed toward highly conserved HIV sequences, it probably fails at handling the diversity of rebounding viruses.

Recent therapeutic vaccines aim at eliciting a broad immune response that is robust enough to target escape variants emerging during ATI. Strategies explored include DC-based and attenuated lentiviral vector based vaccines[298-301]. Clinical studies showed that viral control was associated with enhanced T cell functionality. However, vaccines developed so far showed moderate efficacy only and no decrease of replication competent HIV reservoir has been observed. Though results are encouraging, improved strategies are still needed.

Improvement may result from combinatorial strategies such as combination with potent LRAs or immune modulators. In particular, the broadly immunogenic combination of ALVAC-HIV and Lipo-6T followed by IL-2 injection showed promising results[302,303]. The addition of IL-2 has been shown to impact T cell exhaustion and generally provide better antigen specific response[304]. Similarly, a recently published study on non-human primates supported the potential of broad therapeutic vaccines, especially when coupled with innate immune stimulation[305]. They used an adenovirus serotype 26 (Ad26) and MVA, both

expressing Env, Gag and Pol with or without TLR7 agonist. The Ad26/MVA vaccine strongly increased the magnitude of cellular immune responses and expanded cellular immune breadth, though it did not decrease the set point viral load following ATI. However, the addition of TLR7 resulted in 33% of the monkeys that maintained undetectable viral loads after ATI. Following those promising data, Ad26/MVA vaccine is currently being tested in HIV-infected individuals (NCT03307915).

So far, vaccines that elicit a broad immune response had stronger impact on viral rebound following ATI. Combination with potent LRA's or immune modulators may provide further benefit toward a remission.

1.5.2.2 Immunomodulators

LRAs are very efficient at reactivating HIV from latency but clearance of the virus requires potent and efficient immune response. Major component of the host immune response to HIV are cytotoxic T lymphocytes (CTL) and NK cells[306,307]. Compounds such as the cytokines IL-7, IL-15 and IL-21 have been shown to upregulate the cytotoxic effector function of CTL and NK cells without unspecific activation and proliferation of immune cells, and may be suitable for immunotherapy or as vaccine adjuvant[308-313]. In addition, immune receptors such as TLR-7 were shown to play a role in HIV-1 pathogenesis[137,314-317]. TLR-7 modulation has demonstrated strong antiviral response and has been associated with $CD8^+$ T cell activation in SIV-infected rhesus macaques[318].

$CD8^+$ T cells are largely excluded from B cell follicles. However, a subset of $CD8^+$ T expressing the B follicle homing receptor CXCR5 has been recently identified[319,320]. Tissue resident memory $CD8^+$ T cells may also reach follicules. However, those two CD8 subsets mount only low production of perforin and granzymes, probably to limit the inflammation in the tissue[320]. The exact role of follicular and T_{RM} $CD8^+$ T cells still remains unknown in the context of HIV infection.

As already discussed, chronic infection such as HIV is associated with T cell exhaustion and impaired immune function[204,205]. Immune checkpoint inhibitors including anti-CTLA4 and anti-PD-1 have been evaluated and approved in oncology to relieve $CD8^+$ T cells from exhaustion and help eliminating cancer cells. Similarly, anti-PD-1 may serve as adjuvant in HIV therapy for optimal $CD4^+$ and $CD8^+$ T cell response. Case reports have evaluated the impact of anti-CTLA-4 ipilimumab and anti-PD-1 nivolumab treatment on HIV reservoir from cancer patients that were infected with HIV[249-251,321]. ICI treatment is generally safe in cancer patient infected with HIV, and a positive impact on HIV specific T cells, including increased

functionality and count, have been observed. However, the real impact of ICIs on HIV reservoir remains under debate.

1.5.3 Specific targeting of HIV infected cells

1.5.3.1 Anti-HIV antibodies

The first neutralizing anti-HIV antibodies are detectable about twelve weeks after onset of infection[322]. However, defective humoral immune response leads to the production of poorly functional antibodies[93,323]. In addition, the rapid evolution of HIV envelope leads to successive cycles of antibody production and viral escape. Nevertheless, the presence of highly polyfunctional non-neutralizing antibodies (nNAb) capable of inducing virolysis, phagocytosis and antibody-dependent cell cytotoxicity have been described and were associated with viral control in HIV elite controllers[324,325]. Moreover, the presence of polyfunctional antibodies were a major correlate of protection in the RV144 vaccine trial[326,327]. nNAb protective attributes were poorly observed in animal studies though, probably due to their lack of breadth[328-331].

In contrast, a small percentage of HIV infected individuals produces antibodies with outstanding breadth and potency toward multiple HIV isolates, so-called broadly neutralizing antibodies (bNAb)[332]. Those antibodies are elicited in chronic infected individuals by high viral loads after several years of exposure to HIV virus, and thus are not associated with viral control[333]. Due to their amazing breadth and potency, bNAbs might be good candidates to develop immune therapeutics. In fact, *in vitro* studies demonstrated that, beside neutralization capacity, bNAbs can elicit strong antiviral activity, including ADCC, ADCP and complement dependent cytotoxicity[334-336]. Therefore, bNAbs can direct clearance of virions and mediate killing of HIV infected cells.

In vivo studies performed in macaques and in human have shown that passive immunization with bNAbs confers protection against and even suppresses infection[337-347]. bNAbs strongly reduced viral load in infected individuals, and delayed the onset of viral rebound after ART arrest. However, bNAbs monotherapy leads to viral escape arguing for the need to maximize breadth and antiviral efficacy by combining bNAbs targeting different epitopes on HIV envelope.

Mouse studies strongly suggest that bNAb *in vivo* activity requires FcR mediated effector mechanisms[348,349]. Indeed, mutations in the antibody constant fragment to disable FcyR binding resulted in decreased control of mouse viral loads compared to the wild type antibody. In a context of defective humoral response, triggering innate immune mechanisms such as ADCC are appealing to target HIV infected cells. However, the direct impact of bNAb treatment on HIV reservoir has never been demonstrated[340,344].

1.5.3.2 Bispecific antibodies (BsAbs)

Bispecific antibodies combine the specificity for two antigens present on the same or on different cells. There are two major classes of BsAbs with or without antibody Fc region.

Recently developed BsAbs were based on the specificity of bNAbs and shared a common Fc region to increase the breadth and potency against HIV[350]. *In vitro* neutralization profiles were very encouraging and BsAbs could be used in HIV cure. Other interesting BsAbs are the bispecific T cell engager (BiTE) and dual-affinity re-targeting (DART) molecules, which rely on the activation of $CD3^+$ T cells to mediate cytolysis of the target cell in a MHC I and co-stimulatory molecule independent manner (**Figure 8**).

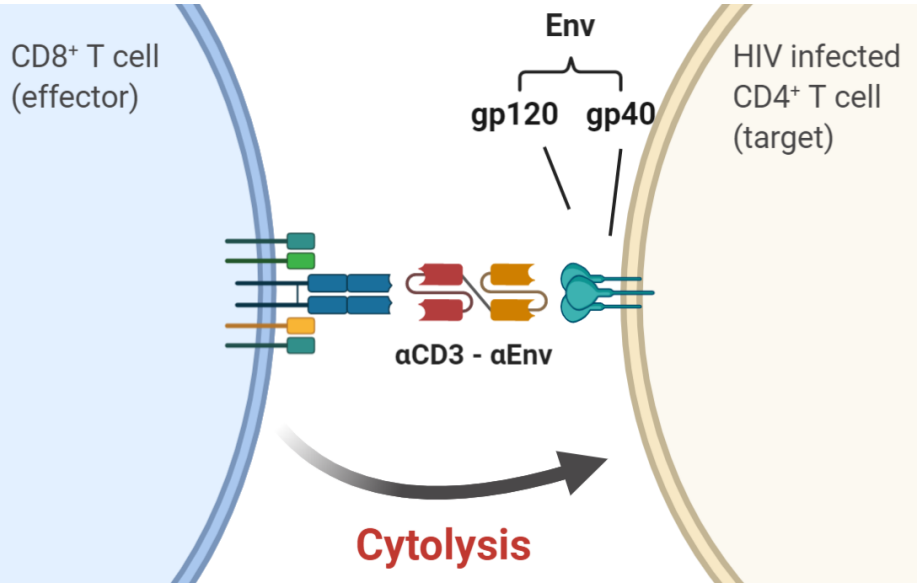


Figure 8: Schematic representation of BiTE targeting. *The engagement of both scFv to gp120 and to CD3, respectively, promotes activation of the effector cell and cytolysis of the target cell.*

BiTE molecules were first developed for applications in oncology and consist of two single chain variable fragments (scFv) targeting CD3 and tumor specific-antigen joined by a single polypeptide linker[351]. A recent study proposed a BiTE combining CD4, b12 or VRC01 to CD3 targeting moiety and evaluated the different scaffold *in vitro*[352]. Similar to previous studies, they observed that using CD4 moiety resulted in infection of CD4- cells. However, all the constructs were efficient at redirecting T cell lysis and inhibited HIV replication in lab strain infected PBMCs.

DART scaffold offers improved stability, manufacturability and potency compared to BiTE[353]. DARTs combining HIV-Env and CD3 specificities were developed and redirected lysis was associated to reduced virus recovery in culture supernatants following stimulation of ART suppressed HIV donor cells [354,355]. Another study using a VRC07 Fab combined to anti-CD3 scFV immunomodulatory protein showed that treatment of latently infected cells from ART

suppressed donors resulted in decreased cell-associated HIV DNA[356]. Moreover, treatment of BaL (lab adapted HIV strain) infected rhesus macaque demonstrated no adverse effect, though the impact on HIV reservoir has not been assessed.

1.5.3.3 Antibody-drug conjugates / immunotoxins

Drug conjugate strategy has been developed predominantly in the cancer field. Drug conjugates consist in a toxin payload that is attached to a carrier such as a monoclonal antibody or an immunologic ligand. The advantage of drug conjugates compared to a drug alone is the increased specificity for the target cell. Highly cytotoxic drugs can be used while sparing normal tissue, thus broadening the therapeutic window[357].

Due to the resistance of HIV reservoir to ART and the multiple immunological failures associated with HIV infection, drug conjugates represent an interesting approach in HIV research as well. The first compound that showed promising results to kill HIV infected cells was a soluble CD4 conjugated to *Pseudomonas aeruginosa* exotoxin A (PE40). However, studies in human showed no virological control and strong liver toxicity[358,359]. Subsequently, 3B3(Fv)-PE38 was developed, derived from 3B3 antibody that targets CD4 binding site on gp120[360-362]. 3B3(Fv)-PE38 was specific, had a low toxicitiy profile and, combined to ART, further reduced viral load in HIV infected humanized mice.

However, targeting gp120 represents several challenge. Indeed, anti-envelope antibodies, even bNAbs, do not neutralize every variants present in infected individuals, which may lead to the selection of escape variants and ultimately therapeutic failure[339,340,342]. Moreover, envelope is expressed at very low levels on infected cell surface and virtually not expressed at all on latently infected cells, representing a major barrier in presence of ART.

Following this, many studies focused on identifying a suitable marker to identify HIV reservoir. Among the most promising candidates, we can cite immune checkpoint molecules, including PD-1, LAG-3 and TIGIT[171,172,363]. Other markers such as and CD32a, CXCR3 and CD30 have been proposed as surrogate marker for HIV as well[364-367]. In this context, one case-report analyzed the impact of Brentuximab-vedotin (CD30) on HIV persistence in a ART-suppressed HIV infected cancer patient[368]. They observed transient decrease in cell associated HIV RNA and DNA, as well as transient decrease of viral load. The reason for the lack of sustained viral control was unknown and may involve cell redistribution from tissue compartment. Moreover, no analysis of HIV reservoir has been performed, which would have provided more information on the treatment impact. Altogether, this study provides good rationale for targeting a non-viral marker to decrease HIV reservoir.

1.5.3.4 Anti-α4β7

As already discussed, the early depletion of $CD4^+$ T cells in gut tissues plays a central role in HIV pathogenesis and the damage are not fully reversed despite effective ART. α4β7 is a gut homing receptor expressed on $CD4^+$, $CD8^+$ T cells, B cells, NK cells and macrophages. Many viruses have been shown to interact with α4β7, and in the context of HIV binding is mediated by gp120 envelope glycoprotein[369,370]. It has been shown that memory $\alpha4\beta7^{high}CD4^+$ T cells were more susceptible to productive infection and, therefore, are important for HIV dissemination[371].

In this context, studies have been performed to evaluate anti-α4β7 in prevention and treatment of SIV infection. Byrareddy and colleagues demonstrated that treatment with anti-α4β7 could reduce mucosal transmission of SIV in macaques[372]. Another study showed that pre-treatment of macaques with anti-α4β7 prior virus inoculation resulted in delayed peak viremia and lower viral load set-point[373]. Moreover, early anti-α4β7 treatment was associated with the preservation of gut tissue $CD4^+$ T cells[374]. Interestingly, ART suppressed SIV-infected macaques treated with anti-α4β7 maintained low to undetectable viremia and restored their CD4 level for up to nine months after treatment interruption[375]. However, the exact mechanisms leading to viral control remains largely unknown and results should be taken in a balanced way since macaques were infected with a weakly infectious SIV strain.

1.5.3.5 Chimeric antigen receptor-modified T cells

Chimeric antigen receptors (CAR) consist in an extracellular domain that binds the target antigen, a transmembrane portion that works as anchor and an endo-domain, mostly based on CD3ζ, that has a signaling function[376]. Binding to the target cell results in the activation and subsequent effector function response of the CAR modified T cells. Cells used for modification have mostly been autologous $CD8^+$ T cells, though interest in using NK cells and even HPSCs is rising[377,378]. CAR T cells have been predominantly developed for oncological applications, with complete remission of hematological malignancies achieved by using autologous CD19-specific CAR T cells[379-381]. However, the success of CAR T cells in treatment of solid tumor was less successful, possibly due to restricted trafficking[382].

As discussed earlier, cytotoxic $CD8^+$ T cell response (CTL) during HIV infection is associated with viral control[86,89]. Moreover, studies have demonstrated that HIV controllers had increased number of HIV-specific $CD8^+$ T cells that had enhanced avidity to HIV antigen and polyfunctional response[209,211,212]. However, HIV escape mutants rise very quickly in response to $CD8^+$ T cell pressure in most individuals and associated persistence of the virus ultimately leads to immune exhaustion[90,239]. In this regard, CAR T cells provide the strong advantage that

stimulation of the cell is independent from MHC-antigen stimulation, provided that the CAR can recognize a variety of HIV variants.

Different strategies have been investigated to target HIV-infected cells using CAR T cells, namely using CD4 CAR or broadly neutralizing antibody (bNAb) CAR on $CD8^+$ T cells (**Figure 9**)[383-385]. CD4 is the natural ligand for HIV envelope and binds gp120 with high affinity[17]. Therefore, escape mutations to CD4 binding are unlikely to happen. In vitro studies have demonstrated that CD4 CAR $CD8^+$ T cells have strong lytic capacity[386]. However, $CD8^+$ T cells expressing CCR5, CD4 CAR $CD8^+$ T cells are potentially vulnerable to HIV infection. On the contrary, bNAbs have the particularity to target highly conserved regions on HIV envelope and potently neutralize various isolates. Though bNAb CAR T cells are potent at killing HIV infected cells and are resistant to infection, naturally occurring bNAb resistant strains may be selected in vivo[384,385]. Therefore, careful evaluation of the reservoir and selection of bNAbs are required for therapeutic success. Studies are currently evaluating CD4 based multispecific CARs that target different conserved epitopes on envelope[387].

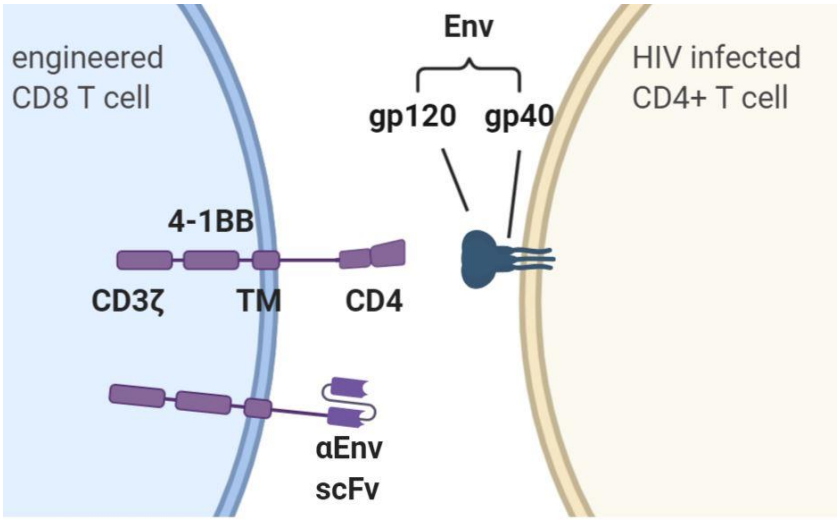


Figure 9: Schematic representation of CD4 and bNAb CARs on $CD8^+$ T cells. *CARs consist in an extracellular domain that targets the antigen on the cell to eliminate, in a trans-membrane domain (TM) and an endo-domain responsible for the signaling function (CD3ζ and 4-1BB co-stimulation).*

Though CAR T cells were performant in vitro, early clinical trials demonstrated safety but little efficacy and no impact on HIV reservoir in vivo[383]. The main challenge of developing CAR T cells for HIV cure is the lack of persistence/expansion of CAR T cells *in vivo*[388]. Studies demonstrated the benefit of low dose IL-2 to favor persistence[389]. Subsequent studies evaluated engineered IL-2 receptor orthogonal pairs to interact with one another only[390]. IL-2 signaling is transmitted without interaction with natural IL-2, thus avoiding off-target toxicity. Moreover, unlike cancer, HIV antigen is expressed at very low levels on infected cells during

HIV infection, and is almost undetectable in ART treated individuals. The lack of antigen stimulation may be detrimental to CAR T cell persistence.

1.5.4 Gene editing

Gene editing consists in the insertion, deletion or disruption of genes using targeted nucleases that bind DNA and create double-strand breaks. Gene editing can be applied to treat many diseases, including cancer, monogenic diseases and infectious diseases, with more than half of trials currently in oncology[391]. Due to the persistence of HIV in latently infected cellular reservoirs, gene editing might be an interesting tool to exploit in order to achieve a cure. Different gene editing strategies have been investigated, mostly focused on HIV genome cutting/excision and CCR5 editing.

Various tools have been developed to edit genomes, including zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and the clustered regularly interspaced short palindromic repeat (CRISPR) and CRISPR-associated (Cas) system[392-394]. ZFNs and TALENs are engineered endonucleases fused to DNA binding domains (proteins) that are specific for a target nucleotide sequence. On the contrary, CRISPR utilizes a short guide RNA (gRNA) that is complementary to the target DNA sequence and recruits Cas9 endonuclease to cleave genomic DNA. The advantage of CRISPR system is that Cas9 can be paired to a variety of gRNAs and does not need unique enzymes for each target sequence[395]. Overall, the three nucleases silence target genes by insertion or deletion during non-homologous end joining repair (NHEJ) occurring after DNA double strand break. To modify cell genome, genes encoding the enzyme system are inserted in a suitable vector such as DNA, RNA and viral vectors for transfection/transduction of the target cells.

HIV genome cutting/excision aims at targeting and disabling HIV genome directly by either excise the provirus from the human genome, or to generate replication incompetent proviruses by introducing deleterious mutations. The 2-LTRs, present at both ends of HIV genome, are a strong advantage to remove integrated HIV provirus. Indeed, a single target sequence is theoretically required to fully excise the provirus, as shown by in vitro studies using CRISPR/Cas9[396-398]. However, the risk of resistance due to non-lethal NHEJ is too high, requiring dual or multiplex gRNAs that target essential conserved regions in HIV genome[399-402].

The most investigated strategy of genome editing in HIV consist in targeting CCR5 co-receptor. Mutation CCR5Δ32 correlates with lower infection rate and slower progression of the disease[263]. Also, cases of remission from HIV infected cancer patients that were transplanted with cells from CCR5Δ32 donor largely contributed to the rise of interest in editing CCR5 gene[264,265]. Many clinical trials (phase I and II) focused on editing CCR5 from autologous

$CD4^+$ T cells or hematopoietic stem using an adenoviral CCR5-targeted ZFN construct, which are then reinfused to the patient[403] (NCT02388594, phase 1/2; NCT02225665, phase 1/2; NCT02500849, phase 1). However, it appears that transduction efficiency is crucial for HIV control. Added to homozygous gene disruption and the small number of cells delivered in the absence of bone marrow transplantation, this strategy still faces many challenges.

One major drawback of targeting CCR5 is that it does not protect from the infection with CXCR4-tropic viruses. Though transmitted/founder viruses usually are CCR5-tropic, about 50% of HIV infected individuals develop CXCR4-tropic viruses during the course of infection[404,405]. The presence of CXCR4-tropic viruses at the time of treatment may favor the selection of that variant and remission would not be achieved, as observed for the Essen patient and in animal models[406,407].

1.5.5 Combination of strategies

Many studies described above have shown the limitations of single therapy to achieve a functional cure. Low reactivation of latently infected cells, low antigen presentation, inefficient immune response and viral escape contribute to the suboptimal efficacy of the described therapies. Therefore, most recent studies combine drugs targeting different approaches to try to optimize the immune response and achieve a functional cure for HIV.

2 Aim of the project

The current project aims at killing HIV infected cells based on PD-1, which serves as a marker for the major $CD4^+$ T cell compartment for HIV-1 infection, replication, and production. Our hypothesis is that the targeted elimination of cells expressing PD-1 marker will significantly reduce the latent reservoir of cells that are capable of producing infectious virus[171,172,268]. Based on well established strategies for killing cells *in vitro* and *in vivo*, the following targeting approaches were investigated (**Figure 10**):

- **Antibody-dependent cell cytotoxicity (ADCC)**
- **Antibody-drug conjugates (ADC)**

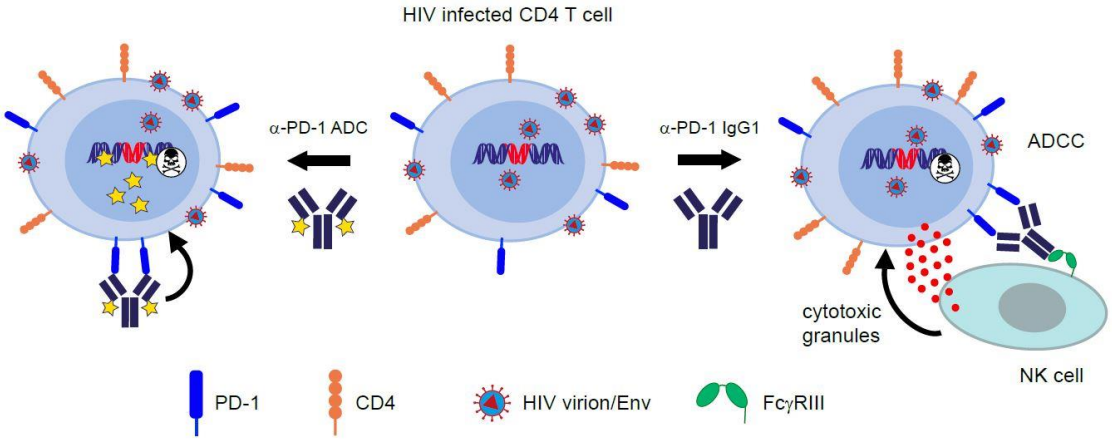


Figure 10: Strategies investigated to achieve a functional cure for HIV.

A panel of anti-PD-1 monoclonal antibodies was generated in our lab by immunizing mice with human PD-1 protein and immortalizing the antibody producing cells by a hybridoma technology. These antibodies were characterized according to their binding affinity and specificity and the best clones were used to generate antibody-drug conjugates or humanized IgG1. Given that ADCs need to be internalized and degraded in the lysosome in order to have their desired killing effect on the targeted cells, two different anti-PD-1 antibodies were selected that bound to different epitopes on the PD-1 protein. This diversity in antibody binding to PD-1 may allow for the selection of an ADC/IgG1 clone with improved killing properties[408]. In support to this premise, prior evidence in our lab has shown that the PD-1 receptor is internalized upon antibody binding and that antibodies are internalized at different rates according to their epitope binding.

The toxin selected for these studies was PNU-159682 (PNU), a very potent metabolite of the antitumor drug nemorubicin[409]. This toxin targets both dividing and non-dividing cells, which is an essential feature in the elimination of the HIV infected latent reservoir that may reside in dormant, non-dividing cells. In their unconjugated form, the use of PNU is highly restricted so

ADCs were generated at Levena Biopharma through chemical conjugation of cysteine residues in the anti-PD-1 antibodies.

3 Results

3.1 Paper I: Anti-PD-1 mediated depletion of PD-1$^+$CD4 T cells leads to the reduction of HIV cellular reservoir (in preparation)

Despite efficient suppression of viral replication by antiretroviral therapy, HIV can persist in cellular reservoirs that are not targeted by ART and treatment arrest irremediably leads to viral rebound and progression of the disease in most infected donors. As a result, continual suppression of HIV requires life-long treatment, which is associated with issues including adverse effects, non-compliance and emergence of drug resistance and high health-care associated costs. Therefore, strategies have been developed to eliminate HIV reservoir cells in an effort to allow infected patients to stop or significantly reduce the need for continual drug regimens.

One strategy may consist in selectively target and kill HIV infected cells using HIV specific markers. Since HIV envelope is poorly expressed on HIV latently infected cells, there is a high interest in better characterizing those cells to find surrogate markers. Notably, recent studies demonstrated that HIV latently infected cells capable of producing infectious particles were enriched in PD-1$^+$CD4 T cells. We therefore hypothesized that targeted depletion of PD-1$^+$CD4 T cells may decrease the frequency of HIV infected cells.

In order to assess the impact of PD-1$^+$CD4 T cell depletion on the HIV reservoir, we evaluated two targeting strategies, namely antibody-dependent cell cytotoxicity and antibody-drug conjugates, on blood mononuclear cells isolated from HIV untreated and ART treated donors. ADCC was assessed for a humanized anti-PD-1 IgG1 and antibody-drug conjugates were generated by conjugation of the nemorubicin metabolite PNU-159682 to mouse or humanized anti-PD-1 antibodies. In HIV untreated donors, we observed that both strategies, ADCC and ADC, increased the frequencies of cells undergoing apoptosis and cell death, which was associated with reduced production of HIV p24 and HIV RNA in culture supernatant. In ART treated donors, we demonstrated that ADCC and ADC targeting significantly reduced the frequencies of HIV infected cells harboring replication competent and infectious viruses.

Overall, these results represent a proof of concept for targeting PD-1$^+$CD4 T cells to reduce HIV cell reservoir.

Targeted killing of PD-1^{+}CD4 T cells *in vitro* specifically depletes HIV producing and latently infected cells from viremic and ART treated patient

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Abstract

Persistence of the human immunodeficiency virus (HIV) in long-lived cellular reservoirs despite antiretroviral therapy (ART) represents an overwhelming barrier to a cure for infected individuals. Recent studies demonstrate that a major portion of this reservoir in both viremic and ART treated individuals comprises CD4 T cells expressing the PD-1 receptor. Here we show that targeted cell killing mediated through anti-PD-1 antibody-depend cellular cytotoxicity (ADCC) reduces virus production by 59% and cells producing replication competent virus by 66% in blood mononuclear cells from viremic and ART treated donors, respectively. Furthermore, toxin coupled anti-PD-1 antibody-drug conjugates (ADC) gave stronger PD-1^{+} cell depletion with an 80% reduced virus production from viremic donor CD4 T cells and an eradication of cells harboring infectious viruses to undetectable levels in ART treated donors. These studies provide evidence that anti-PD-1 ADCC and ADC therapies directly eliminate HIV infected cells and represent viable strategies that may contribute towards a functional HIV cure.

Introduction

The advances in HIV research and especially the advent of potent anti-retroviral drugs have led to a strong and long-lasting viral suppression to levels below the limit of detection in the peripheral blood of most treated patients. As a result, the prognosis of HIV infected individuals over the last 10-20 years has improved immeasurably in terms of quality of life and life expectancy. Despite the efficacy of ART at suppressing viral replication, patients are not cured since reservoirs of infected cells persist and are not targeted by antiretroviral therapy (ART)[1-5]. As a result, HIV infected patients face life-long ART and treatment interruption invariably results in viral rebound and disease progression.

The HIV-1 retrovirus archives a proviral DNA copy in all productively infected cells, which poses a significant barrier for immune mediated viral eradication. Several reservoirs of infected cells persist in patients even after decades of complete viral suppression with ART[5]. The first defined cellular reservoir of HIV-1 infected cells in blood was a small pool of long-lived latently infected resting memory CD4 T cells[2,6,7]. These cells have a reduced capacity to produce viral particles, which facilitates their escape from immune surveillance[8,9]. The major peripheral reservoir of infected cells was further defined as central memory and transitional memory CD4 T cells, which persists through low-level antigen-driven proliferation and homeostatic proliferation[10]. Additionally, memory CD4 T cells expressing the PD-1 receptor have significantly elevated levels of integrated HIV DNA. More recently, studies have shown that lymph node T follicular helper cells and more specifically PD-1^{+}CD4 T cells represent a major cellular reservoir for replication competent and infectious HIV-1 viruses in viremic and in ART treated aviremic infected individuals[11,12]. Likewise, studies have shown that lymphoid organs act as sanctuary sites where residual ongoing virus replication replenishes the HIV latent cell reservoir and allows for viral evolution[13,14]. Within these privileged anatomic tissues, lower ART drug concentrations as well as poor accessibility to HIV-specific cytotoxic CD8 T cells contribute to the survival of these HIV-1 reservoir cells, which represent a major barrier for viral eradication[15,16].

Over the last 20 years, numerous innovative strategies dubbed “Shock and Kill” have been evaluated to eliminate the HIV-1 cellular reservoir. Primary strategies include the reactivation of the latent reservoir to reveal infected cells from their hidden locations or dormant state (Shock), and enhanced cell killing approaches that boost the host immune response in order to facilitate the elimination of infected cells (Kill). Latency reversing agents (LRAs) tested have included 1) cytokine therapy (e.g. IL-2, IL-2/OKT3, IL-2/IFNγ) that acts through immune activation[17-19], 2) histone deacetylase inhibitors that reverse

heterochromatin formation at the HIV long-terminal repeat (LTR), which can maintain the provirus in a transcriptionally suppressed state[20,21], 3) protein kinase C antagonists such as bryosostatin-1 that activate NF-κB in the HIV LTR promoter[22], 4) bromodomain inhibitors that promote HIV Tat transcriptional activation through facilitated interaction with P-TEFb[23] and 5) cyclophosphamide therapy to eliminate regulatory T cells and induce T cell growth factors such as type I interferons[24]. Despite low level viral reactivation for some of these strategies, latency reversing agents tested in HIV infected individuals failed at significantly reducing the frequency of HIV-infected cells *in vivo*[25]. Several case report studies have also tested the potential benefit of using immune checkpoint inhibitors including anti-PD-1 or anti-CTLA-4 monoclonal antibodies as immunotherapies[26-28]. These strategies use human IgG4 antibodies that lack Fc-mediated functional activity and act through receptor-ligand blockade to reinvigorate exhausted HIV-specific CD8 T cells as a way to enhance the elimination of infected cells. At present, these studies have shown only modest effects at decreasing the HIV reservoir size, though further investigation is needed. More recently, several antibodies against HIV envelope have been characterized for their efficiency in recognizing and targeting infected cells through antibody-dependent cellular cytotoxicity (ADCC) activity[29-32]. Furthermore, several antibody-derived molecules have been generated to deplete HIV infected cells, including Dual-Affinity Re-Targeting antibodies (DART) and Bispecific T-cell Engager (BiTE)[33-36], which recruit and activate T cells towards HIV envelope expressing cells, immunotoxins, composed of an antibody linked to a protein toxin, and antibody-drug conjugates (ADC) that act both through direct killing of targeted cells[37-39]. The use of ADCs is well validated in the clinical setting for oncology, where an antibody targets a surface receptor overexpressed on cancerous cells[40-42]. Internalization of the toxin-linked antibody, trafficking to the lysosomes and proteolytic digestion releases the toxin and kills the cancerous cell. Therefore, these approaches provide clinically validated therapeutic options that could be applied to an HIV cure strategies.

Herein, we propose a novel strategy to deplete HIV reservoir cells using PD-1 as a surrogate marker for HIV latently infected cells. We hypothesized that a targeted depletion of PD-1^{+}CD4 T cells may result in a reduced frequency of HIV infected cells capable of producing infectious viruses. In this study we therefore evaluated 1) the *in vitro* activity of a humanized anti-PD-1 IgG1 for antibody-dependent cell cytotoxicity and 2) anti-PD-1 antibodies coupled to the nemorubicin metabolite PNU-159682 (PNU)[43] for the impact of those therapeutic strategies on HIV latently infected cell reservoir.

Results

ADCC mediated killing of PD-1 expressing cells

PD-1 expressing CD4 T cells represent an important cellular reservoir for the production and persistence of HIV in infected individuals. Using a strategy that is frequently applied in clinical oncology[40,42], we evaluated the ADCC mediated killing of PD-1 positive cells using anti-PD-1 human IgG1 antibodies (*h*PD-1 IgG1). This directed immune mediated cell killing of target cells requires the binding of the Fc portion of the antibody to Fc receptors expressed on effector cells including natural killer (NK) cells.

The ADCC activity of an *h*PD-1 IgG1 was first evaluated using a PD-1^{+} Jurkat target cell and a NFAT/FcγRIII effector cell line in a commercial reporter assay. Incubation of target cells with effector cells in the presence of increasing concentrations of *h*PD-1 IgG1 resulted in a dose-dependent activation of the FcγRIII mediated reporter (*Sup Fig 1A*). In contrast, the same anti-PD-1 antibody produced as an IgG4 subclass, which binds only weakly to the FcγRIII receptor, did not induce effector cell activation. Effector cell activation by the *h*PD-1 IgG1 Ab was also shown to be dependent on PD-1 expression by the Jurkat target cell.

ADCC mediated killing of PD-1 positive cells was next evaluated in an assay using anti-CD3/CD28 stimulated PD-1 high CD4 T cells and autologous NK effector cells isolated from healthy donors. Co-culture of target PD-1 high CD4 T cells and NK effector cells in the presence of *h*PD-1 IgG1 Ab resulted in a significant increase in cell killing as determined by the level of Aqua positive CD4 T cells compared to untreated, isotype control and *h*PD-1 IgG4 antibody treatments (*Sup Fig 1B*).

Taken together, these studies support the specific ADCC mediated killing of PD-1 expressing cells with our *h*PD-1 IgG1 antibody.

ADCC mediated depletion of HIV producing cells

To investigate the impact of an anti-PD-1 ADCC therapy on HIV-1 infected cells, *h*PD-1 IgG1 was incubated with blood mononuclear cells (PBMC) from either viremic untreated or ART treated HIV infected donors (Table 1). Samples from viremic donors were collected ~1.2 years post diagnosis (ranging from 0.05 to 5.41 years) with approximately 30% of CD4 T cells expressing the PD-1 receptor, while ART treated donors were on therapy for ~1.8 years (ranging 0.08 to 18 years) with a mean 30% PD-1 expression on

CD4 T cells. ADCC functional activity of *h*PD-1 IgG1 on donor cells was assessed after a 5 day incubation in cell culture by monitoring the frequencies of Annexin V and Aqua positive CD4 T cells, corresponding to apoptotic and dead cells, respectively (*Fig 1A-B*). Compared to untreated, isotype control or *h*PD-1 IgG4 antibodies, PBMCs treated with *h*PD-1 IgG1 showed modest but significant 1.7- to 2.3-fold increased levels of apoptosis and cell death in CD4 T cells from both viremic (*Fig 1C-D, Sup Fig 2A-B*) and ART treated (*Fig 1E-F, Sup Fig 2C-D*) HIV-1 positive donors ($P<0.0015$). Direct depletion of PD-1 expressing cells could not be reliably monitored due to competitive binding of anti-PD-1 fluorescently labeled flow cytometry antibodies with the treatments and/or treatment induced PD-1 receptor internalization. However, the increased *h*PD-1 IgG1 ADCC cell killing corresponds to a low 34% (14%-165%) and 14% (5%-31%) reduction in PD-1 positive CD4 T cells from viremic untreated and ART treated HIV donors, respectively.

The effect of the *h*PD-1 IgG1 ADCC mediated killing on the minor subpopulation of HIV infected cells was assessed by monitoring the production of HIV RNA and viral p24 in the culture supernatants. In viremic HIV positive donors tested, *h*PD-1 IgG1 treatment resulted in a significant 59% decrease (ranging from 41% to 77%) of HIV RNA production and 43% decrease (ranging from 30% to 58%) in p24 production in culture supernatant compared to untreated, isotype control and *h*PD-1 IgG4 control samples (both $P<0.001$) (*Fig 2*).

In samples from ART treated HIV infected donors, no p24 could be detected in the cell culture supernatants following the 5-day treatments. This can be attributed to the low frequency of infected cells and the presence of latently infected CD4 T cells in ART treated donors. Given the limited number of HIV infected cells in the PBMCs of ART treated donors, the *h*PD-1 IgG1 and control treated samples were evaluated using a limiting dilution format of the quantitative viral outgrowth assay (QVOA) assay (*Fig 3A*). This assay monitors the ability of *h*PD-1 IgG1 treated infected cells to produce virus capable of infecting activated CD4 T cells from an HIV negative healthy donors. Performed with multiple replicates, the QVOA can determine the frequency of cells harboring replication-competent virus per million cells (RUPM) by monitoring the production of HIV RNA in cell culture supernatants[44]. Of note, depletion of PD-1 positive cells from ART treated donors with *h*PD-1 IgG1 significantly reduced the frequency of infected cells by 66% ($P<0.0232$) compared to the control conditions (*Fig 3B*). The observed decreases in virus production and frequency in HIV infected cells are all the more encouraging given that only cells expressing high levels of a surface receptor are efficiently killed in ADCC therapies[45].

Characterization of anti-PD-1 antibody-drug conjugates (ADC)

To achieve more robust depletion of PD-1$^+$ cells, we conjugated two different antibody clones with a toxin to develop an ADC therapeutic strategy. Given that internalization of the receptor/ADC complex is essential for cell killing, antibodies targeting different epitopes on PD-1 and with different affinities were evaluated to identify agents with improved activity[46,47]. The *h*PD-1 IgG1 antibody binds PD-1 at the interface of the PD-1 /PD-L1 interaction site (0.3 nM IC_{50} for cell surface PD-1) while a second mouse anti-PD-1 antibody (*m*PD-1 IgG) binds an alternate epitope overlapping with PD-1 residue 96-104 (1.8 nM IC_{50} for cell surface PD-1) and does not compete with the binding of *h*PD-1 IgG1[48].

The *m*PD-1 IgG and *h*PD-1 IgG1 antibodies were conjugated with the highly toxic nemorubicin metabolite PNU-159682 (PNU) through maleimide-based (MA) conjugation of thiol residues in the anti-PD-1 antibodies (*Fig 4*). The linker and toxin payload consist of a cathepsin B protease-sensitive valine-citrulline dipeptide (vc) followed by the para-amino-benzoyloxycarbonyl (PAB) self-immolative spacer attached to PNU through a N-formyl-N, N'-dimethylethylendiamine (DMEA) spacer (MA-PEG4-vc-PAB-DMEA-PNU159682 linker-payload)[49,50]. Drug-antibody ratios for each coupled antibody were 1.5:1 for the *m*PD-1 ADC and 3.4:1 for the *h*PD-1 ADC according the manufacturer specification sheet.

The cell killing specificity of the ADCs was assessed using wild type and PD-1 expressing Jurkat cell lines treated with toxin-conjugated or unconjugated versions of the anti-PD-1 antibodies. Following 3 days in culture, ADC mediated activity was evaluated by the frequencies of Annexin V and Aqua positive cells. Jurkat cells, expressing high levels of PD-1, were still effectively targeted by mPD-1 and hPD-1 ADCs at concentrations of 0.2 and 0.01 µg/ml, respectively. These toxin-induced effects were specific given that only minimal Annexin V staining was observed in wild type Jurkat cells with ADC concentrations up to 5 µg/ml (*Sup Fig 3*).

Anti-PD-1 ADC depletion of virus producing HIV infected CD4 T cells

The impact of the anti-PD-1 ADCs on HIV infected cells was first evaluated using isolated CD4 T cells from viremic untreated donors (**Table 1**). ADCs and unconjugated anti-PD-1 antibodies were incubated with isolated CD4 T cells for five days, after which cell apoptosis and cell death were evaluated by flow cytometry, and HIV p24 antigen was quantified in culture supernatant.

Given the lower expression level of PD-1 on donor CD4 T cells relative to the Jurkat PD-1 stables (*Sup Fig 4*), an ADC concentration response was tested to identify optimal

conditions for selective cell killing. Doses of 10 µg/ml *m*PD-1 and 5 µg/ml *h*PD-1 ADCs were determined to have both acceptable off-target toxicity profiles and a strong reduction in levels of HIV p24 antigen released by infected cells following a 5 day incubation in cell culture (*Sup Fig 5*).

Anti-PD-1 ADC treatments were extended to isolated CD4 T cells from a panel of HIV infected viremic untreated donors (**Table 1**). Flow cytometry analysis (*Fig 5A-B*) demonstrated that *m*PD-1 and *h*PD-1 ADCs lead to 2.7- and 3.8-fold increase in cell apoptosis (Annexin V+) and 2.2- and 3.5-fold increase of cell death (Aqua), respectively, compared to the controls (P<0.001) (*Fig 5C-F; Sup Fig 6*). This *m*PD-1 and *h*PD-1 ADC treatment mediated cell killing corresponds to a low 25% (13%-96%) and moderate 84% (46%-204%) reduction in PD-1 positive CD4 T cells from HIV donors, respectively, and resulted in strong 84% (54%-88%) and 86% (70%-91%) reductions of HIV p24 antigen production in culture supernatants, respectively (P<0.001) (*Fig. 6*).

Anti-PD-1 ADC depletion of latently infected CD4 T cells from HIV infected ART treated donors

The strong ADC mediated depletion of PD-1$^+$ cells was next evaluated for their effect in targeting infected cells from ART treated donors (**Table 1**). As with previous experiments, *m*PD-1 ADC treatment of isolated CD4 T cells led to a significant increase in the frequencies of apoptotic cells (3.2-fold; P< 0.001) and cell death (2.9-fold; P<0.001) compared to the unconjugated controls, corresponding to a partial 38% (9%-43%) reduction of PD-1 positive CD4 T cells (*Fig 7, Sup Fig 7*). Similarly to ADCC-mediated depletion, no p24 could be detected in the cell supernatants following the 5-day treatments. The frequencies of cells harboring replication competent (RUPM) and infectious (IUPM) viruses were then evaluated for each condition through detection of HIV RNA and HIV p24 antigen in a QVOA assay (*Fig 8A*)[44]. Since infected cells from ART treated donors may be in a transcriptionally inactive latent state, CD4 T cells incubated with antibody controls or ADC for 5 days were subsequently stimulated with anti-CD3/CD28 to enhance viral reactivation and infection of the allogeneic target cells. In the 14-day QVOA, the *m*PD-1 ADC treatment resulted in a strong reduction of cells harboring HIV replication competent viruses by 88% (P<0.001). Importantly, we observed a dramatic depletion of cells capable of producing infectious virus to levels below the limit of detection (P<0.001) compared to the control conditions (*Fig 8B-C*).

Taken together, data indicate that targeting CD4 T cells from HIV donors with anti-PD-1 ADCs contributes to the reduction of HIV latently infected cells.

Discussion

HIV persists in cellular reservoirs that are not targeted by ART[1-3]. Significant effort has been made to identify cellular markers associated with these reservoirs that could provide a basis for a selective targeted therapy[51]. The most evident marker for infected cells is the HIV envelope protein, which is displayed on the cell surface prior to viral budding. However, HIV latency results in very low and/or stochastic viral antigen expression leading to inefficient *in vivo* elimination of these infected cells, even by potent neutralizing antibodies targeting envelope[52-54]. Recently, many surface proteins have been proposed as surrogate marker for HIV latently infected cells including PD-1, LAG-3 and TIGIT immune checkpoint inhibitory molecules, CD30 and CD32a[10-12,55-58].

In the present paper, we evaluated the potential of PD-1 targeting to decrease the frequencies of HIV infected cells in blood mononuclear cells isolated from HIV viremic and ART treated donors. We evaluated the ADCC activity of *h*PD-1 IgG1 and two ADCs targeting different epitopes on PD-1 that were conjugated to the nemorubicin metabolite PNU-159682[43]. Cytotoxicity was assessed by flow cytometry and the impact on the HIV reservoir by the production of HIV RNA or p24 antigen in culture supernatants, or by a quantitative viral outgrowth assay.

Anti-PD-1 ADCC and ADC treatment strategies both resulted in significant increased frequencies of apoptotic and dead CD4 T cells from viremic untreated HIV infected donors. In parallel with this increased cell killing, viral HIV RNA or p24 levels were significantly reduced in the culture supernatants of ADCC and ADC treated cells. Given that infected CD4 T cells from viremic HIV infected donors actively produce viral particles, these results support the direct killing of virus producing cells by the anti-PD-1 IgG1 through ADCC and by the ADCs.

In contrast with viremic donors, CD4 T cells from ART treated HIV infected donors have lower frequencies of infected cells in the blood, low levels of cell associated HIV viral transcripts and undetectable production of virus as determined by p24 levels in the cell supernatants of unstimulated cells[12]. Therefore, patients treated for multiple years with a highly suppressive ART have infected cells that are primarily in a transcriptionally silent latent state. ADCC and ADC treatment of cells from ART treated donors induced increased frequencies of apoptotic and dead CD4 T cells that were associated with significantly reduced production of both, replication competent and infectious viruses. These results are consistent with previous reports that the HIV cellular reservoir in ART treated patients

expresses elevated levels of PD-1 and unambiguously shows that both, anti-PD-1 ADC and IgG1 mediated ADCC are able to target and kill HIV-infected cells[10-12].

In comparing the therapeutic efficiency of the two strategies in depleting HIV infected cells, ADCC was overall less effective than ADC in our experimental setup. This could be attributed to the lower level of cell killing observed through ADCC that depleted only a portion of the PD-1 expressing cells. This was most evident in cells from ART treated donors where it is estimated that 13% of PD-1+ CD4 T cells were killed with the ADCC therapy and 38% using the ADC. Reduced cell killing efficacy may be due to the use of resting NK effector cells that underrepresent the full potential of this ADCC therapy. The fact that HIV infection impairs the functionality of NK cells and that ART only partially restores this activity also contributes to the reduced efficacy of the anti-PD-1 ADCC treatment *in vitro*[59,60]. A second limitation of the anti-PD-1 ADCC strategy for depleting HIV infected PD-1$^+$ CD4 T cells is that antigen density is crucial for NK cell cytotoxic activity[45]. In this context, targeted killing of PD-1 high cells may have been favored over PD-1 low, leading to a suboptimal depletion of PD-1 positive cells. These factors may explain the moderate reduction of virus production by 59% and cells producing replication competent virus by 66% in blood mononuclear cells from viremic and ART treated donors, respectively.

ADCs with the PNU toxin coupled to either a mouse or human IgG1 anti-PD-1 antibody both induced a strong selective killing of PD-1 expressing cells. Antibodies binding different epitopes on PD-1 were evaluated since the binding site and the potential for receptor dimerization has been reported to influence internalization and the trafficking to the lysosome required for ADCs to undergo proteolytic cleavage and toxin release[46]. In the case of the toxin coupled antibodies tested, both ADCs were equally effective at inducing PD-1$^+$ cell depletion resulting in ~ 80% reduced virus production from viremic donor CD4 T cells. Several classes of toxins are used in the oncology field for ADCs and include drugs targeting tubulin filaments and DNA alkylating agents[61,62]. Our selection of PNU-159682 was motivated by the sub-nanomolar activity against many cancer cell lines and the specific killing of both dividing and non-dividing cells that would be essential to target latently infected CD4 T cells that are in a quiescent state[43,50]. Importantly, the efficacy of the *m*PD-1 PNU therapy is compelling in reducing the presence of HIV replication competent cells by 88% and the eradication of cells harboring infectious viruses to undetectable levels in ART treated donors.

Although our *in vitro* studies provide a strong proof of principle for both, anti-PD-1 IgG1 mediated ADCC and anti-PD-1 ADC strategies in the killing of HIV infected cells, *in vivo*

efficacy will present additional challenges. Indeed, many studies have underlined the importance of the lymphoid organs as sanctuary sites for HIV persistence[11,12,14]. The access of antibodies to lymph node germinal centers, where T_{fh} cells and other PD-1 high infected CD4 T cells reside, will be critical for the *in vivo* efficacy of the treatment.

ADC and ADCC approaches both have advantages and limitations when considering their use as a clinical agent for the treatment of HIV infected patients. Antibody-drug conjugates are very efficient and do not rely on a pre-existing immune response. Moreover, the use of a highly potent toxin such as PNU-159682 allows for low doses of ADC to be administered to specifically kill the target cell while sparing healthy tissues. Under the protection of ART to prevent re-infection of cells, intermittent ADC dosing of a patient would allow for minimal effects on healthy tissues and the gradual accumulation of toxin in the reservoir of infected cells leading to their death. However, despite high specificity for their target, ADCs are associated with some strong side-effects that are mostly caused by the spontaneous release of the toxin[63].

The unconjugated IgG1 antibody used for ADCC is devoid of toxin-associated adverse effects, but their function relies on effector cells including NK cells. Given that the functional activity of NK cells is partially compromised in HIV infected patients, ADCC activity may need to be enhanced with cytokine priming such as IL-12, IL-15 or IL-21 therapy[64-66]. A second consideration for the *in vivo* efficacy of an anti-PD-1 ADCC therapy in HIV infected patients is the accessibility of NK cells to tissues that harbor latently infected cells. Lymph node germinal centers contain T_{fh} cells and other PD-1 high infected CD4 T cells that represent a major cell reservoir for HIV[11,12]. Though some reports mentioned the presence of NK cells in lymph node from African Green Monkeys, this has not been demonstrated in human[67].

A potential limitation concerning the anti-PD-1 ADCC and ADC therapies is the indiscriminant killing of all PD-1 expressing cells including cells not infected with HIV. PD-1 is the archetypal marker of exhaustion on CD4 and CD8 T cells but also an activation marker on T cells, NK cells and other immune cell populations[68]. We propose that the depletion of CD4 and CD8 exhausted T cells would have a minimal effect on a patient's virus specific immune response since these cells have a sub-optimal functional activity. However, HIV specific CD8 T cells may also express PD-1 as an activation marker and contribute to the killing of infected cells in viremic patients. The negative impact of depleting PD-1^{+}CD8 T cells would be minimized by only using anti-PD-1 depleting strategies on HIV patients that have been on fully suppressive ART for multiple years. Under these conditions,

patients have undetectable levels of circulating virus and thus low level of viral antigens would be present to activate and increase PD-1 expression on HIV specific CD8 T cells. Future experiments to support our *in vitro* studies will use HIV infected humanized mouse models to provide an *in vivo* proof of principle validation for the anti-PD-1 depletion strategies.

In conclusion, we demonstrated that anti-PD-1 mediated ADCC and ADC treatments significantly reduced the frequencies of cells harboring replication competent and infectious HIV viruses in blood cells from viremic and ART treated donors. Our data confirmed that anti-PD-1 depletion strategies directly eliminate HIV infected cells and represent viable strategies that may contribute toward a functional cure for HIV infected patients.

Material and methods

HIV-positive donors, ethic statement and cell isolation

The present study was approved by the Institutional Review Board of the Centre Hospitalier Universitaire Vaudois, and all individuals gave written informed consent. Blood mononuclear cells used for the *in vitro* functional assays were obtained following leukapheresis performed on ten viremic untreated HIV-positive donors and five ART treated HIV-infected donors. Inclusion criteria were detectable virus production after 5 days in culture for HIV untreated donors, and less than five years under ART or blip, as well as reactivation under viral outgrowth condition for ART treated donors. Blood mononuclear cells were isolated by ficoll gradient and cryopreserved in liquid nitrogen.

Blood mononuclear cells from healthy donors were isolated from apheresis filter purchased from the centre de transfusion (CTS) as previously described and cryopreserved in liquid nitrogen. When required, CD4 T cells and NK cells were isolated from blood mononuclear cells using immunomagnetic negative selection kits according the manufacturer protocol (STEM CELL).

Cell lines

Wild type Jurkat and PD-1+ Jurkat cells were obtained/purchased from ATCC and BPS Bioscience, respectively.

Cell culture

Blood mononuclear cells and wild type Jurkat cells were cultured in Roswell Park Memorial Institute (RPMI) medium (GIBCO Life Technologies) containing 10% heat-inactivated FBS (BioWest), 100 IU/ml penicillin, and 100 µg/ml streptomycin (BioConcept), which will be referred as R10. To grow PD-1 Jurkat cells, R10 was supplemented with 200 µg/ml hygromycin B (Corning) and 1 mg/ml geniticin (Gibco Life Technologies). Incubation of cells were performed at 37°C with 5% CO2.

Antibodies

Novel mouse and humanized anti-PD-1 Ab were developed as previously described[48]. Promising clones were selected for conjugation to the nemorubicin metabolite PNU159682, which was performed by Levena biopharma. Those antibodies were evaluated in ADCC and ADC assays. Purified mouse IgG1 (clone MG1-45; BioLegend) or human IgG4 (clone ET904; Eureka therapeutics) isotype controls were used as negative controls.

The following antibodies were used for flow cytometry: Allophycocyanin (APC)–H7–conjugated anti-CD3 (SK7), PE-CF594–conjugated anti-CD4 (RPA-T4), Pacific Blue (PB)- or PerCP/Cy5.5-conjugated anti-CD8 (RPA-T8 and SK1, respectively), PB-conjugated anti-CD14 (M5E2), AlexaFluor700 (AF700)-conjugated anti-HLA-DR (G46-6) antibodies, Pe- or APC-conjugated Annexin V (BD Biosciences); AF700-conjugated anti-CD4 (RPAT4), Pe-conjugated anti-CD56 (HCD56), Brilliant violet 421 (BV421)-conjugated anti-PD-1 (EH12.2H7) antibodies (BioLegend); AF488-conjugated anti-CD19 (HIV19) (eBioscience).

For stimulation in the viral outgrowth assay, purified NA/LE anti-CD3 (UCHT1; BD Biosciences) and anti-CD28 (CD28.2; BD Biosciences) were used.

FcγRIII stimulation assay

FcγRIII stimulation was evaluated with using Promega ADCC reporter bioassay (Promega, Ref. G7010) according the manufacturer protocol. Briefly, ADCC effector cells (NFAT/FcγRIIIa Jurkat cells) were co-cultured with PD-1+ Jurkat at a ratio 2.5:1 in presence of various concentrations of *h*PD-1 IgG1 or IgG4. After 6 hours incubation, luciferase activity was revealed using One-Step Luciferase Assay System (BPS Bioscience) according the manufacturer protocol. The luminescence was recorded on a Synergy H3 plate reader.

ADCC assay against stimulated $CD4^+$ T cells

$CD4^+$ T cells and NK cells were isolated by negative selection from healthy blood mononuclear cells (STEM CELL). $CD4^+$ T cells were stimulated for 48 hours with Dynabeads Human T-Activator CD3/CD28 (Gibco by Life Technologies) according the manufacturer protocol. In parallel, NK cells were stimulated for 24 hours with 100U/ml IL-2 (Miltenyi). PD-1 expression level of stimulated CD4+ T cells was evaluated by flow cytometry before the assay using fluorescently conjugated antibodies against CD4, PD-1 and Live/Dead Aqua. Stimulated NK and CD4+ T cells were co-cultured at a ratio 5:1 in presence of *h*PD-1 IgG1 or IgG4. After 6 hours incubation, cells were collected and stained with fluorescently conjugated antibodies against CD4, CD56, Annexin V and Live/Dead Aqua. The stained cells were run on an LSRII flow cytometer (BD Biosciences).

ADCC evaluation on *ex-vivo* HIV infected cells

Blood mononuclear cells from HIV infected donors were cultured at 10^6 cells/ml in presence of 10 U/ml IL-2 and 5 µg/ml *h*PD-1 IgG1 or IgG4. After 5 days, cells were collected and stained with fluorescently conjugated antibodies against CD3, CD4, CD8, Annexin V and live/dead Aqua. The stained cells were run on a LSRII flow cytometer (BD Biosciences). In parallel, the presence of p24 antigen in culture supernatants was assessed by ECL

COBAS HIV Ag (Roche; Switzerland). The presence of HIV-1 RNA in culture supernatants was assessed by COBAS AmpliPrep/TaqMan HIV-1 Test (Roche; Switzerland).

ADC assays

W/T or PD-1 expressing Jurkat cells were cultured in presence of various concentration of *m*PD-1 or *h*PD-1 ADC. After 3 days of culture, cells were collected and stained with fluorescently conjugated Annexin V and live/dead Aqua (Life Technologies) cell marker. The stained cells were run on an LSRII flow cytometer (BD Biosciences).

Total CD4+ T cells were isolated from HIV infected donor blood mononuclear cells by negative selection (STEM CELL). CD4+ T cell purity was evaluated by flow cytometry using fluorescently conjugated antibodies against CD3, CD4, CD8, CD56, CD19, CD14, HLA DR and live/dead Aqua. Similarly, basal PD-1 expression and cell death/apoptosis were evaluated using fluorescently conjugated antibodies against CD4, PD-1, Annexin V and live/dead Aqua. Isolated CD4+ T cells were then resuspended at 10^6 cells/ml, supplemented with 50 U/ml IL-2 (Miltenyi Biotec) and incubated with 10 µg/ml *m*PD-1 or 5 µg/ml *h*PD-1 antibody or ADC. After 5 days of culture, cells were collected and stained with fluorescently conjugated antibodies against CD4, Annexin V and live/dead Aqua cell marker. The stained cells were run on an LSRII flow cytometer (BD Biosciences). In parallel, the presence of p24 antigen in culture supernatants was assessed by ECL COBAS HIV Ag (Roche; Switzerland).

Quantitative viral outgrowth assay

Cells from ADC or ADCC assay that were performed on ART suppressed individuals were collected and washed 3 times thoroughly. Different cell concentrations (five-fold limiting dilutions, i.e. 5×10^5, 1×10^5, 2×10^4 and 4×10^3) were cultured with allogeneic fresh CD8-depleted blood mononuclear cells (10^6 cells/ml) (CD8 immunomagnetic beads, MACS Miltenyi) from HIV-uninfected individuals in quintuplicate. These cells were stimulated for 5 days with anti-CD3 and anti-CD28 mAb-coated plates (10 µg/ml) in presence of IL-2 (50 U/ml). One plate was prepared for each ADC/ADCC condition and one uncoated plate was added for the untreated condition as negative control. Supernatants were collected at days 0, 5 and 14. Medium was replaced at day 5, and the culture was resupplemented with R10 + IL-2 (50 U/ml). The presence of p24 antigen was assessed in culture supernatants by ECL COBAS HIV Ag (Roche; Switzerland). The presence of HIV-1 RNA in culture supernatants was assessed by COBAS AmpliPrep/TaqMan HIV-1 Test (Roche; Switzerland). Wells with detectable p24 (≥1 ECL unit/ml) were referred to as p24-

positive wells. Wells with detectable HIV-1 RNA (≥50 HIV-1 RNA copies/ml) were referred to as HIV-1 RNA-positive wells. When required, culture supernatants were diluted (1/5 or 1/10) in basement matrix buffer (RUWAG Handels AG). RUPM, based on HIV RNA detection, and IUPM, based on p24 detection, were calculated by using conventional limiting dilution methods using 'extreme limiting dilution analysis' (ELDA) (http://bioinf.wehi.edu.au/software/elda/)[44].

Data processing and statistical analysis

Flow cytometry data were analyzed with FlowJo software. Frequencies were plotted using GraphPad Prism and statistical significance (P-values) were calculated using multiple t-tests. Dose-response curve were fitted to a 4PL curve using GraphPad PRISM software. Mean frequencies of infected cells were calculated with the "extreme limiting dilution analysis" (ELDA) (http://bioinf.wehi.edu.au/software/elda/), and P-values determined with a Chi-squared two-tailed analysis.

Figures

Table 1: Clinical data

Patient ID	Years post diagnosis	Years on ART	ART regimen	Viremia	CD4 count	% PD-1$^+$ CD4$^+$ cells
B03b[a,b,c]	1.10	-	-	155000	547	29
B09[a,c]	1.66	-	-	530000	398	22
MP034[a]	0.28	-	-	5000	549	24
MP117[a,b,c]	5.41	-	-	54000	504	30
MP125[a]	0.16	-	-	17000	511	33
MP135[a]	0.52	-	-	1600000	176	27
MP140[c]	0.08	-	-	360000	427	22
MP148[b]	0.06	-	-	20000	717	34
MP153[a,b]	0.05	-	-	77000	439	19
MP154[b]	0.07	-	-	510000	544	23
MP009[a,c]	26.68	18.00	TDF,FTC, ATV/r	30	441	34
MP057[a,c]	0.20	0.08	DTG, ABC, 3TC	<20	791	14
MP088[a]	2.43	1.67	DTG, ABC, 3TC	<20	1261	27
MP103[a,c]	1.92	1.62	DTG, ABC, 3TC	<20	217	40
MP134[a,c]	1.93	1.83	DTG, ABC, 3TC	<20	795	22

a) ADC *m*PD-1 PNU

b) ADC *h*PD-1 PNU

c) ADCC assay *h*PD-1 IgG1

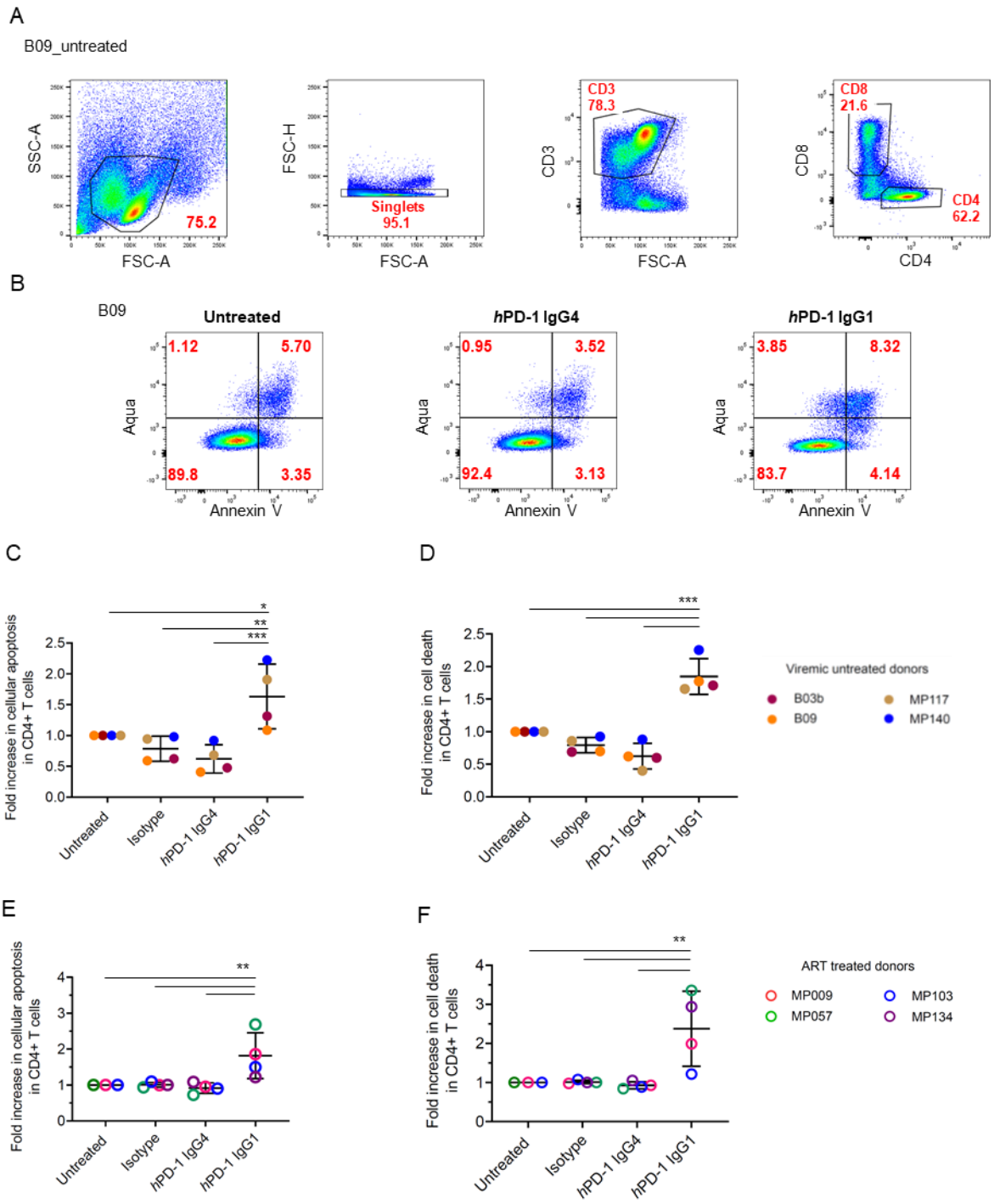


Figure 1: hPD-1 IgG1 ADCC-mediated depletion of cells from HIV viremic untreated and ART treated donor cells. *(**A**) Gating strategy. (**B**) Representative flow cytometry data for the evaluation of Annexin V and Aqua positive populations. Fold increase apoptotic cells (Annexin V⁺) (**C**) and dead cells (Aqua⁺) (**D**) compared to untreated condition on day 5 after 5 µg/ml hPD-1 antibody treatment in viremic untreated donors. Fold increase apoptotic cells (**E**) and dead cells (**F**) compared to untreated condition on day 5 after 5 µg/ml hPD-1 antibody treatment in ART treated donors. Means are represented and error bars indicate SD. (**<0.01; ***P<0.001; t-test)*

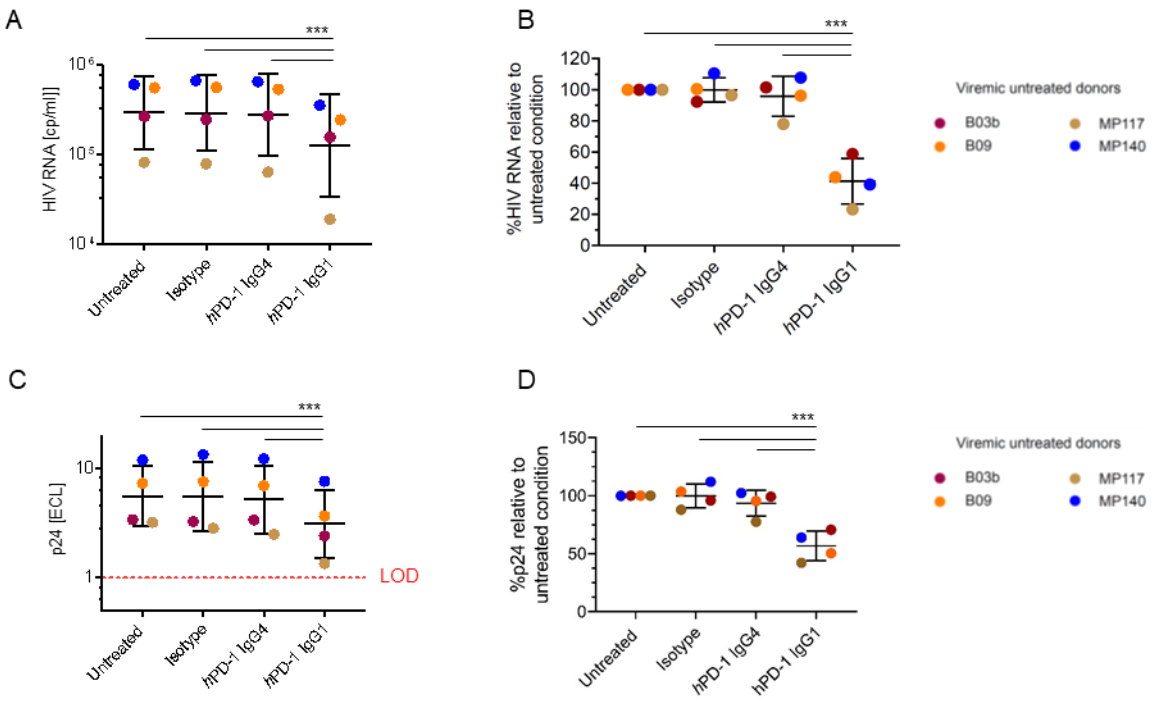


Figure 2: Impact of hPD-1 IgG1 treatment on virus production from viremic untreated HIV donors. *(**A**) Production of HIV RNA detected in culture supernatants after 5 µg/ml hPD-1 antibody treatment and (**B**) calculated percentage relative to untreated condition. (**C**) Production of viral p24 detected in culture supernatants after 5 µg/ml hPD-1 antibody treatment and (**D**) calculated percentage relative to untreated condition. (**A**, **C**) Geometric means are represented and error bars indicate geometric mean SD. (**B**, **D**) Means are represented and error bars indicate SD. (***P<0.001, t-test)*

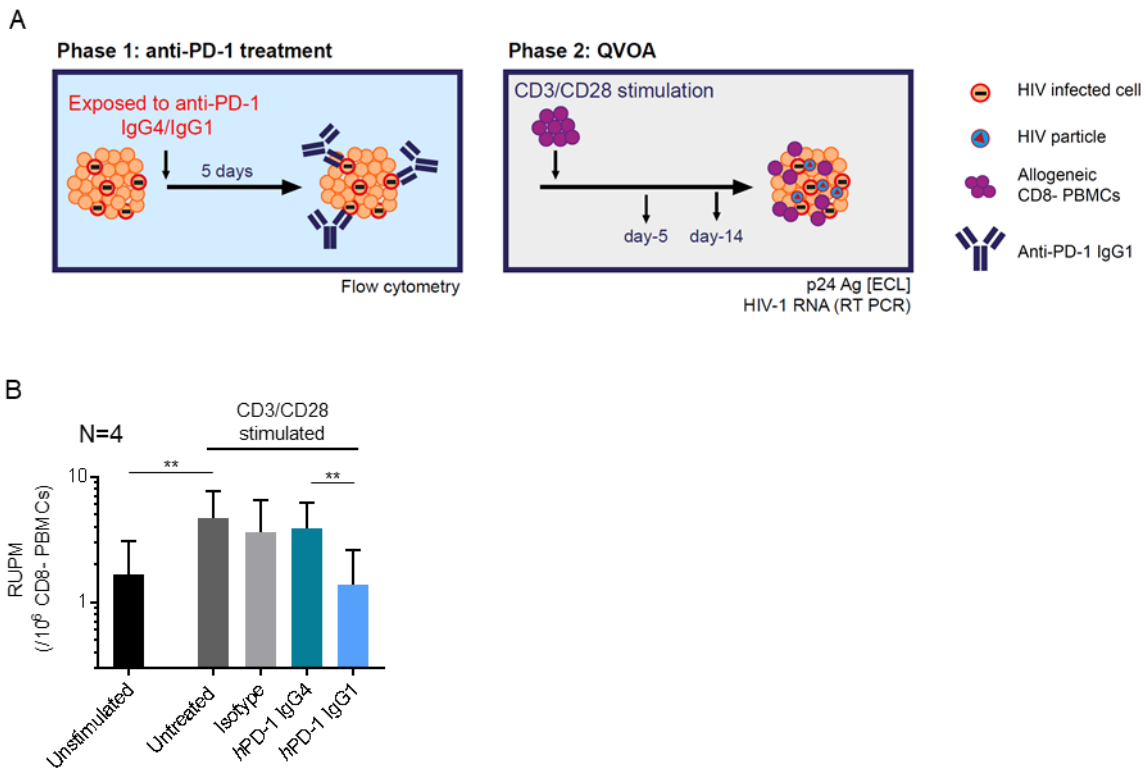


Figure 3: Impact of hPD-1 IgG1 treatment on HIV reservoir from ART treated donors. *(**A**) Schematic representation of the experimental design performed with cells from ART treated donors. (**B**) Mean frequencies of infected cells harboring replication competent (RUPM) viruses for the different treatment conditions. Frequencies were calculated using a maximum likelihood method (ELDA). (**P<0.01, Chi-squared 2-tailed analysis)*

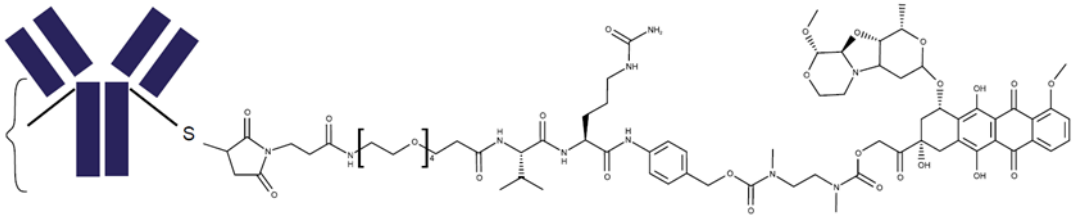


Figure 4: Schematic representation of an ADC. *Anti-PD-1 antibody is conjugated to PNU-159682 toxin.*

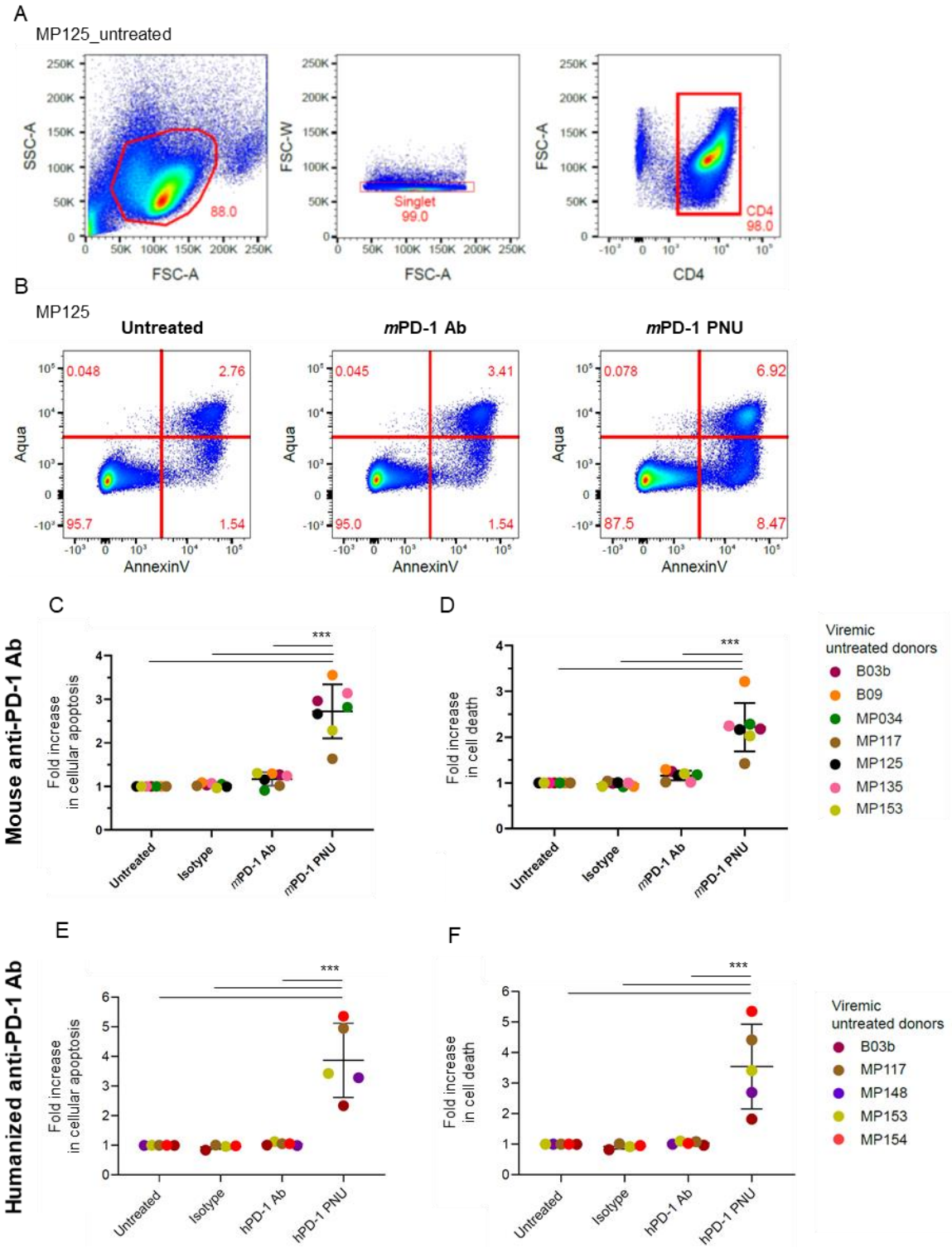


Figure 5: ADC targeting of isolated CD4⁺ T cells from viremic untreated HIV donors. *(**A**) Gating strategy. (**B**) Representative flow cytometry data for the evaluation of Annexin V and Aqua positive populations. Fold increase apoptotic cells (Annexin V⁺) (**C**) and dead cells (Aqua⁺) (**D**) compared to untreated condition on day 5 after 10 µg/ml mPD-1 antibody treatment. Fold increase apoptotic cells (**E**) and dead cells (**F**) compared to untreated condition on day 5 after 5 µg/ml hPD-1 antibody treatment. Means are represented and error bars indicate SD. (***P<0.001; t-test).*

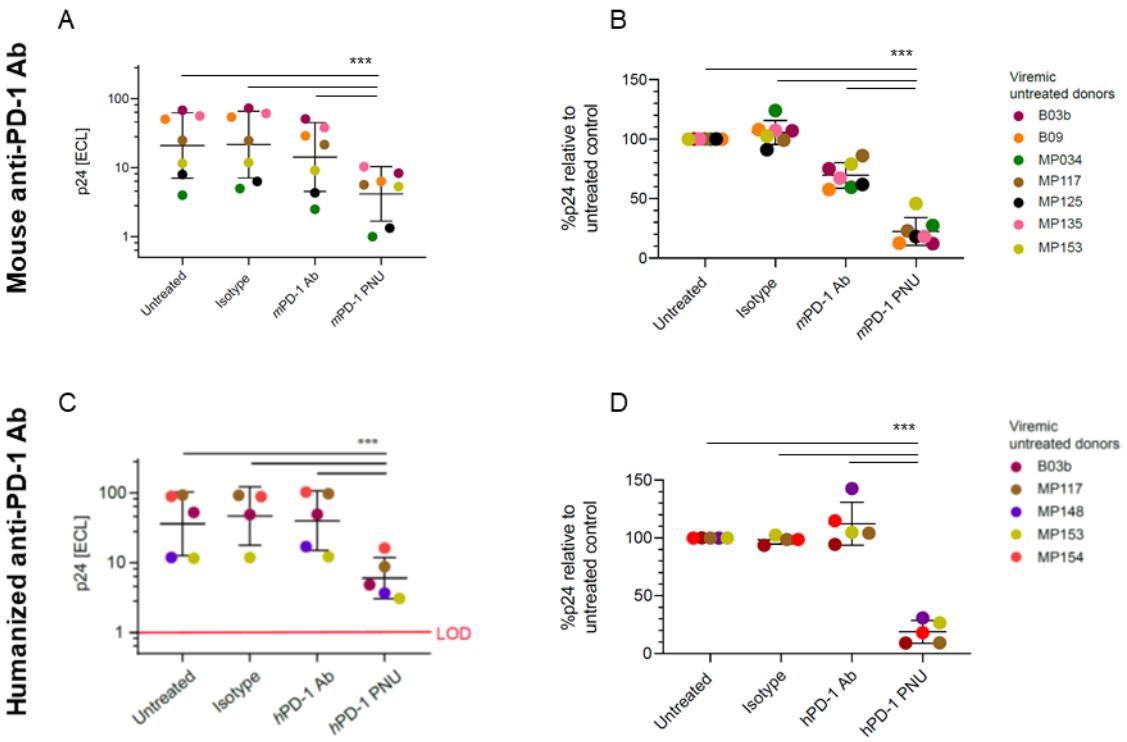


Figure 6: Impact of ADC treatment on viral p24 production measured on day 5. *(**A**) Production of p24 antigen detected after 10 µg/ml mPD-1 antibody treatment and (**B**) calculated percentage relative to untreated condition. (**C**) Production of p24 antigen detected after 5 µg/ml hPD-1 antibody treatment and (**D**) calculated percentage relative to untreated condition. (**A, C**) Geometric means are represented and error bars indicate geometric mean SD. (**B, D**) Means are represented and error bars indicate SD. (***P<0.001; t-test)*

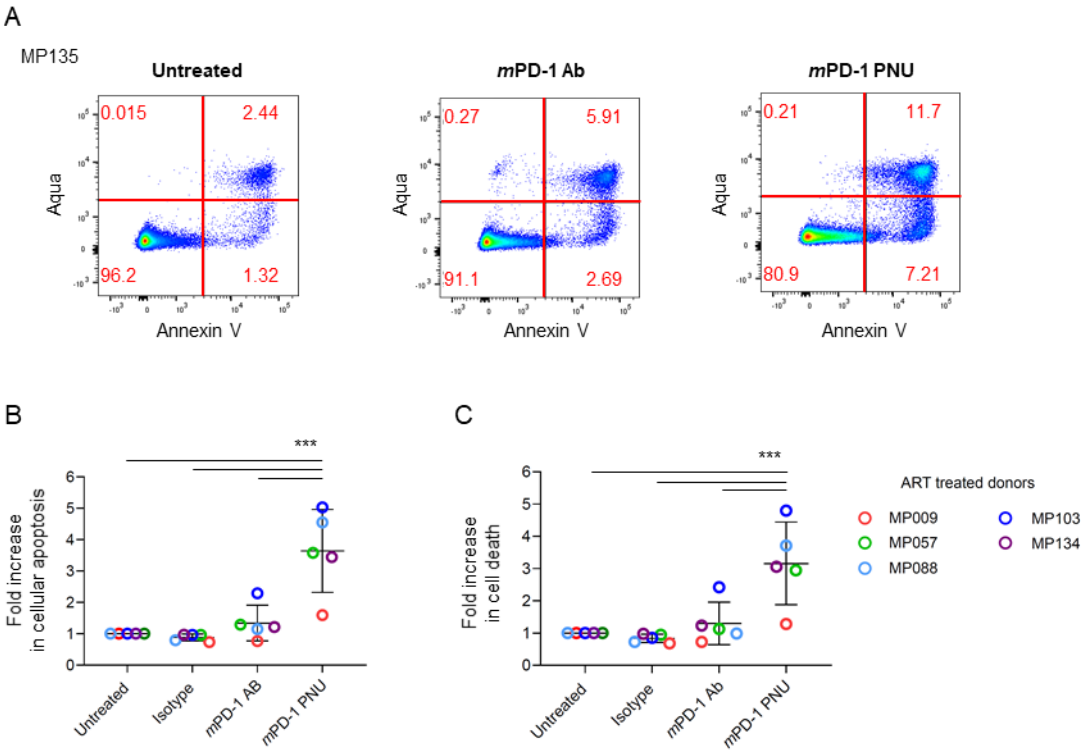


Figure 7: ADC targeting of isolated CD4⁺ T cells from ART treated HIV donors. *(**A**) Representative flow cytometry data for Annexin V and Aqua evaluation in different treatment conditions. Fold increase apoptotic cells (Annexin V⁺) (**B**) and dead cells (Aqua⁺) (**C**) compared to untreated condition on day 5 after 10 µg/ml mPD-1 antibody treatment. Means are represented and error bars indicate SD. (***P<0.001; t-test)*

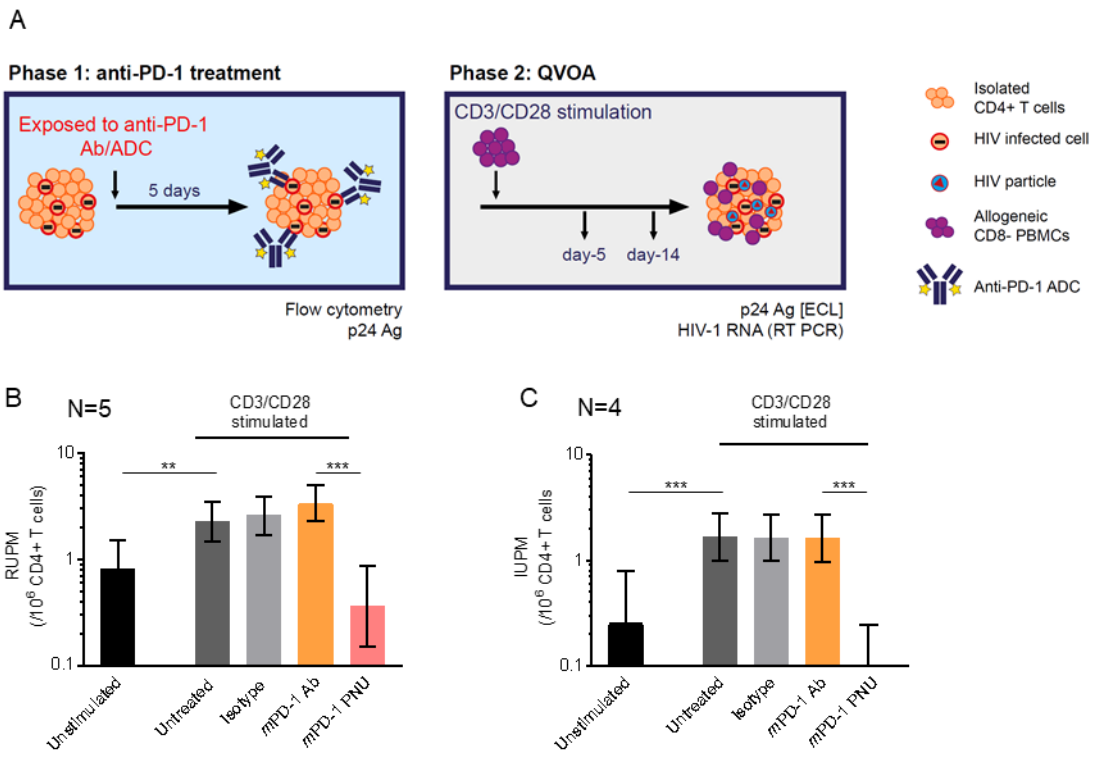
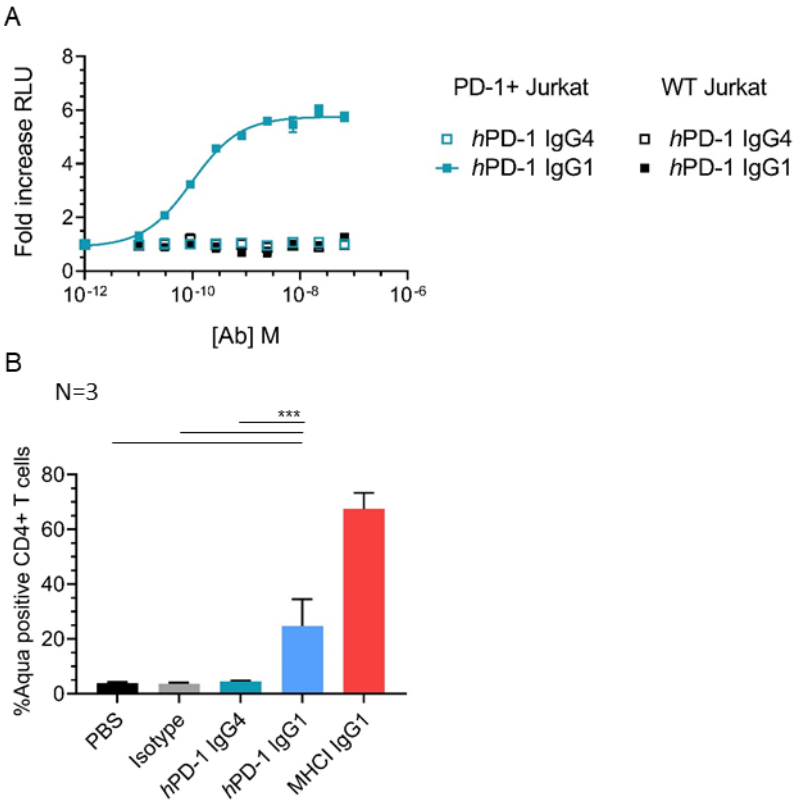
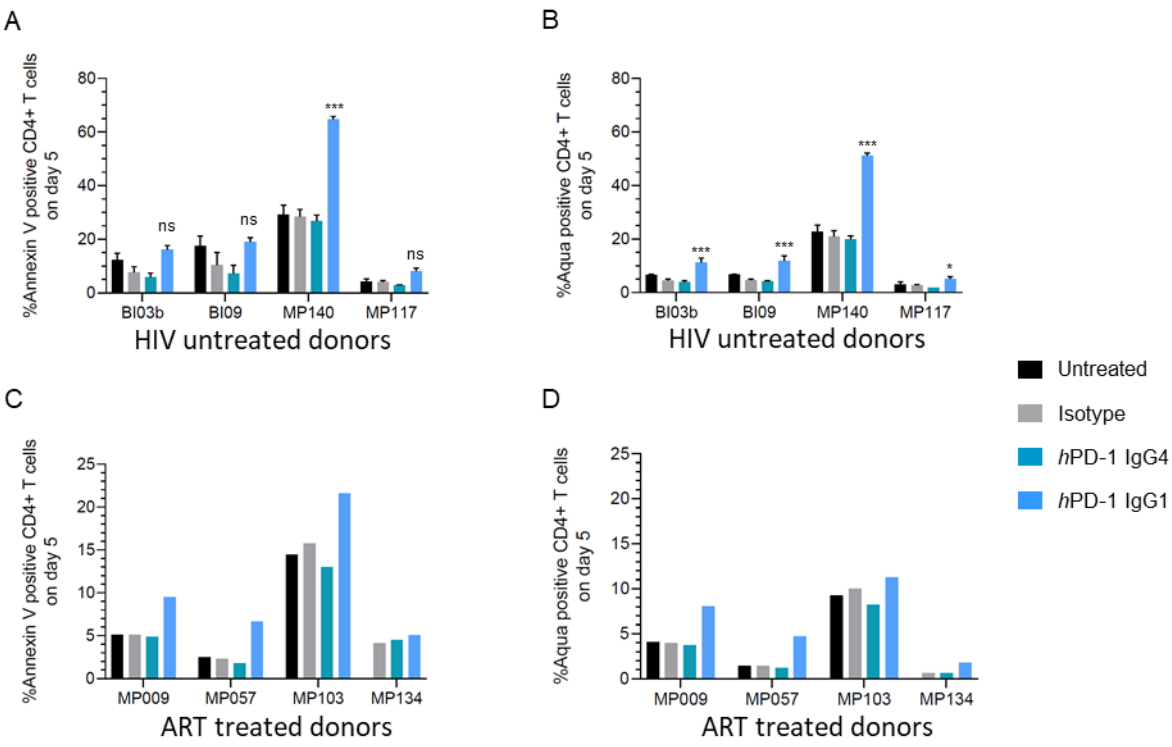


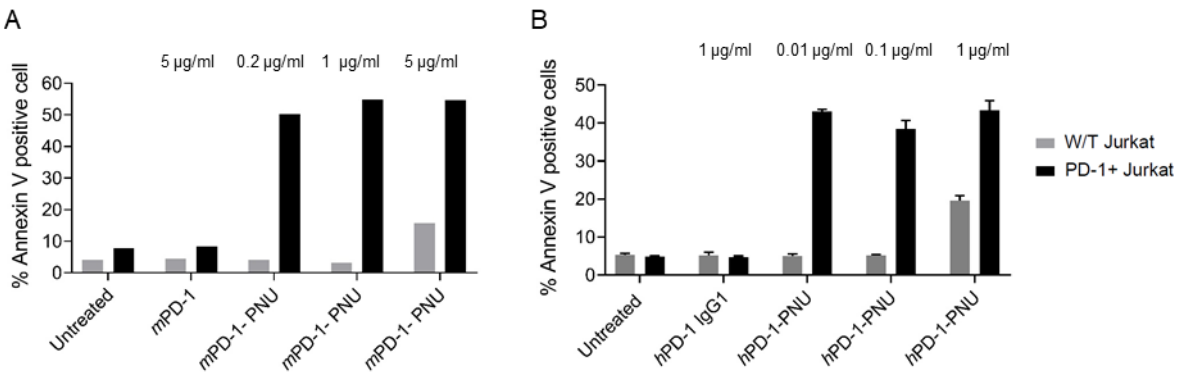
Figure 8: Impact mPD-1 antibody treatment on HIV reservoir. *(**A**) Schematic representation of the experimental design. (**B, C**) Calculated mean frequencies of infected cells harboring replication competent (RUPM) and infectious (IUPM) viruses for the different treatment conditions. Frequencies were calculated using a maximum likelihood method (ELDA). (**P<0.01; ***P<0.001; Chi-squared 2-tailed analysis)*



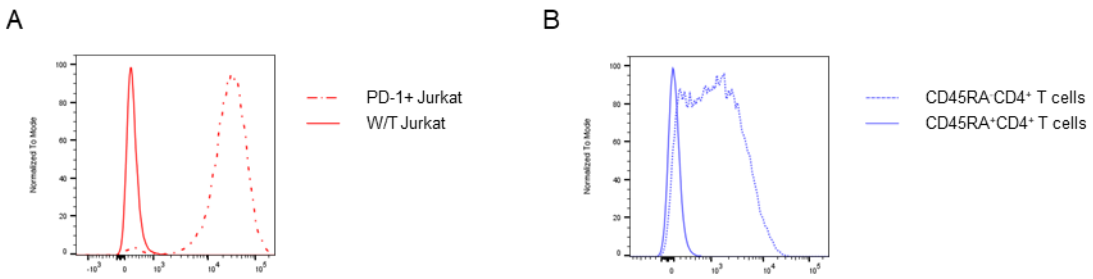
Supplementary Figure 1: Validation of hPD-1 IgG1-mediated ADCC activity. *(**A**) Evaluation of ADCC activity using a luciferase reporter assay. Effector cells were NFAT/FcγRIII Jurkat cells, and target cells were PD-1⁺ Jurkat cells. Mean of duplicates. (**B**) Effect of hPD-1 Ab treatment on isolated NK cells and CD4⁺ T cells from healthy donors in a co-culture assay. Mean of three biological replicates are represented and the error bars indicate SEM (***P<0.001, t-test).*



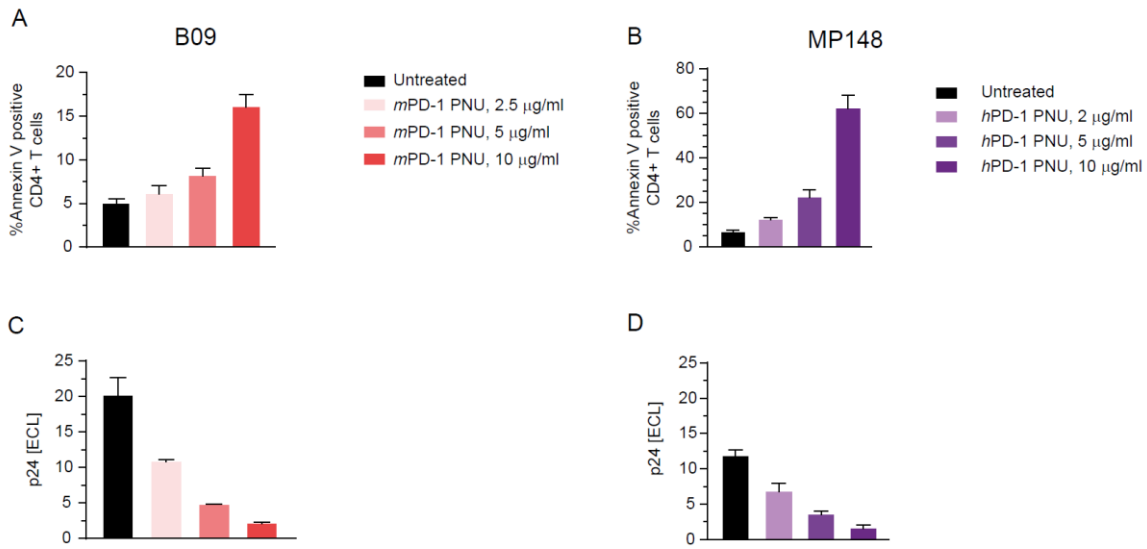
Supplementary Figure 2: ADCC targeting of isolated peripheral mononuclear cells from viremic untreated and ART treated HIV donors. *(A) Percentage Annexin V and (B) Aqua positive cells on day 5 after 5 µg/ml hPD-1 antibody treatment for each individual donors. Means of technical replicates are represented and error bars indicate SD. Significance was calculated for ADC treatment compared to the other conditions (nsP>0.05, *P<0.05, **P<0.01, ***P<0.001; t-test). (C) Percent Annexin V and (D) Aqua positive cells on day 5 after 5 µg/ml hPD-1 antibody treatment for each individual donors. (No replicate was performed).*



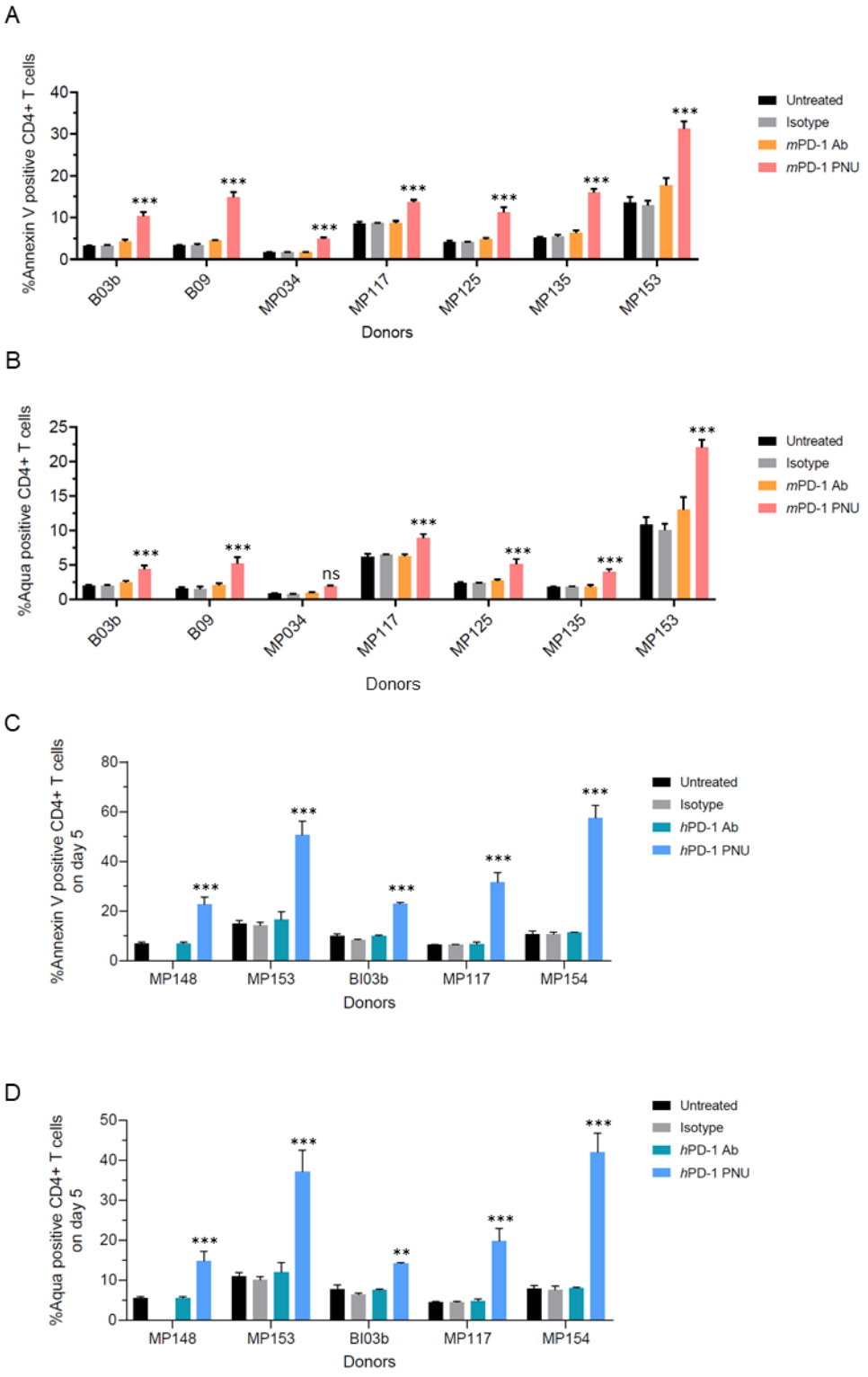
Supplementary Figure 3: Specificity and toxicity of anti-PD-1 ADC. *(**A**) Percent Annexin V positive cells on day 3 after mPD-1 ADC treatment. (**B**) Percent Annexin V positive cells on day 3 after hPD-1 ADC treatment.*



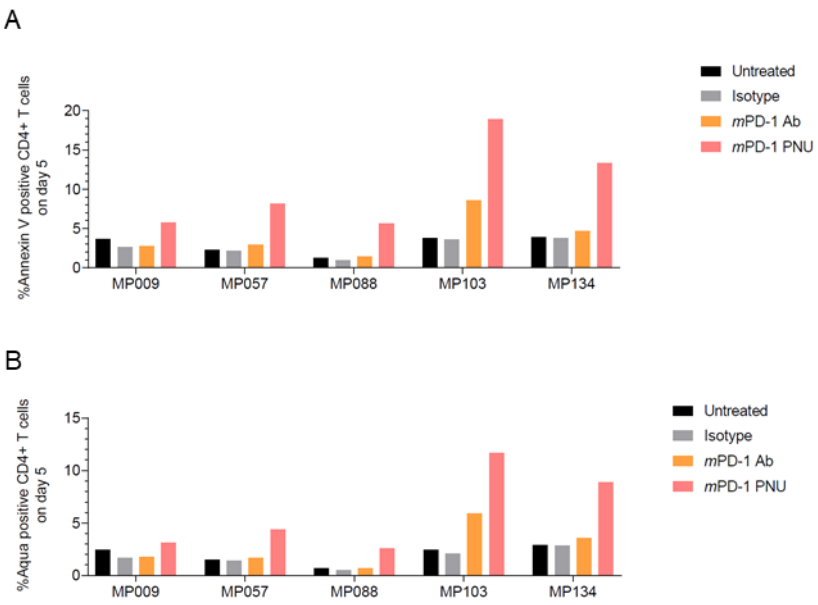
Supplementary Figure 4: PD-1 expression. *(**A**) PD-1 expression in W/T vs PD-1⁺ Jurkat cells and (**B**) in naive (CD45RA⁺) vs memory (CD45RA⁻) CD4⁺ T cells.*



Supplementary Figure 5: Anti-PD-1 ADC titration. *Percent Annexin V positive CD4⁺ T cells on day 5 after various concentrations of (**A**) mPD-1 PNU and (**B**) hPD-1 PNU ADC treatment. HIV p24 antigen produced in culture supernatants on day 5 after various concentrations of (**C**) mPD-1 PNU and (**D**) hPD-1 PNU ADC treatment. Means of technical triplicates are represented and error bars indicate SD.*



Supplementary Figure 6: ADC targeting of isolated CD4⁺ T cells from viremic untreated HIV donors. *Percent Annexin V (A) and Aqua (B) positive cells on day 5 after 10 µg/ml mPD-1 antibody treatment for each individual donors. Percent Annexin V (C) and Aqua (D) positive cells on day 5 after 5 µg/ml hPD-1 antibody treatment for each individual donors. Means of technical replicates are represented and error bars indicate SD. Significance was calculated for ADC treatment compared to the other conditions (nsP>0.05, *P<0.05, **P<0.01, ***P<0.001; t-test).*



Supplementary Figure 7: ADC targeting of isolated CD4⁺ T cells from ART treated HIV donors. *Percent Annexin V (A) and Aqua (B) positive cells on day 5 after 10 µg/ml mPD-1 antibody treatment for each individual donors. (No replicate was performed).*

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3.2 Paper II: T cell exhaustion in HIV infection

T cell exhaustion is a progressive condition that includes increasing loss of cell effector function and is associated with increased expression levels of immune checkpoint inhibitory molecules on the cell surface. The upregulation of immune checkpoint inhibitory molecules belongs to the normal process of cell stimulation and aims at dampening the activation signal to limit immune function after clearance of the pathogen. However, the persistence of antigen and chronic stimulation as in cancer or chronic infection leads to progressive T cell functional defects and suboptimal control of the pathogen. The purpose of this review is to summarize recent knowledge on T cell exhaustion and its implication in HIV infection. In particular, this review discuss the role of T cell exhaustion in HIV disease progression and the defect of T cell function that persists despite ART suppressed viremia. This review also discuss the role of exhaustion-specific markers in the maintenance of HIV latency and the implication for HIV persistence in cellular reservoir. Finally, suggestions about how to exploit these markers in HIV cure strategies are presented.

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INVITED REVIEW

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T-cell exhaustion in HIV infection

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Abstract

The T-cell response is central in the adaptive immune-mediated elimination of pathogen-infected and/or cancer cells. This activated T-cell response can inflict an overwhelming degree of damage to the targeted cells, which in most instances leads to the control and elimination of foreign invaders. However, in conditions of chronic infection, persistent exposure of T cells to high levels of antigen results in a severe T-cell dysfunctional state called exhaustion. T-cell exhaustion leads to a suboptimal immune-mediated control of multiple viral infections including the human immunodeficiency virus (HIV). In this review, we will discuss the role of T-cell exhaustion in HIV disease progression, the long-term defect of T-cell function even in aviremic patients on antiretroviral therapy (ART), the role of exhaustion-specific markers in maintaining a reservoir of latently infected cells, and exploiting these markers in HIV cure strategies.

KEYWORDS

chronic viral infection, HIV, immune checkpoint inhibitors, PD-1, T-cell exhaustion

1 | GENERAL INTRODUCTION ON T-CELL EXHAUSTION

The T-cell activation paradigm proceeds in a highly organized process involving three signals consisting of antigen recognition, receptor costimulation, and a termination signal that are required for the tight regulation of a strong functional and proliferative response.

Signal one in T-cell activation represents the specific recognition through the T-cell receptor (TCR) of their cognate antigen presented by professional antigen-presenting cells (APCs) including dendritic cells, macrophages, and B cells.

Signal two is the costimulatory signal where receptors on the T cells bind to their counterpart ligands. CD28 is the primary costimulatory receptor for T cells that acts through interaction with its ligands, CD80 and CD86 expressed on APCs. Costimulation is essential for T-cell activation since signal one TCR/antigen recognition in the absence of signal two drives the cell toward an anergic and/or tolerogenic state.[1] Additional costimulatory receptors that can enhance T-cell activation include CD27, OX40 (CD134), ICOS (CD278), CD40L (CD154), CD226 that bind to CD70, OX40L (CD252), B7-H2 (CD275), CD40, and CD155/CD112, respectively, on APCs.[2] The concentration of costimulatory and ligand molecules can vary significantly such that either no positive signal is sent or a paired interaction provides a strong supporting signal two to the T cells. Soluble pro-inflammatory cytokines including IL-12 and type I interferons contribute to a fully activated T-cell response.

Signal three arises in the days following T-cell activation, the effector phase of the immune response, and eventually the elimination of the pathogen. Signal three is responsible for terminating the immune response. Inhibitory receptors, also called immune checkpoint inhibitors (ICIs), exert their influence on T-cell activation at this stage where under normal conditions, ICIs including programmed cell death receptor 1 (PD-1), cytotoxic T lymphocyte antigen-4 (CTLA-4), LAG3 (lymphocyte activation gene protein), and TIM3 (T-cell immunoglobulin domain and mucin domain-containing protein 3) are transiently

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upregulated on the surface of effector T cells within hours to days following T-cell activation. Here, their purpose is to attenuate T-cell activation and limit immune function at the end stages of an acute infection when a pathogen is controlled. Upon clearance of antigen, the expression of these ICIs on memory T cells declines to normal levels over time. Termination of the T-cell response is an essential self-limiting mechanisms to help preserve self-tolerance. However, in the case of chronic infection or cancer when the immune response is incapable of clearing the foreign antigen and there persistent chronic stimulation, T cells can enter a dysfunctional state called exhaustion.

T-cell exhaustion is a progressive condition with increasing loss in effector function coinciding with increased expression levels and assortment of ICIs. PD-1 is recognized as the master inhibitory regulator of T-cell function but with increased degrees of exhaustion comes elevated levels of additional ICIs including CTLA-4, TIM3, LAG3, T-cell immunoreceptor with Ig and ITIM domains (TIGIT), 2B4 (CD244), and CD160[3,4] (Figure 1). T-cell exhaustion mediated through PD-1 relies on interaction with its cognate ligands, PDL-1 and PD-L2, which regulate the delicate balance between immune defense and the protection of healthy tissue. Immune cells, non-immune endothelial, and epithelial cells constitutively express PDL-1, while PDL-2 expression is limited to APCs. The expression of PDL-1 is further upregulated after activation, which modulates the immune responses against self and foreign antigens.[5] In a likewise fashion, the interaction of additional ICIs with their respective ligands exert a further level of control and suppression of T-cell function.

2 | FUNCTIONAL PROFILE

In exhausted CD8 T cells, the increased expression of ICIs leads to a hierarchical loss of function that begins with reduced IL-2 secretion, cytokine polyfunctionality, and diminished proliferative potential. Functional defects progress to the loss in cytotoxic capacity and TNF-α secretion with highly exhausted cells having reduced ability to produce IFN-γ.[6-8] Although T-cell exhaustion was first identified in CD8 T cells, it is now accepted that CD4 T cells are also subject to exhaustion leading to reduced production of IL-2, IFN-γ, and TNF-α[9-11] along with reduced CD4 T-cell help.[12] In addition to persistent antigen and pathogen burden, the loss or reduction of CD4 T-cell help is an important factor that contributes to the initial establishment of exhaustion.[13-15] The precise nature of optimal CD4 T-cell help needed to antagonize exhaustion is unclear, but is likely to involve the production of IL-2 and IL-21 cytokines that support CD8 T-cell response both directly and indirectly through activation of APCs.[16]

Aside from a blunted immune response against infected or cancerous cells, exhausted T cells have a poor or varied response to homeostatic cytokines including IL-7 and IL-15 responsible for the maintenance of memory T cells. In the case of complete clearance of a viral pathogen or in adoptive transfer studies to antigen-free mice, exhausted T cells are poorly maintained through self-renewal.[17-19] Instead, persistence of exhausted T cells occurs through continual antigen signals that promotes proliferation.[20] Exhausted T cells can persist in vivo for years but in the final stages of exhaustion with high antigen stimulation, there is a loss of the virus or tumor-specific cells through apoptosis.[7,21] Further characteristics of T-cell exhaustion include altered transcription factor expression, metabolic profile and epigenetic modifications that are distinct from other memory T-cell subsets.

3 | TRANSCRIPTIONAL PROFILE

The transcriptional profile of exhausted CD4 and CD8 T cells are significantly different from all memory T-cell subsets. Although several transcriptional factors are correlated with exhausted T

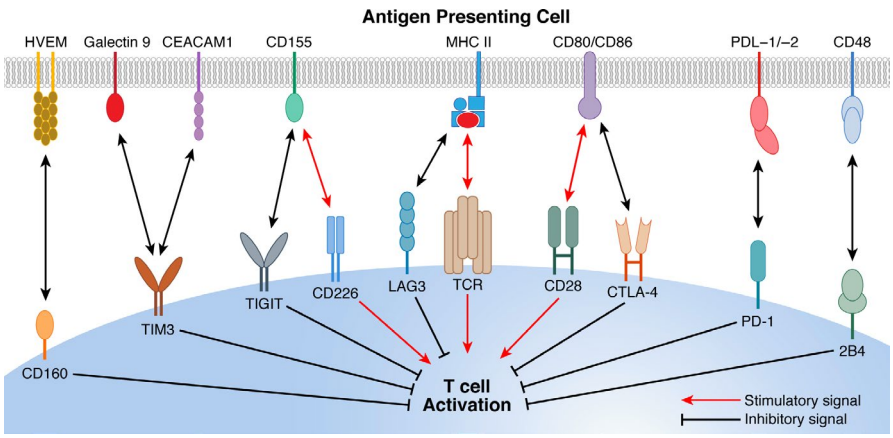

FIGURE 1 T-cell immune checkpoint inhibitors and related ligands. TCR interaction with the antigenic peptide-MHC complex displayed by professional APCs delivers the primary signal for T-cell activation. The CD28 co-receptor and other costimulatory receptors enhance the T-cell stimulatory signal following interaction with their corresponding ligands. Immune checkpoint inhibitors including PD-1, CTLA-4, LAG3, TIGIT, TIM3, CD160, and 2B4 act to suppress T-cell signal following interaction with their related ligands expressed on APCs

cells including NFAT, Batf, IRF-4, T-bet, Eomes, and Blimp-1, no master regulator of exhaustion has been identified. NFAT activation during chronic infection along with a corresponding low nuclear translocation of AP-1 induces strong transcriptional activation of ICIs including PD-1, LAG3, and Tim3.[22-25] Signaling through the PD-1 receptor induces the upregulation of the Batf transcription factor, which in turn inhibits AP-1 activation and contributes to the sustained high levels of PD-1. Indeed, exhausted CD8 T cells from chronically infected HIV donors have elevated levels of Batf with higher levels observed in patients that experience disease progression compared to those that spontaneously controlled their HIV viral loads.[24] In the chronic lymphocytic choriomeningitis virus (LCMV) mouse infection model, IRF-4 contributes to T-cell exhaustion in consort with Batf and NFAT and reduction of IRF-4 expression restores the functional properties of exhausted antigen-specific T cells.[26] T-bet and Eomes transcription factors are individually important for the development of KLRG-1+ terminal effector CD8 T cells in response to inflammation and the maintenance of memory cells, respectively. In contrast, both are essential for the development of exhaustion as demonstrated in studies involving the genetic deletion of either[20] and both are upregulated in exhausted T cells with different viral infections.[27] Although necessary for exhaustion, high levels of the T-bet directly repress the transcription of the PD-1 gene. It is therefore interesting that in combined expression with Eomes, the T-bet^{high} Eomes^{dim} population represent an intermediate exhausted subset with progenitor capacity and a sustained virus-specific CD8 T-cell response during chronic infection.[28,29] In HIV-infected donors, exhausted T cells with a skewed balance toward T-bet^{dim} Eomes^{high} expressional profile represent a highly functionally exhausted state with elevated levels of multiple ICIs including PD-1, CD160, and 2B4 on CD8 T cells.[30-32] Blimp-1 is a primary transcription factor for the differentiation of germinal center B cells; however, it is also upregulated in exhausted T cells and correlates with the protein expression levels of ICIs. Similar to Batf, Blimp-1 is upregulated in progressor patients with chronic HIV infection compared to non-progressors that control HIV viral loads.[33] Blimp-1 expression in CD4 T cells mediates the production of IL-10, which can further contribute to the dysfunctional state of exhausted T cells during chronic viral infection.[34] Exhausted T cells may coexpression pairs of transcription factors including Blimp-1 and Eomes, however, expression is not uniform where combinations of different factors may coexist. Therefore, along with the phenotypic and functional profiles, exhausted T cells consist of a heterogeneous population with varied expression of transcription factors.[12,22,35] This diversity of exhausted T-cell populations is exemplified in a recent study using high dimensional mass cytometry to analyze cells from chronic HIV-infected donors and in human tumors. Using phenotypic, functional, transcription factor, and ICI coexpression patterns, nine distinct exhausted CD8 T-clustered cell populations were identified.[36]

4 | METABOLIC ABNORMALITIES AND EXHAUSTION

The metabolic profile of T cells shifts from the use of the oxidative phosphorylation pathway in naive cells to glycolysis in the acute phase of an infection when activated effector cells have increased bioenergetics needs. Once the infection is cleared, memory cells revert to a quiescent state that uses oxidative phosphorylation and gains the additional metabolic ability for fatty acid oxidation.[37] With chronic antigen stimulation and the onset of exhaustion, the suppression of the glycolysis pathway ensues with reduced cellular glucose uptake and signs of a dysregulated mitochondrial function.[38] The utilization of endogenous fatty acids by exhausted cells may dictate the available energy reserves under conditions of ICIs engagement.[39] Metabolic pathways implicated in this defective state include transcriptions control through Foxo1[40] and PGC1α.[41]

5 | EPIGENETICS AND EXHAUSTION

Epigenetic modifications associated with T-cell exhaustion begins within the first 2-3 weeks of chronic LCMV infection. This programming is irreversible, even in adoptively transferred studies with uninfected antigen-free mice where T cells maintain their exhausted transcriptional and functional profile.[12,27,42] The epigenetic landscape of exhaustion has been studied by comparing CD8 T cells during acute and chronic phases of LCMV infection. Changes consisted in large reorganizations of chromatin-accessible regions resulting in altered access to transcriptional start sites. These modifications were associated with the induction of multiple genes including Pdcd1 (PD-1), Havcr2 (Tim3), and Batf which are known to be upregulated in exhausted CD8 T cells. The epigenetic modifications in Pdcd1 include histone acetylation in the promoter and proximal enhancer region followed by full demethylation in these regions that is maintained in exhausted cells and allows for consistent high PD-1 expression. These epigenetic changes are also present in several chronic viral infections including HIV, CMV, and EBV.[43] Indeed, the vast majority of this epigenetic program linked to exhaustion in mice (approximately 80%) was also successfully mapped to tetramer positive CD8 T cells of treatment naive chronically infected HIV donors.[44-46] As such, epigenetic modifications play a key role in maintaining T cells in an exhausted state.

6 | T-CELL EXHAUSTION IN HIV INFECTION (CAUSES OF EXHAUSTION, PHENOTYPIC MARKERS OF EXHAUSTION, AND FUNCTIONAL EXHAUSTION)

T-cell exhaustion was first described in mouse models with chronic LCMV infection where antigen-specific CD8 T cells progressively lost their effector functions and developed a reduced capacity to kill virally

infected cells.[47-50] Subsequent to these studies, it became clear that the same principles of T-cell exhaustion occurred in human chronic viral infections including HIV, HCV, HBV, and HTLV-1 as well as cancer.

Chronic HIV infection occurs in most patients in the presence of persistent high levels of virus replication and is associated with a loss of immune control of virus replication. Virus-specific CD8 T cells partially suppress HIV viral replication in the initial stages of infection. In a similar manner, SIV-infected macaque studies showed that CD8 T-cell depletion leads to a dramatic increase in viral load.[51] However, with persistent high levels of viral antigen, HIV-specific T cells become exhausted and lose their capacity to kill efficiently infected cells. In addition to high levels of viral antigen, the strong pro-inflammatory immune activation during HIV infection and a compromised T-cell homeostasis during HIV infection contribute to the development of T-cell exhaustion.[52,53]

In HIV-1 infection, PD-1 expression on virus-specific T cells is the primary marker of exhaustion that correlates with disease progression. Pivotal studies showed that PD-1 expression correlated with impairment of CD8 T cells functionality, viral load, and reduced the CD4 T-cell counts.[54-56] Importantly, cytomegalovirus-specific CD8 T cells from the same HIV-infected donors did not upregulate PD-1 and preserved the functional capacity to produced high levels of cytokines. This demonstrated that the T-cell defects were HIV-specific and driven primarily by the high level of antigen that induced exhaustion. Longitudinal studies show that following ART initiation, PD-1 expression levels gradually decrease on HIV-specific CD8 T cells. Long-term non-progressors (LTNPs) or viremic controllers were also evaluated and show lower levels of PD-1 expression on HIV-specific T cells that exhibited a stronger effector functions as compared to progressors[54,55] (Figure 2). Aside from PD-1, other ICIs including LAG3, Tim3, TIGIT, 2B4, and CD160 expressed independently or combined lead to more

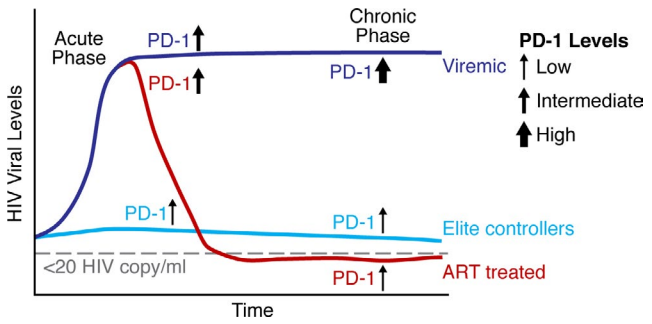

FIGURE 2 HIV Infection and progression of exhaustion. During the acute phase of HIV infection, the majority of individuals experience a dramatic increase in HIV viral load and increased levels of the PD-1 receptor on HIV-specific T cells. In contrast, elite controllers maintain low viral load levels and consequently, T cells express low levels of PD-1. Untreated patients with high viral load progress to the chronic phase of HIV infection where persistent elevated levels of HIV antigen result in T-cell exhaustion with high expression levels of PD-1. ART in most patients significantly inhibits viral replication, resulting in decreased plasma viral load to levels below the limit of detection with standard assays. With low levels of viral antigen present, PD-1 expression decreases to lower levels on HIV-specific T cells

pronounced stages of CD8 T-cell exhaustion.[54,57-62] Coexpression of PD-1 with TIGIT was shown to correlate with disease progression in both HIV-infected patients and SIV infection model.[58] PD-1 and LAG3 expression either alone or in combination with CD38 expression also correlates with the with plasma viral load of patients and was predictive of the time to disease progression.[63] Similarly, simultaneous expression of PD-1, CD160, 2B4, and LAG3 on CD8 T-cell populations correlated directly with HIV load and inversely with the multiplicity of functional outputs exhibited by HIV-specific CD8 T cells[64] (Figure 3).

The progressive loss of CD8 T-cell function starts with an initial loss of proliferative capacity, cytotoxic potential, and a restricted IL-2 production. In more pronounced stages of exhaustion associated with chronic exposure of T cells to viral antigen, T cells eventually lose the ability to produce IFN-γ.[55,56,65,66] HIV-specific CD8 T cells with elevated levels of PD-1 also show greater susceptible to apoptosis that was attributed to lower levels of the pro-survival Bcl-2 and higher levels of CD95/Fas surface receptor compared to the PD-1 low T cells.[67]

The focus of T-cell exhaustion is often on the loss of CD8 T cells function that is primarily responsible for the killing infected cells. However, CD4 helper T cells also exhibit functional defects during HIV infection. Exhausted CD4 T cells exhibit a reduced HIV-specific proliferative capacity and a loss in polyfunctional cytokine response that centers on reduced IL-2 production.[68,69] Exhausted virus-specific CD4 T cells also express PD-1 with elevated levels correlating with disease progression, viral loads and reduced CD4 T-cell count.[70] As such, PD-1 is a common regulator of exhaustion on HIV-specific CD4 and CD8 T cells. In contrast, the CTLA-4 ICI is more selectively upregulated on exhausted CD4 T cells that correlates with disease progression and T-cell dysfunction.[71] Conversely, the ICIs 2B4 and CD160 that are characteristically upregulated on exhausted CD8 T cells are virtually absent from exhausted CD4 T cells.[72] As with CD8 T cells, expression of multiple ICIs including PD-1, CTLA-4, and TIM3 is associated with a more pronounced state of functional CD4 T-cell exhaustion.[73] A characteristic feature of CD4 and CD8 exhausted T cells is both experience at least partial restoration of antigen-specific proliferative and functional activity following antibody-mediated ICI blockade therapy.

Aside from increased levels of ICIs during HIV infection, an activated T-cell phenotype with upregulated levels of CD38 on CD8 and CD4 T cells is a well-established predictive marker for disease progression.[74] Additional activation markers upregulated during HIV infection include CD38/HLA-DR coexpression on CD8 T cells and signs of ongoing replication as determined by Ki-67+ T cells.[75] T cells also exhibit impaired T-cell maturation characterized by reduced expression of CD28 and high levels of CD27 costimulatory molecules, suggesting a decreased effector phenotype.[76] Decreased levels of CD28 on HIV-specific CD8 T cells are associated with shorter telomere lengths and reduced proliferation.[77] HIV-specific CD8 T cells with elevated levels of CD27 also have reduced Granzyme A and perforin cytotoxic activity compared to CD27 low effector T cells.[78] Moreover, HIV-specific memory CD8 T cells were also found to have a preterminally differentiated phenotype (CD45RA-CCR7-), when compared to CMV-specific cells that instead expressed a terminally differentiated (CD45RA + CCR7-) phenotype.[79,80]

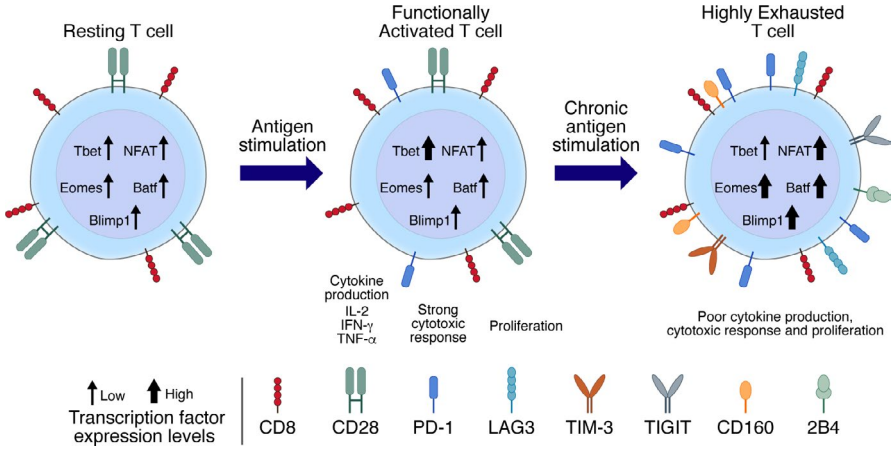

FIGURE 3 Phenotype, transcriptional, and functional profile of T cells progressing to exhaustion. Antigen-specific stimulation of resting T cells leads to a functionally active T-cell response with increased cell surface PD-1, upregulated expression of the T-bet transcription factor and a strong functional and proliferative response. Chronic stimulation with high levels of antigen drive T cells into an exhausted state characterized by high levels of PD-1, an increased expression of additional immune checkpoint inhibitors and a pronounced T-cell dysfunction. Compared to functionally active T cells, highly exhausted T cells have elevated expression of transcription factors including NFAT, Batf, Eomes, and Blimp-1 with decreased levels of T-bet

Cytokines including IL-10 are also implicated in T-cell exhaustion during HIV infection. IL-10 production is part of the body's response to chronic inflammation established primarily through strong upregulation of the type I and II IFN-related genes and pathways. Tregs accumulate at the sites of chronic HIV infection and play a direct role in the promotion of T-cell exhaustion through production of IL-10 that inhibits T-cell proliferation.[81] Aside from Tregs, multiple cell types contribute to IL-10 production and patients with elevated plasma levels of IL-10 correlate with rapid disease progression and impaired CD4 T-cell help. In vitro blockade of IL-10 increases proliferation of HIV-specific CD4 and CD8 T cells and increases production of cytokines by CD4 T cells.[82,83]

Overall, HIV-specific T-cell exhaustion established in the early stages of infection represents an almost insurmountable barrier to the immune mediate control of viral load and the elimination of HIV-infected cells. Individuals who can spontaneously control HIV infection are rare, representing ≤1% of those infected.[84] However, a recent longitudinal study provides evidence that T-cell exhaustion plays an important role in the loss of viral control by these rare HIV controllers. These studies showed that just prior to increases in patient viremia, PD-1 levels increased on HIV-specific CD8 T cells and these cells exhibited reduced in vitro capacity to kill HIV-infected cells.[85] As such, a delicate balance may exist between T-cell–mediated control of viral infection and the progressive development of T-cell exhaustion.

7 | T-CELL EXHAUSTION IN OTHER VIRAL INFECTIONS

Pathogens have evolve through natural selection to evade immune-mediated elimination by exploiting the IC pathways needed by the host to maintain peripheral tolerance and limits immunopathology under physiologic conditions. A key advantage in the discovering T-cell exhaustion using the LCMV mouse infection model was the different viral laboratory strains and/or different viral inoculum that induce either an acute infection that could be resolved or a persistent chronic infection.[47-49] Although CD8 T-cell dysfunction was known to be an essential feature of exhaustion, the identification of PD-1 as a key player during chronic infection was revealed much later through gene expression analysis. These studies showed that in the early stage of infection, PD-1 was upregulated to similar levels in mice infected with either Armstrong (acute) or clone 13 (chronic) LCMV viruses. Clearance of Armstrong strain lead to a rapid downregulation of PD-1 on virus-specific CD8 T cells. However, in chronic clone 13 strain LCMV infection, virus-specific CD8 T cells showed sustained increase PD-1 levels that progressively lost their functionality. In this model, transient depletion of CD4 T cells has no effect on an acute viral strain that can be resolved within 2 weeks. However, in studies using a chronic LCMV variants that requires >3 months to be contained and cleared by the LCMV-specific CD8 T-cell response, even transient depletion of CD4 T cells at the time of infection resulted in a complete loss of CD8 T-cell response to control the virus.[14] These studies provide support for the concept that loss of CD4 T-cell help can enhance conditions that lead to CD8 T-cell exhaustion. An important concept validated in the LCMV model was that antibody-mediated blockade of the PD-1/PDL-1 interaction reversed signature featured of T-cell exhaustion that allowed for proliferation and increased functionality of LCMV-specific T cells that lead to the killing of infected cells and decrease viral load.

Human cases of viral hepatitis have common features with LCMV infection where virus is either cleared by the immune system or leads to chronic infection in 10% and 70% of HBV- and HCV-infected patients, respectively. The important role of virus-specific CD8 T cells was demonstrated in non-human primate models for

HBV and HCV infection where depletion of CD8 cells lead to a prolonged viremia that only declined when CD8 T cells returned.[86,87] The two primary mechanism for chronic hepatitis infection in patients is viral mutation that leads to escape from antiviral CD8 T cells and through exhaustion where CD8 T cells lose their effector function. A strong and early CD4 T-cell helper response in HCV infection is also associated with viral clearance in patients. In contrast, development of HCV-specific CD4 T cells with limited proliferative potential at an early stage following infection resulted in the evolution of a chronic infection.[88] Studies performed with blood mononuclear cells from chronically infected HBV patients showed that blockade of the PD-1 pathway resulted in enhanced proliferation and functionality of HBV-specific CD8+ T cells.[10,89] Furthermore, a HBV-related chronic hepadnaviral infection model in woodchucks showed that PD-1 blockade in combination with an antiviral agent and therapeutic DNA vaccination restored virus-specific CD8+ T cells functionality and enhanced a continual immune-mediated viral control.[90] PD-1 blockade alone had a moderate therapeutic result in patients with chronic HCV infection where 20% of those treated showed a significant drop in viral load.[91] However, this result may be due to the nature of exhaustion associated with HCV since in vitro studies showed that hepatic PD-1 + CTLA-4 + virus-specific CD8 + T cells where only functionally restored with a combination of CTLA-4 and PD-1 blockade and not with either therapy administered alone.[92]

Human T-cell lymphotropic/leukemia virus type 1 (HTLV-1) causes adult T-cell leukemia/lymphoma and in both asymptomatic carriers and ATL patients there is an inverse correlation between HTLV proviral load and the functionality of CD8 T cells in response to viral antigens. Decreased functionality correlated directly with PD-1 levels expressed on CD8 T cells and blockade through PDL-1 significantly increased anti-HTLV functionality. Of note, CD8 T-cell functionality and levels of HTLV-1 provirus did not correlate with expression levels of TIM3, LAG3, or CTLA-4, indicating that different chronic viral infections or the extent of exhaustion may favor the upregulation of different ICI subsets.[93,94]

8 | MAJOR SIGNALING PATHWAYS INVOLVED IN T-CELL EXHAUSTION

T-cell activation takes place in a highly organized process where the antigenic peptide-MHC complex on APCs binds to its cognate T-cell TCR complex forming the core of the cell-cell interaction. This primary contact sets into motion the reorganization of cell surface and cytosolic molecules leading to the formation of the immunological synapse. It is during this contact with the APC that ICIs exert their inhibitory effect to suppress signaling events needed for a fully activated T-cell response.

Following immunological synapse formation, Lck phosphorylation of TCR-associated CD3 ζ-chains initiates the intracellular signaling cascades with ZAP-70 propagating the TCR signal through phosphorylation of downstream intermediaries including PLC-γ1.[95,96] T-cell costimulation through CD28 is needed for robust, functional T-cell response and acts through phosphatidylinositol 3-kinase (PI3K) to activate the PI3K/AKT/mTOR signaling network. Both the TCR and CD28 costimulatory pathways converge to upregulate the activation of AP1, NFAT, and NFκB, which induce the transcription of T-cell immune response genes such as IL-2, IRF-4, and pro-survival factors.[97] The strength, affinity, and duration of the initial TCR-peptide-MHC complex as well as the presence of costimulatory receptors dictate the level of response from the different signaling cascades and degree of their contributions on transcription factor activation.

ICIs exert their regulatory effect on T cells through a variety of mechanism. Several receptors including PD-1 have intracellular domains containing immunotyrosine inhibitory motif (ITIM) and/or immunotyrosine switch motif (ITSM). These ITIM or ITSM motifs can recruit SHP tyrosine phosphatase proteins and other adapter into the vicinity of the TCR in order to disrupt positive signaling events. ICIs that suppress T-cell activation through the ITIM/ITSM mechanism includes PD-1, 2B4, LAIR-1, and KLRG-1.[98] A second mechanism of T-cell regulation through inhibitors receptors is by direct competition with costimulatory receptors. CTLA-4 is the best studies example that can bind tightly to the CD80 and CD86 on APCs. In so doing, CTLA-4 outcompetes CD28 for interaction with its natural ligands and increases the T-cell activation threshold, especially for antigens that do not effectively induce T-cell activation.[99] ICIs including LAG3 and TIM3 have varied intracellular domains distinct from the classical ITIM/ITSM motifs and that recruit a different subset of molecules that are suppressive of T-cell signaling. Finally, negative regulation can occur via receptors including TIGIT and BTLA that operate through a combination of different mechanism. TIGIT not only possess an intracellular ITIM motif on its cytoplasmic tail but also acts through a receptor competition mechanism in binding the CD155 and CD112 ligands on APCs that can activate T cells through interaction with CD226.[98]

PD-1–mediated inhibition of T-cell signaling is the best-studied ICI pathway involved in T-cell exhaustion. During T-cell activation, the PD-1 receptor migrates into T-cell/APC contact region where it forms microclusters upon binding with PDL-1. This recruitment brings the SHP2 and SHP1 phosphatases into membrane proximal region of the TCR and costimulatory receptors, inducing dephosphorylation of TCR-linked signaling proteins CD3ζ, ZAP-70, PLC-γ1,[95,96] and the CD28 pathway involving PI3K and the associated downstream cascade through the AKT pathway. The PD-1/SHP2 complex attenuates phosphorylation events associated with PKCθ, PI3K/AKT/mTOR, and Ras/MAPK/Erk signaling pathways needed for optimal T-cell activation, proliferation, survival, and an altered cellular metabolism.[100,101] The dynamics of PD-1 with CD28 and intracellular partners has been observed in fluorescence microscopy imaging studies where PD-1 exists in microclusters on the cell surface and is recruited along with SHP2 phosphatase into the immunological synapse to suppress phosphorylation events during TCR activation.[3,95,96,102-104] One caveat with many of these mechanistic studies is that PD-1 expression on T cells was achieved through

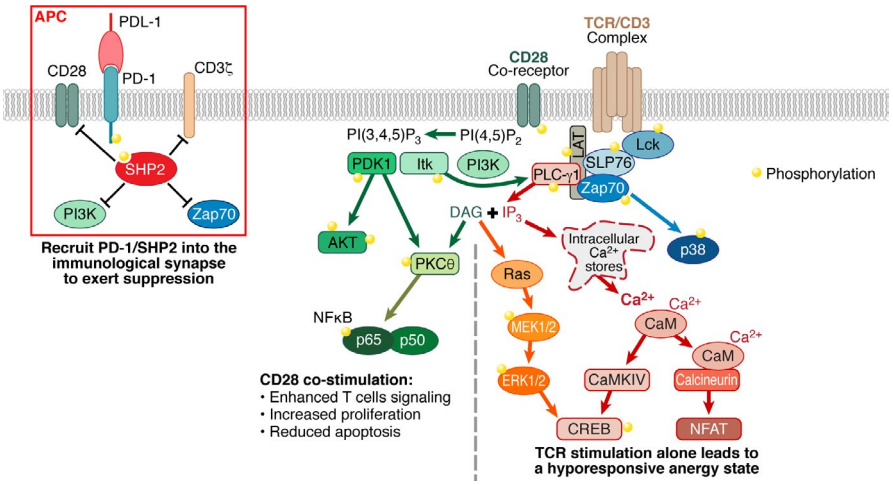

FIGURE 4 Signaling in T-cell activation and PD-1–mediated suppression. T-cell activation through the TCR/CD3 complex leads to the phosphorylation of proximal signaling molecules that trigger downstream activation of NFAT, CREB, and p38 pathways. In the absence of a costimulatory signaling through CD28, TCR activation of T cells leads to a hyporesponsive, anergic state. Signaling through the CD28 costimulatory receptor enhances activation of the PI3K/AKT/NFκB pathways that support T-cell proliferation and reduce apoptosis. Interaction of PD-1 with PDL-1 during T-cell activation recruits the PD-1/SHP2 tyrosine phosphatase complex into the vicinity of TCR/CD3 and the CD28 co-receptor. This recruitment results in the dephosphorylation of membrane proximal PI3K, ZAP-70 and the intracellular domains of CD28 and the CD3ζ chain, which suppresses T-cell activation. In PD-1 high exhausted T cells from chronically infected HIV donors, the ligation of PD-1 with PDL-1 results in reduced phosphorylation of TCR proximal Lck and Zap-70 and downstream Erk1/2 in the Ras-MEK1/2-Erk1/2 pathway. PD-1 suppression of T-cell activation also results in reduced calcium mobilization that is upstream of CREB and NFAT transcription factors. In the CD28 costimulatory receptor pathway, ligation of PD-1 with PDL-1 significantly inhibits the PI3K/AKT/NFκB pathway with reduced phosphorylation of PDK1 and AKT

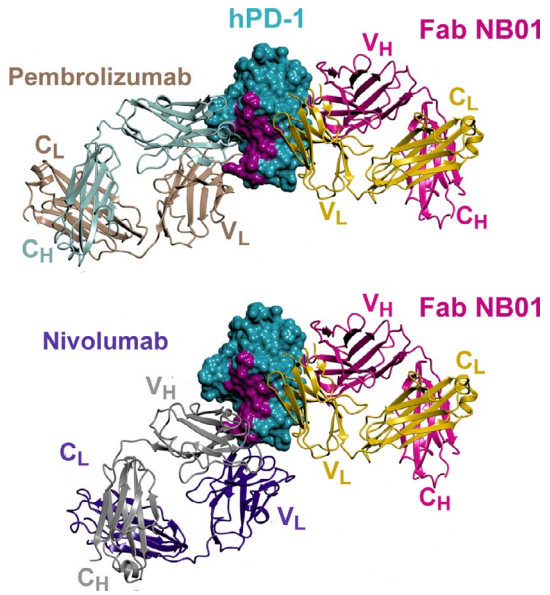

FIGURE 5 Antagonistic antibodies binding the PD-1 receptor. The structure of human PD-1 (hPD-1) in complex with the anti-PD-1 NB01 Fab was solved by molecular replacement using crystals that diffracted to 2.2 Å resolution (6HIG). Molecular modeling by Cα superpositioning of hPD-1 coordinates with the pembrolizumab (PDB 5GGS) and the nivolumab (PDB 5GGR) confirms that NB01 Fab binding to PD-1 does not interfere with the binding of either pembrolizumab or nivolumab anti-PD-1 Abs. The hPDL-1–binding surface (PDB 4ZQK) on hPD-1 is colored in purple and is distinct from the binding epitope of the NB01 Fab that is non-blocking of the PD-1/PDL-1 interaction

prolonged in vitro T-cell stimulation as opposed to exhausted T cells produced in vivo in response to chronic infection.

Our own laboratory recently reported a direct evaluation of signaling in truly exhausted T cells with elevated levels of PD-1 from a chronically infected HIV donor.[105] T-cell activation in concert with PD-1 ligation by PDL-1 suppressed signaling event in both the TCR and CD28 pathways. TCR proximal phosphorylation of the Lck and ZAP-70 was inhibited along with the downstream Ras-MEK1/2 pathway monitored through Erk1/2 phosphorylation. Calcium flux experiments using exhausted T cells also showed a reduced degree of calcium mobilization in stimulations performed in the presence of PDL-1, which leads to reduced activation of the NFAT pathway.[106] The suppression of the CD28 signaling pathway resulted in significantly reduced phosphorylation of PDK1 and its direct target, AKT, phosphorylated at position T308. Formation of the full catalytically activation AKT, phosphorylated at S473 by mTORC2, was also inhibited in the exhausted T cells (Figure 4).

An important validation of the signaling pathways involved in PD-1/PDL-1–mediated T-cell exhaustion is to monitor the restoration of specific phosphoprotein levels following anti-PD-1 therapy. In classical anti-PD-1 antibody therapy, relief of T-cell exhaustion is achieved through PD-1/PDL-1 blockade. However, we recently reported the discovery and validation of a novel class of antagonistic anti-PD-1 antibody that are non-blocking of the PD-1/PDL-1 interaction. Biochemical and structural studies demonstrated that these antibodies bound to the opposite face of the PD-1 protein relative to the PD-1/PDL-1 interaction site. The region on PD-1 targeted by these non-blocking antibodies is highly conserved across six different species and may potentially represent

a binding site for a yet to be identified alternate PD-1 ligand or a region important for transmitting the negative regulatory effect of PD-1. Structural modeling and competitive binding studies with cell surface PD-1 also showed that the non-blocking anti-PD-1 antibody NB01 could bind PD-1 concomitantly with either pembrolizumab or nivolumab blocking anti-PD-1 antibodies (Figure 5). Consistent with blocking and non-blocking antibodies both exerting distinct immune-enhancing functional activity through PD-1, treatment with combinations of the two antibody classes resulted in synergistic functional recovery of proliferation and IFN-γ production from exhausted HIV-specific CD8 T cells. In signaling studies performed in the presence of PD-1/PDL-1 suppression, both blocking and non-blocking anti-PD-1 antibodies partially restored signaling phosphorylation of PDK1 and AKT at positions T308 and S473 in the CD28 pathway. These results are consistent with two recent studies showing that that anti-PD-1–mediated tumor suppressive activity is primarily dependent of the CD28 costimulatory receptor.[103,104] Both classes of anti-PD-1 antibodies also restored calcium mobilization that is downstream of the TCR activation pathway (Figure 4).

Aside from the signaling relationship between PD-1 and the CD28 pathway, immunoprecipitation studies performed with the pembrolizumab blocking anti-PD-1 antibody further demonstrated that in stimulated T cells, PD-1 exists in a complex that includes CD28, SHP2, PI3K, and phosphorylated Lck Src protein. Importantly, our antagonistic non-blocking anti-PD-1 antibody pulled down PD-1 in complex with SHP2 and phosphorylated Lck Src, but showed significantly reduced interaction with CD28 and associated PI3K. This indicates that elevated levels of PD-1 on a T cell may suppress T-cell activation by two mechanism. One through PDL-1–mediated recruitment of PD-1 into the immunological synapse and other through a PD-1 cis-complex with the CD28 costimulatory receptor. The later colocalization would bring CD28-associated intracellular kinases essential for T-cell costimulation into contact with PD-1–associated SHP2 phosphatase, resulting in a direct inhibition of the CD28 costimulatory signaling (Figure 6).

The coexpression of additional ICIs with PD-1 in exhausted HIV-specific T cells reflects an increased level of T-cell dysfunction that is linked to the suppression of T-cell signaling. These ICs include TIM3, TIGIT, and Lag3 that acting on distinct or overlapping points of the signaling cascade relative to PD-1. TIM3 suppresses T-cell activation through Lck, Fyn, and PI3K[107,108] TIGIT recruits SHIP1 into the signaling complex which blocks further signal transduction to PI3K, MAPK pathways, and NFκB[109] and LAG3 is structurally similar to the CD4 co-receptor and acts through a poorly defined mechanism.[110] With their combined inhibitory effect, it is not surprising that T cells with elevated levels of these ICIs are almost completely functionally defective and incapable of mounting an effective immune response.

9 | RECOVERY OF T-CELL FUNCTION: EFFECT OF ART

Upon initiating ART, the majority of patient have a dramatic reduction of viral load and HIV-1 productively infected cells in both the periphery and lymph nodes.[111] However, despite a spectacular efficacy in reducing morbidity and mortality associate with HIV infection, these drugs only inhibit viral replication and cannot cure those infected. ART maintains the level of plasma HIV-1 RNA below the limit of detection for most treated patients. However, long-lived latently infected memory CD4 T cells persist and the potential for residual virus replication prevent the eradication of infected cells.[112] Even after decades of viral suppression, interruption of ART in patients invariably leads to a rapid viral rebound. In this regard, the continued immune dysfunction, while on ART is evident by the inability of the virus-specific immune response to delay significantly the reemergence of the HIV virus. The major causes of this immune dysfunction in HIV are T-cell exhaustion that prevents a functional control of the virus and necessitates life-long ART in most infected patients to suppress viremia and prevent disease progression.

Patient under fully suppressive ART with undetectable HIV-1 plasma viral loads gradual experience a downregulation of immune ICI expression on T cells, although frequencies remain higher compared to HIV-uninfected individuals. A similar trend is observed CD38 + HLA-DR + T cells, which represent an increased immune activation state.[113,114] The functionality of HIV-specific T cells improves with ART treatment with both CD4 and CD8 T cells producing increased levels of IL-2 following stimulation.[115,116] However, PD-1, TIM3 and LAG3 expression on the T cells of ART-treated patients was shown to be associated with the time to viral rebound in studies following standardized treatment interruptions.[117] This demonstrates that the extent of T-cell exhaustion contributes an underlying immune dysfunction that is unable to control even the low levels of virus produced from the latent HIV reservoir in the absence of ART. Our own unpublished results confirm a persistent functional exhaustion of HIV-specific CD8 T cells in long-term suppressive ART patients where PD-1 blockade significantly restores IFN-γ production by twofold. Similarly, combined blockade studies targeting TIGIT and PDL-1 restored the proliferative capacity of virus-specific CD8 T cells with enhanced proliferation compared to either single blockade.[58]

Early initiation of ART is clearly beneficial to patients on multiple levels. Early adoption of ART in the acute phase of viral infection correlates with a lower burden of latent HIV-1 reservoir and reduced systemic inflammation.[118-122] Since the development of T-cell exhaustion is a progressive condition with hierarchical loss of functionality, early ART initiation represents the best opportunity to preserve a patient's HIV-specific response before more pronounced and effectively irreversible T-cell dysfunction is established.

10 | INFLUENCE ON ICIS IN PERSISTENCE OF HIV

Multiple observational studies have demonstrated a clear association between expression of ICIs and the HIV reservoir. Amongst these, a pivotal study indicated that central memory CD4 T cells expressing PD-1 were enriched in HIV-infected cells, thus providing the first evidence for ICI expression on HIV-infected cells.[123] However, the first demonstration

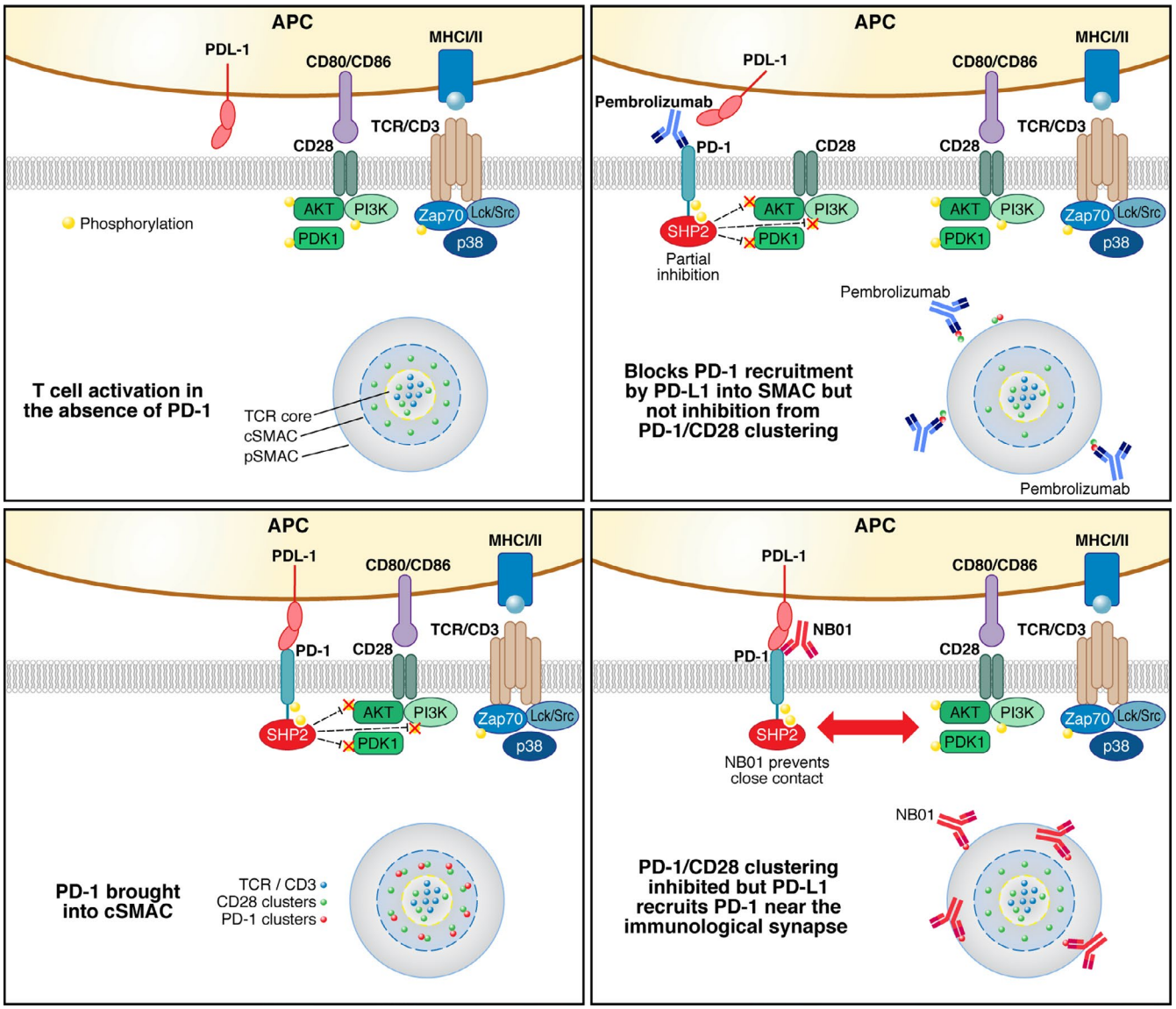

FIGURE 6 Anti-PD-1 antibody-mediated restoration of exhausted T cells. (A) In the standard activation model of T cells expressing low levels of PD-1, the immunological synapse forms with the TCR/CD3 complex at the core, surrounded by the CD28 costimulatory receptor within the cSMAC. This distribution forms a close complex of signaling molecules that enhances the T-cell activation cascade. (B) In PD-1 high T cells, PDL-1 binding to PD-1 recruits the PD-1/SHP2 complex into the cSMAC that effectively suppresses signaling through the SHP2 phosphatase-mediated dephosphorylation of TCR/CD3 and CD28 proximal signaling molecules. (C) Use of blocking anti-PD-1 antibodies such as pembrolizumab partially restores T-cell signaling through limiting PD-1/PDL-1–mediated recruitment of the PD-1/SHP2 complex into the cSMAC. The exclusion of SHP2 from the cSMAC reduces dephosphorylation of signaling molecules including ZAP-70, Lck, PI3K, and AKT. However, our studies show that in activated T cells, pembrolizumab binding to PD-1 pulls down a complex that includes SHP2, CD28, and PI3K following T-cell activation. As such, PD-1 may partially suppress T-cell activation by recruiting CD28 away from the cSMAC and suppressing CD28 costimulation through the SHP2 phosphatase. (D) Our newly discovered anti-PD-1 antibody NB01 is non-blocking of the PD-1/PDL-1 interaction and has equivalent antagonistic activity compared to pembrolizumab in restoring T-cell signaling and antigen-specific functional and proliferative activity to exhausted HIV-specific T cells. Based on immunoprecipitation studies, our proposal is that non-blocking anti-PD-1 antibodies act through inhibiting close contact of the PD-1/SHP2 complex with CD28 and associated signaling molecules following T-cell activation. As such, CD28 is free to migrate into the cSMAC and enhance T-cell activation

that PD-1 expressing CD4 T cells might also be the source of replication competent virus was provided with the isolation and analysis of PD-1^{+} and PD-1^{hi}/Tfh cells from subjects with non-progressive infection and low viremia.[124] Since then, many studies have shown a significant correlation between the frequency of PD-1^{+} CD4 T cells with HIV persistence during ART in blood[125,126] and in tissues.[127] However, the direct evidence of a clear relationship between HIV reservoir and PD-1 expression came from isolated memory CD4 T cells from blood and lymph node of HIV-infected aviremic ART-treated individuals. These studies demonstrated that inducible replication competent HIV was found to be highly enriched in lymph node PD-1^{+} CD4 T cells, containing the Tfh cell population.[128] Recently, a further enrichment in HIV infection

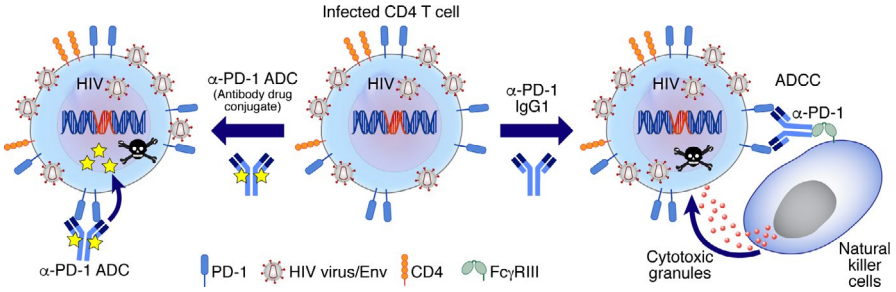

FIGURE 7 Targeted killing of PD-1–positive HIV-infected cells. PD-1 expressing CD4 T cells represent the primary compartment of HIV-infected cells that produce replication competent virus. Targeted killing of these PD-1 positive cells represents a novel therapeutic strategy that can deplete the HIV reservoir of infected cells. Antibody drug conjugates (ADC) use a toxin-conjugated antibody that binds to the cell surface PD-1 receptor. Internalization of PD-1 results in ADC degradation within the lysosome, resulting in toxin release that specifically kills the PD-1 expressing cell. An alternate strategy for the targeted killing of HIV infected PD-1 positive cells is through antibody-dependent cellular cytotoxicity (ADCC) with an IgG1 anti-PD-1 antibody. The Fc portion of the antibody binds to the FcγRIII receptors expressed on effector cells including natural killer (NK) cells. NK cells release cytotoxic granules that kill the PD-1–positive HIV-infected CD4 T cell

was shown in cells that express multiple ICIs simultaneously including PD-1, TIGIT, and LAG3, suggesting that these inhibitory receptors not only suppress T-cell activation but consequently suppress HIV transcription,[129] and therefore favor HIV latency.[130] These observations have prompted the investigation of whether ICI signaling through inhibition of T-cell activation facilitate the establishment of latent HIV infection. Indeed, Evans *et al* recently demonstrated that PD-1 blockade prior to in vitro HIV infection decreased the frequency of latently HIV-infected cells in their in vitro model of HIV latency, highlighting the potential of ICIs blockade to disrupt latency.[130] In addition, we recently observed that PD-1/PDL-1 interactions strongly inhibited TCR-mediated reactivation of HIV transcription and viral production from lymph nodes memory CD4 T cells. Furthermore, PD-1 blockade with anti-PD-1 monoclonal antibody treatment reactivated HIV replication from primary latently infected cells in vitro.[131] These illuminating results revealing the association between HIV persistence and ICIs expression are now being further explored in in vivo studies in individuals with HIV and cancer. Several case report studies tested the potential benefit of using ICI blockers, that is, anti-PD-1 or anti-CTLA-4 monoclonal antibodies to (a) potentially reverse HIV latency in CD4 T cells, thereby allowing the expression of HIV proteins on the cell surface and to (b) reinvigorate HIV-specific CD8 T cells from their exhausted state to potentiate the elimination of reactivated HIV-infected cells. While several reports highlighted a potential reactivation of HIV reservoir markers,[132-134] only one study reported a subsequent decrease in HIV reservoir size.[132] Taken together, these revelations highlighted the enrichment of HIV replication competent virus within ICIs expressing CD4 T cells. Further investigation is needed to determine if targeting these T cells and relieving exhaustion could break latency and eliminate the HIV reservoir.

11 | EXPLOITING PD-1 TARGETING TO PURGE THE HIV RESERVOIR

Immunotherapy through PD-1 blockade represents a major breakthrough that has provided a significant clinical benefit to patients for the treatment of different cancers.[135-137] In vitro studies using the cells of HIV-infected patients have established a clear proof of principle benefit in using anti-PD-1 or PDL-1 antibodies to relieve exhaustion and enhance HIV-antigen-specific functionality and proliferation. Our own in vitro studies show that the combination of classical blocking anti-PD-1 antibodies with novel antagonistic anti-PD-1 antibodies that are non-blocking of the PD-1/PDL-1 interaction synergize to relieve functional exhaustion of HIV-specific CD8 T cells and represent an exciting option for HIV immunotherapy.[105] In vivo PD-1 blockade studies with SIV-infected macaques demonstrated a rapid expansion and functional quality of virus-specific CD8 T cells in both the blood and gut tissue. PD-1 blockade reduction of plasma viral load and impressively prolonged the survival of SIV-infected macaques.[138] Anti-PD-1 therapy combined with ART vs ART alone in SIV-infected monkeys also had a more rapid suppression of viral loads and delayed rebound after a standardized treatment interruption.[139] Despite the success of these studies and others at boosting the immune-mediated antiviral activity, SIV-infected monkeys were not able to maintain immunological control of the SIV virus. As such, relieving T-cell–mediated exhaustion through anti-PD-1 blockade is unlikely to be successful as a monotherapy. Although results are preliminary for several clinical studies employing PD-1 blockade, the patients tested thus far have only shown a modest response at best.[132-134] This indicates that immunotherapy targeting several ICIs in combination with other strategies to reactivate the virus from latently infected cells may be needed to purge the HIV reservoir.

The HIV virus has developed a considerable stealth in evading detection from a patient's immunological response. Antibody-mediated immunotherapy targeting ICIs can address T-cell functional exhaustion. However, a limitation is the lack of access of HIV-specific cytotoxic CD8 T cells to privileged anatomic compartments including lymphoid organs where persistent viremia and/or residual virus replication may occur in memory CD4 T cells.[140,141] Approaches for the targeted killing of infected cells would provide an orthogonal method of eliminating the highly heterogeneous latent population of infected cells. Passive immunization using broadly neutralizing antibodies (bNabs) against the HIV-1 Envelope protein may contribute to the killing of infected cells through

antibody-mediated effector function. However, a recent clinical study was unable to show a benefit in reducing HIV-1 persistence in ART suppressed patients with a combined bNab therapy.[142] A strategy currently under evaluation by our group exploits the fact that PD-1 + CD4 T cells from blood and lymph nodes represent the major cell reservoir for replication competent and infectious HIV in chronic and in long-term antiretroviral-treated subjects. An anti-PD-1 antibody drug conjugate (ADC) was developed with a PNU toxin and in vitro studies show specific induction of apoptosis and cell death in PD-1 positive cells. Anti-PD-1 ADC treatment of CD4 T cells from chronically infected HIV-1 donors significantly reduced viral production relative to a control anti-PD-1 antibody. This therapy was also effective in aviremic ART donors, purging the majority of cells from that were capable of producing infectious virus (Figure 7).[131] Although these data are very encouraging, a primary consideration with all of therapeutic approaches to eliminate the HIV reservoir will be safety. Considerable strides have led to the development of highly potent ART that effectively suppressed viral loads in most patients for decades with limited adverse events and liabilities. As such, the bar will be set high to demonstrate a clear medical need for the use of curative strategies that present any dangers to the long-term health of patients.

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CONFLICT OF INTEREST

All authors declare no conflict of interest with this review.

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4 Discussion

HIV persists in cellular reservoir that are not targeted by ART[123,410,411]. A lot of effort has been made to identify markers that characterize HIV reservoir and could provide a support for selective therapeutic targeting[412]. The most obvious marker for HIV infected cells is HIV envelope protein (Env), which is displayed on the infected cell surface. Antibodies against Env can be detected very early in HIV infection and are able to neutralize autologous viral variants. However, Env evolves rapidly to escape neutralizing antibodies (NAb) leading to successive cycles of antibody production and viral escape[232,322,413].

Though most neutralizing antibodies are highly specific against autologous viruses, studies have identified antibodies from the sera of some HIV-1 infected individuals that can neutralize diverse HIV-1 isolates, the so called broadly neutralizing antibodies (bNAbs). bNAbs have high level of mutations and are detected in the late course of HIV infection, reflecting chronic immune responses to HIV and persistent hypermutation and selection[414]. Passive immunization with bNAbs has been shown to effectively protect against HIV infection in animal models and to decrease viral loads in infected animals and humans[337-342,344,345,415-418].

In addition to neutralization, bNAbs accomplish a variety of effector functions associated with the fragment crystallizable region (Fc), such as antibody-dependent cell mediated cytotoxicity (ADCC), antibody-dependent cellular phagocytosis (ADCP) and activation of the complement cascade[334,349]. Though passive immunization with bNAbs prolonged viral control after ART interruption in HIV infected individuals, HIV virus ineluctably rebounded associated with the selection of bNAb resistant HIV variants[338,341]. Also, one study showed no difference in HIV reservoir as described by the frequency of cells harboring replication competent and infectious viruses (IUPM) before and after bNAb treatment[344].

HIV latency leads to very low HIV antigen expression, resulting in inefficient targeting[419]. Therefore, many surface proteins have been proposed as surrogate marker for HIV latently infected cells, such as immune checkpoint molecules[171,172,268,363]. Studies have demonstrated that lymph node PD-1^{+}CD4 T cells was the population contributing the most to HIV reservoir, as described by the frequency of infected cells capable of producing replication competent and infectious viruses, in untreated and ART treated donors[171,172]. Another study showed that HIV reservoir cells were characterized by the individual or co-expression of PD-1, LAG-3 and TIGIT[363].

The attempt to fine-tune the characterization of HIV reservoir and develop targeting strategies as specific as possible lead to the discovery of CD32a, though highly controversial, and CXCR3 as new markers for HIV cell reservoir[364-366]. Studies on cancer patients have even

investigated the impact of brentuximab vedotin treatment, an anti-CD30 ADC, on HIV reservoir[368].

In the present project, we evaluated the potential of PD-1 targeting to decrease the frequencies of HIV infected cells in peripheral mononuclear cells isolated from HIV viremic and aviremic ART treated donors. We used a mouse (*m*PD-1) and a humanized (*h*PD-1) anti-PD-1 targeting different epitopes that were conjugated to the nemorubicin metabolite PNU159682[409]. In parallel, we evaluated the antibody-dependent cellular cytotoxicity activity of *h*PD-1 IgG1. Cytotoxicity was assessed by flow cytometry, and the impact on HIV reservoir by the production of p24 HIV antigen in culture supernatants or by a quantitative viral outgrowth assay (qVOA).

We observed that both, IgG1 mediated ADCC and ADC strategies to target PD-1^{+}CD4 T cells, resulted in significant increased frequencies of cell undergoing apoptosis and cell death on viremic untreated donors. In accordance with depletion, p24 production was significantly decreased in the culture supernatants, suggesting that anti-PD-1 IgG1 or ADC have a targeted effect on virus production.

To look at the impact on HIV reservoir, we next evaluated the efficacy of PD-1 targeting with IgG1 or ADC on ART treated donor cells. We observed that anti-PD-1 IgG1 and ADC treatment also increased the frequencies of apoptotic and dead CD4^{+} T cells, which was associated with the reduction of replication competent and infectious viruses production, as assessed by the qVOA. Consistent with previous observations on HIV reservoir, our data unambiguously shows that both, anti-PD-1 IgG1 and ADC, are able to target and kill HIV-infected cells[171,172,268].

We observed that ADCC was overall less effective than ADC in our experimental setup. Papers have demonstrated that priming NK cells with cytokines such as IL-12 or IL-15 enhanced *in vitro* ADCC activity and that the use of resting effector cells may lead to the underevaluation of ADCC activity *in vitro*[420,421]. Moreover, HIV infection has been shown to impair the functionality of NK cells, though ART partially restores this activity[422,423]. Since we used resting peripheral mononuclear cells from HIV individuals, NK cells may have suffered from insufficient priming for optimal killing activity.

In addition, we observed a rapid internalization of PD-1 receptor upon binding with anti-PD-1. Receptor internalization is beneficial in the context of ADC but might be detrimental in the context of ADCC, where high surface antigen density is required for NK cytotoxic activity[424]. In this context, fast internalization of PD-1 receptor and a favored targeted killing of PD-1 high cells over PD-1 low cells may have led to a suboptimal depletion of PD-1 positive cells.

We did not observe any association between antigen expression and efficacy in either ADC or ADCC strategies. Indeed, neither the percentage of PD-1 positive cells, nor the mean

fluorescence intensity of PD-1 expression (MFI) correlated with the percentage of cell apoptosis or cell death. A previous study conducted with brentuximab vedotin, an anti-CD30 ADC approved in oncology, also failed to demonstrate direct correlation between CD30 expression on target cells and treatment efficacy[425]. The lack of correlation may reflect that the rate of internalization and enzymatic cleavage may vary between cells, representing heterogeneous populations[426,427].

Although our *in vitro* studies provide a good proof of principle for targeting PD-1 as a non-viral cell-surface marker to kill HIV-infected cells, *in vivo* efficacy represents additional challenges. Indeed, many studies have underlined the importance of the lymphoid organs as sanctuary sites for HIV persistence in untreated and ART treated HIV infected patients[96,171,172]. The access of antibodies to lymph node germinal centers, residence place of T_{FH} cells, is critical for the *in vivo* efficacy of the treatment.

Drugs targeting CD19, CD20 or CD30 and used in lymphoma treatment are efficient and target cells that are present in lymphoid organs[428-431]. However, cancer treatment does not rely on the use of immunotherapy as a monotherapy but as an adjuvant to radiotherapy and/or chemotherapy. Also, studies have shown that cells residing in tissues were more resistant to targeted depletion compared to circulating cells and that higher doses are required to saturate lymphoid tissues[432-434]. Therefore, *in vivo* studies are required to assess the safety and efficacy of anti-PD-1 treatment as a cure for HIV.

Off-target toxicity may be a concern since our strategy aims at depleting PD-1 positive cells without increasing the specificity for $CD4^+$ T cells. mAb IgG1 and ADCs used in oncology target antigens that are specifically expressed at high levels on cancer cells compared to healthy tissues, thus favoring the elimination of cancer cells[435]. PD-1, however, is an activation marker for T lymphocytes and expression level is even higher on $CD8^+$ T cells than on $CD4^+$ T cells, so that a depletion of PD-1^+CD8 T cells is expected as well. Studies on rituximab (anti-CD20 IgG1) have shown that B cell depletion was transient and highly dose dependent[432,436]. Therefore, the evaluation of drug dosage and interval between administrations will be crucial to have the desired effect on HIV reservoir and minimize side-effects.

We developed anti-PD-1 antibodies because anti-PD-1 have already been extensively studied and approved for cancer treatment[437,438]. Therefore, the advent of new anti-PD-1 in the clinic might be facilitated compared to unknown molecules. However, the aim of immunomodulators in the oncology is to relieve $CD8^+$ T cells from exhaustion and facilitate the elimination of tumor cells by the immune system[439]. Since ADCC, ADCP or CDC Fc effector functions are not required for that purpose, anti-PD-1 treatment approved in the oncology are

IgG4 antibodies[440,441]. Therefore, no data is currently available on the use of anti-PD-1 IgG1 or ADC.

Both approaches, ADC and ADCC, have their advantages and withdrawals. Antibody-drug conjugates were first developed in the oncology field, with four ADCs approved so far and many more in development[442]. ADC strategy aims at specifically deliver cytotoxic compounds to cancer cells while sparing healthy tissues. This strategy strongly relies on the overexpression of cancer-specific antigen on the cell surface. Highly cytotoxic drugs are conjugated to potent monoclonal antibodies, thus conferring a better clinical profile in terms of efficacy and of toxicity. The main advantage of ADCs is that the efficacy does not rely on a pre-existing immune response. However, ADCs are associated with a wide range of side-effects that may be acceptable in the context of cancer, but unacceptable in the context of HIV, considering the actual safety and efficacy of ART[443,444].

In contrast, unconjugated antibodies may have no toxin-associated toxicity, but their function relies on immune mediated response, including complement activation, phagocytosis and NK cell activity. Many reports relate the presence of functional compromised NK cells in HIV infected untreated donors. Though efficient ART can restore NK functionality to some extent, ADCC activity may be optimized with cytokine priming[311,343,445]. NK cells also have to reach lymph node germinal centers to target T_{FH} cells, described as the major cell reservoir for HIV[171,172]. Though one study mentioned the presence of NK in lymph node from African Green Monkeys, nothing similar has been demonstrated in human[446].

In conclusion, we demonstrated that treatment with both, anti-PD-1 IgG1 and anti-PD-1 ADC, significantly reduced the frequency of cells harboring replication competent and infectious viruses in blood. Our data confirmed that PD-1 is a suitable marker to target HIV reservoir and validated our strategies. Therefore, our very encouraging result support the initiation of experiments in animal models.

5 Future perspectives

The present study shows the efficacy of humanized anti-PD-1 IgG1 and anti-PD-1 ADC at targeting HIV reservoir on PBMCs or $CD4^+$ T cells isolated from blood. We are therefore interested in testing our compounds *in vivo*, namely in using the HIV-1 infected NSG humanized mouse model.

The data presented above provide compelling evidence that PD-1 depletion results in a decrease of HIV reservoir cells, as characterized by cells harboring replication competent and infectious viruses. Now that the ADC experimental system is validated, we could expand our investigation to test additional therapeutic agents. Improved specificity for HIV-infected cells would be possible through the generation of bispecific antibodies targeting both PD-1 and HIV-1 Env, or PD-1 and CD4. Indeed, PD-1 is not an exclusive marker for HIV infected cells and the inclusion of Env or CD4 specificity for the ADC may reduce off target toxicity on healthy $PD\text{-}1^+$ cells including PD-1 expressing $CD8^+$ T cells. Improved efficacy at depleting HIV cellular reservoir might be possible through combinatorial therapies with latency reversing agents such as IL-15 agonist. The possible synergistic activity between reactivation of HIV infected cells associated to the upregulation of surface PD-1 and the depletion of PD-1 positive cells might further deplete HIV cell reservoir and enhance the chance of achieving an HIV functional cure.

Our future work may lead to further validation of immune therapeutics for pre-clinical and clinical testing in the attempt to achieve a functional cure for HIV-1.

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