Part A – Applicant

A.1 Applicant

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Part B – Scientific proposal

B.1 BASIC DETAILS

B.1.1 Title

Towards improving cytotoxic T cell function in acute and chronic HIV-1 infection: tackling T cell exhaustion with the help of an *in vitro* exhaustion system

B.1.2 Abstract

T cell exhaustion is a state of immune dysfunction that is integral to the failure of cytotoxic T cells (CTLs) to control HIV-1 infection. Opposingly, highly functional HIV-specific CTLs are indicative of a good disease prognosis and often lifelong HIV-1 control. In this context, we hypothesize that directly tackling T cell exhaustion could constitute the missing link in the current landscape of HIV-1 cure strategies. Here, we aim to test a wide array of current and novel therapies aimed at reversing or preventing T cell exhaustion – such as checkpoint blockade, exogenous co-stimulation, transcription and epigenetic modulators, and $CD4^+$ T cell help. While some of these therapeutic approaches are well established in other fields (e.g. cancer immunotherapy), little similar progress is achieved in the HIV-1 cure field. As an experimental basis, we will design, optimize, and validate an *in vitro* exhaustion system that can reliably generate *bona fide* exhausted HIV-specific CTLs, which will serve as testing platform for the abovementioned treatment strategies. Overall, this project has the potential to identify the best therapeutic approach(es) of tackling T cell exhaustion, which due to the high translational potential of the *in vitro* exhaustion system, will undoubtedly play an important role in future combinatorial HIV-1 cure strategies. Within the HIV-1 pandemic context, while other options aiming to replace the dysfunctional HIV-specific T cell response are being explored, improving the function of the exhausted T cells is the only research avenue that could lead to an effective, *equitable*, functional cure that would benefit the key populations most burdened by the HIV-1 pandemic.

B.1.3 Layman's summary

The human immunodeficiency virus type 1 (HIV-1) pandemic has been ravaging global human health for more than 40 years, yet no effective cure is in sight. In this context, many researchers throughout the years have placed their hopes on a type of immune cell, called *cytotoxic T cells*, which are the body's primary warriors against the invading virus, and can – in some very few cases – provide unique (life-long) protection. However, more often than not, these cytotoxic T cells rapidly lose their powerful abilities to find and kill the infected cells (harbouring the HIV-1 virus), and this worsening of the protective response is attributed to what is known as *T cell exhaustion*. Indeed, this phenomenon appears in numerous chronic viral infections and cancers, where the ongoing 'battle' with the invading virus or the rapidly growing cancer puts too much pressure on the cytotoxic T cells, leading to a sort of immunological 'burnout'. With this project, we aim to directly tackle this state of T cell 'burnout' in HIV-1 infection,

with the view that once reversed (or even prevented), cytotoxic T cells alone could once more effectively control the HIV-1 virus. First, we will attempt to reinvigorate the exhausted state of the T cells, by taking inspiration from the current advances in the cancer immunotherapy field; this research avenue could one day immensely benefit chronically infected HIV-1 patients. Second, we will attempt to prevent the very onset of the T cell exhaustion state – which could one day promise newly diagnosed HIV-1 patients an early cure. To reach these aims, we will first design and optimize a lab-based assay that can generate exhausted cytotoxic T cells similar to those generated during an actual HIV-1 infection. Importantly, the advantage of this experimental approach is that it could very accurately identify the potential of numerous existing and novel therapies that tackle T cell exhaustion, and this project will thus lay the groundwork for future HIV-1 cure studies in animals or humans. In the end, since cytotoxic T cells are the main cellular 'players' involved both in the long-term control of HIV-1 infection as well as in the failure to control HIV-1 infection, tackling the root of the issue – i.e. T cell exhaustion – would undoubtedly provide the missing link in the current landscape of HIV-1 cure strategies.

B.1.4 Keywords

T cell exhaustion, HIV-1 infection, functional HIV-1 cure, *in vitro* exhaustion system;

B.2 SCIENTIFIC PROPOSAL

B.2.1 Research topic

(a) Theoretical background

T cell exhaustion is a type of immune cell dysfunction strongly associated with the failure of cytotoxic T cells (i.e., CTLs or $CD8^+$ T cells) to eliminate infected or transformed cells in chronic viral infections and cancers, respectively. Exhausted T cells are thought to arise from chronic antigenic stimulation via the T cell receptor (TCR) leading to the gradual loss of T cell proliferation, cytokine secretion, and killing ability, and ultimately to the clonal deletion of the exhausted cells (Wherry, 2011). Within the field of HIV-1 research, despite intensive global efforts aimed at finding a cure for HIV-1 infection for the past four decades, no durable treatment option capable of inducing HIV-1 remission in the absence of combined antiretroviral treatment (cART) has been found (Fenwick et al., 2019). Here, we propose that aiming to improve the HIV-specific cytotoxic T cell compartment by directly tackling T cell exhaustion could provide the 'missing piece' of the solution to the HIV-1 cure conundrum.

Broadly speaking, the lack of an HIV-1 cure stems from the impossibility of most HIV-1-positive individuals to naturally resolve an ongoing HIV-1 infection. While the timely administration of cART (the standard of care nowadays) has managed to significantly improve patients' life quality and lifespan, lifelong treatment regimens are needed to control virus replication and AIDS onset (Perdomo-Celis et al., 2019). From this perspective, the discovery of elite controllers (ECs), i.e., a small proportion (<1%) of HIV-positive individuals capable of controlling HIV-1 infection in the absence of cART, has served as an unbridled study model for HIV-1 cure. Most importantly, it appears that immune control in ECs is primarily mediated by a very potent HIV-specific CTL response, thus allowing an early control of viral replication post-infection (McBrien et al., 2018; Warren et al., 2019). Opposingly, a lack of HIV-1 control is associated with 'faulty' HIV-specific CTLs (qualitatively rather than quantitatively), high viral loads, and high viral escape (Rogan & Connors, 2021). Interestingly, there appears to be a positive feedback loop in both cases. In ECs, an early, strong, and functional HIV-specific CTL response 1) can stop viral replication early post-infection, 2) can limit latent reservoir formation, 3) can limit viral escape and thus loss of T cell recognition of viral epitopes, 4) can limit the chronic stimulation/ inflammation – both direct and bystander – that affects HIV-specific and bulk $CD8^+$ T cells, thus preserving their function. Opposingly, HIV-1 progressors show a stunted HIV-specific $CD8^+$ T cell response, which worsens in the setting of chronic antigenic stimulation due to unbound viral replication and viral escape (Rogan & Connors, 2021; Warren et al., 2019). Interestingly, treated HIV-1 progressors show a partial recovery in the extent of (dys)function that characterizes their HIV-specific CTL compartment, unlike untreated HIV-positive patients; this recovery is likely due to the control of viral replication by successful cART administration, leading to a lowering of viral antigens that reinforce the exhaustion phenotype (Cockerham et al., 2016; Perdomo-Celis et al., 2019). While it remains unclear what causes the initial potent HIV-specific CTL response in ECs, the desirability of a good $CD8^+$ T cell compartment as part of any HIV-1 cure strategy remains uncontested. In this regard, the majority of the most promising cure options developed so far have been unsuccessful by either failing to raise a potent HIV-specific CTL response or by relying on the patient's own suboptimal CTL compartment. For instance, the "shock and kill" strategy using latency reversal agents to reactivate the latent HIV-1 reservoir has failed due to a lack of killing (and possibly recognition) by the patients' CTL response of the latently reactivated $HIV\text{-}1^+$ cells (Thorlund et al., 2017). Similarly, numerous attempts to design a therapeutic vaccine capable of enhancing the $CD8^+$ T cell response so that it controls viral replication in the absence of cART (the 'functional' cure strategy) have overall led to a (quantitative rather than qualitative) increase in HIV-specific CTLs, with no corresponding effect in lowering HIV-1 viral loads following cART treatment interruption (ATI) (Lévy et al., 2021; Stephenson, 2018).

Taken together, it is clear that 1) aiming to improve the quality of patients' HIV-specific $CD8^+$ T cells is key to any HIV-1 cure strategy, and 2) T cell exhaustion is tightly linked to HIV-1 pathogenesis and the chronic infection setting (Hoffmann et al., 2016). With this project, I will assess whether directly tackling T cell exhaustion could constitute a novel strategy to improve the function of HIV-specific CTLs in both the acute phase as well as the chronic phase of HIV-1 infection. Explicitly, I will first investigate whether reversing T cell exhaustion during the chronic HIV-1 infection phase could reinvigorate the function of exhausted HIV-specific $CD8^+$ T cells, and thus contribute to existing HIV-1 cure strategies. Secondly, I will investigate whether preventing the onset of T cell exhaustion altogether early during acute HIV-1 infection is feasible, thereby skewing the balance between HIV-1 control and HIV-1 progression shortly following diagnosis.

To enable the study of these two research goals, we will develop an *in vitro* exhaustion system that allows the reliable generation of human-derived *bona fide* exhausted HIV-specific T cells. Traditionally, T cell exhaustion has been studied *in vivo* using the chronic lymphocytic choriomeningitis virus (LCMV) infection model in mice

(Barber et al., 2006; Gallimore et al., 1998; Zajac et al., 1998). Importantly, this method has been used to address fundamental questions related to the onset and maintenance of *murine* T cell exhaustion – and later corroborated with findings from chronic infection and cancer setting in humans (McLane et al., 2019). However, the LCMV model is unsuited to the study of exhaustion reversal and prevention strategies for human use, mainly due to mouse-human incompatibilities in terms of immune systems and drug targets, but also due to the complex multifactorial infection process that is inherent to LCMV and that makes it difficult to untangle the role and influence of certain therapies and/or drugs studied. Instead, here I propose an *in vitro* exhaustion system based on repeated daily stimulation of healthy human T cells with cognate antigen, which is based on the core understanding that T cell exhaustion stems from persistent TCR-mediated stimulation (Bucks et al., 2009; Wherry, 2011; Zhao et al., 2020). Indeed, our previous findings (unpublished data) have indicated that only 5 days of *in vitro* stimulation with cognate peptide of healthy human $CD8^+$ T cells (transduced with a recombinant, peptide-specific TCR) can lead to substantial phenotypic and partial functional exhaustion (**Figure 1**). Here we will first extend these observations to two HIV-specific T cell platforms, namely HIV-specific recombinant TCR (rTCR) T cells transduced with a lentiviral vector, and HIV-specific T cells enriched from healthy donor (HD) peripheral blood (PB) using HIV-1 peptide pool stimulation. Second, based on the growing body of knowledge of T cell exhaustion in the cancer setting, we will test various strategies to improve the 'power' of the *in vitro* exhaustion system to generate dysfunctional CTLs, such as by mimicking the nutrient-poor, oxygen-poor, and/or immunosuppressive environment that is characteristic of solid tumours. Together, this experimental setting will allow the streamlined *in vitro* testing of various (existing and novel) therapeutic strategies targeting T cell exhaustion. Overall, I hypothesize that directly tackling T cell exhaustion in HIV-1 infection – either by reversing or even preventing its onset – could greatly improve the quality of the HIV-specific $CD8^+$ T cell response, and thus contribute to current HIV-1 cure efforts.

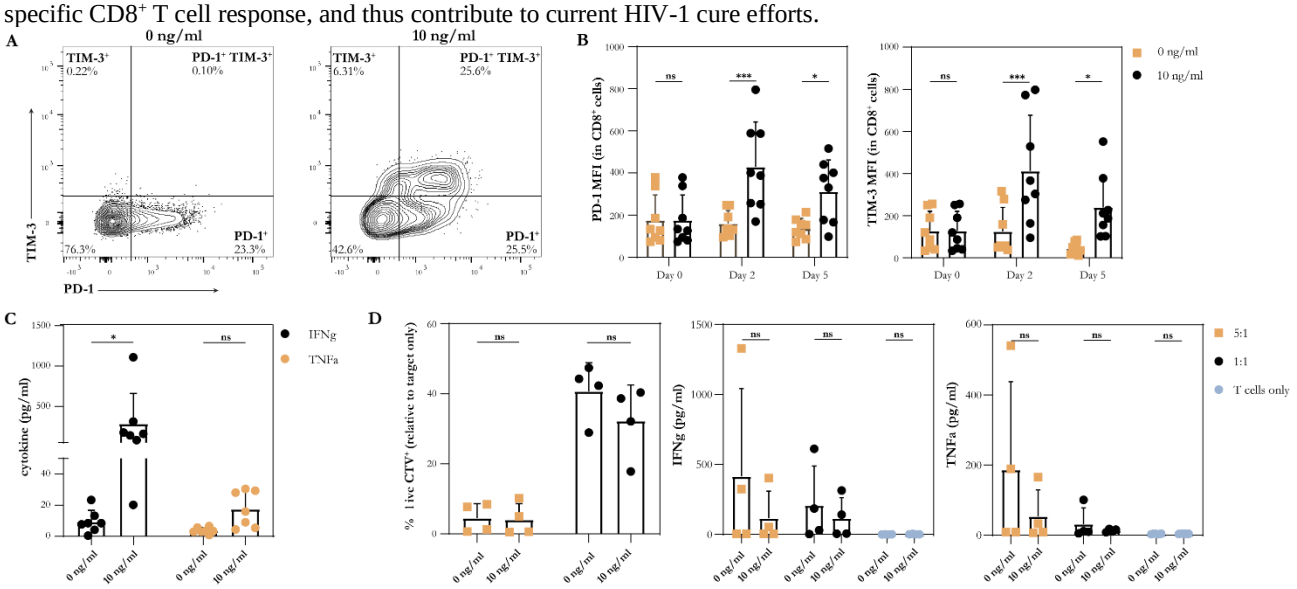

Figure 1: Pilot data of *in vitro* T cell exhaustion system. A: Representative flow cytometry plots of inhibitory receptor expression on *in vitro* stimulated T cells at day 5. B: Upregulated PD-1 and Tim-3 expression at day 5 post-stimulation. C: Relatively low cytotoxic cytokine secretion following *in vitro* stimulation regimen. D: *In vitro* killing assay and *de novo* cytotoxic cytokine secretion during overnight incubation.

(b) Objectives

To test this hypothesis, we will divide the research work in three chronological objectives (**Figure 2**), namely:

- ***Objective 1***: Generate different platforms for T cell exhaustion and design phenotypic, functional, and transcriptomic T cell panels for validation
- ***Objective 2*****:** Improve the *in vitro* exhaustion system to better recapitulate T cell exhaustion by optimizing length of stimulation and testing various T cell exhaustion media, and validate the *in vitro* exhaustion system by comparing the *in vitro* exhausted T cells to *bona fide* exhausted T cells from HIV^+ individuals
- ***Objective 3*****:** Test various exhaustion reversal and exhaustion prevention strategies on *in vitro* exhausted T cells

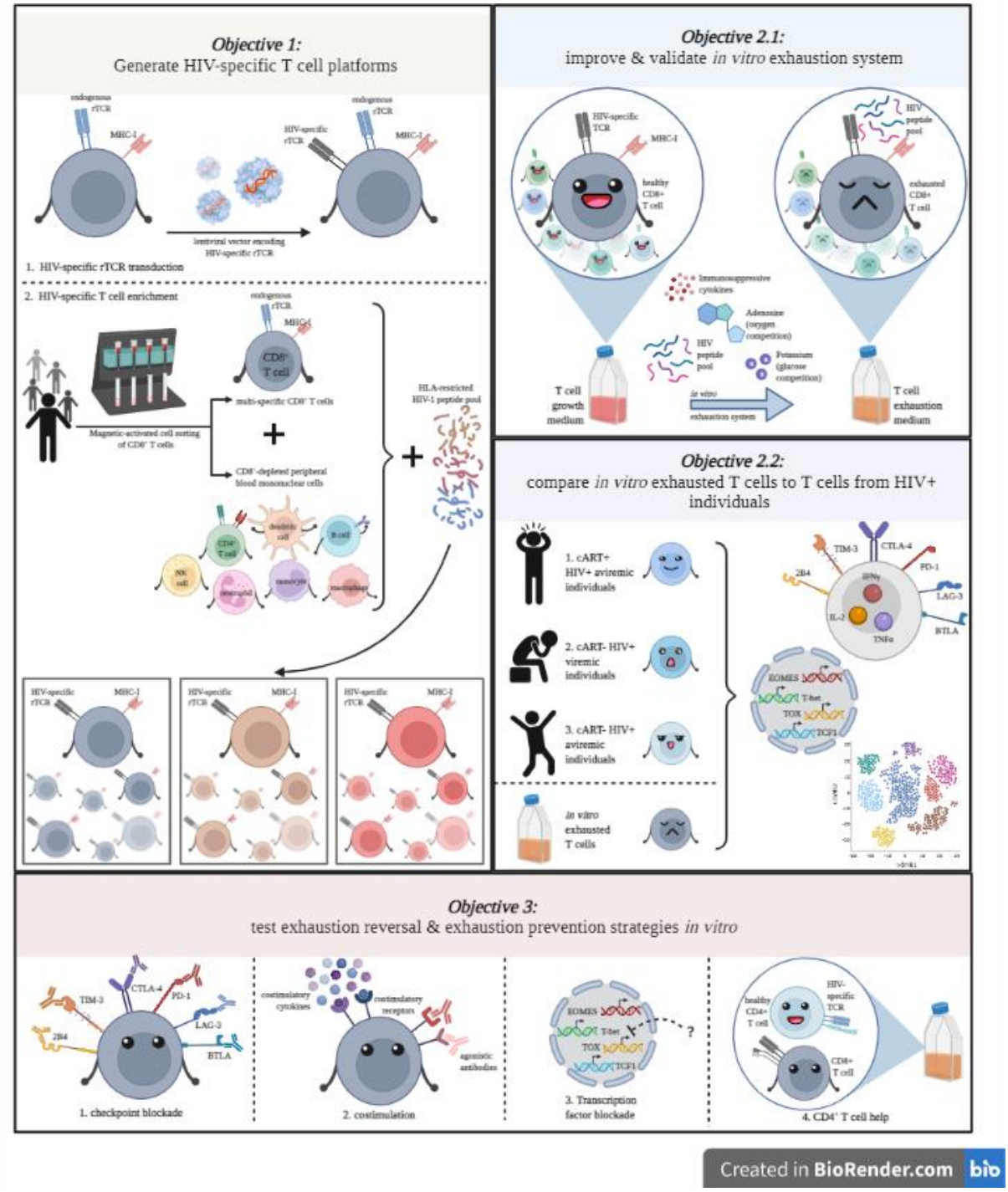

Figure 2: Graphical overview of objectives of proposed project.

(c) **Originality, Innovation & Urgency**

The real urgency behind this proposed project stems from the fact that all HIV-1 cure options hinge on a functional CTL response. Indeed, both HIV-1 cure approaches based on therapeutic vaccination strategies or latency-reversing agents require a potent HIV-specific $CD8^+$ T cell response to successfully target and eliminate the viral reservoir. While other options aiming to replace rather than improve the existing HIV-specific T cell response are being explored (e.g. adoptive T cell therapy), such expensive therapeutic options could never reach the key populations most burdened by the HIV-1 pandemic (UNAIDS, 2021). Thus, researching the potential of existing and novel strategies to revert (if not prevent!) the T cell dysfunction characteristic of chronic HIV-1 infection could pave the way to an *equitable* HIV-1 cure. Since no reliable, accessible, and affordable system to date allows the streamlined testing of HIV-1 exhaustion reversal and prevention strategies *in vitro*, most studies rely on the chronic LCMV

infection model or humanized mouse models; however, neither *in vivo* model fully recapitulates the human situation. While ultimately the best end-point assessment of any functional HIV-1 cure strategy is through clinical trials incorporating cART treatment interruption (ATI) to allow the evaluation of HIV-1 viral loads post-ATI (Stephenson, 2018), an 'earlier', standardized lab-based testing platform is needed to permit the extensive testing of new drugs and strategies aiming to reinvigorate exhausted T cell function. Overall, a validated, standardized *in vitro* exhaustion system would immensely help the HIV-1 cure field in its quest to improve the patients' own T cell function, both during acute and chronic HIV-1 infection.

T cell exhaustion has been undoubtedly implicated in the lack of immune resolution and hardship to cure of many chronic viral diseases as well as cancers at the end of the 1990s (Gallimore et al., 1998; Salek-Ardakani & Schoenberger, 2013; Zajac et al., 1998). Nevertheless, the chronic infection research fields, including that of HIV-1, have been considerably lagging with regards to reversing the detrimental effects that T cell exhaustion poses on the resolution of chronic infection (Vigano et al., 2020). One such (Nobel-worthy) example is checkpoint blockade immunotherapy, which has proven incredibly promising at decreasing tumour load and increasing patient survival by (partially) reversing T cell exhaustion (McLane et al., 2019). Nonetheless, there is little similar advancement in the field of HIV-1 treatment and cure. On the one hand, *in vitro* and animal studies strongly support a therapeutic targeting of the PD-1/PD-L1 inhibitory axis, which was altogether shown to improve T cell function and reduce viral loads (Saeidi et al., 2018). On the other hand, due to fears of immunological side effects (such as greater immune cell activation and thus higher chance of perpetuating the infectious HIV-1 cycle and increasing general inflammation levels), relatively few (cancer-free) HIV-positive individuals have been administered checkpoint blockade, and insights from these studies have been mixed (Castelli et al., 2021). For instance, a few case-studies to date have noted positive results regarding the decrease of latent viral reservoirs upon checkpoint blockade administration, yet little attention was paid to the possible improvement of the T cell compartment (Chen et al., 2020). More clinical work has been performed in HIV-positive cancer patients, where both anti-PD-1 and anti-CTLA-4 therapy have been found safe and well-tolerated, yet less consistency was noted with regards to immunological and virological end-points (Castelli et al., 2021; Chen et al., 2020). Clearly, more work is needed to grasp the clinical potential of checkpoint blockade immunotherapy in HIV-1 infection, especially with regards to the reversal of T cell exhaustion. Meanwhile, the cancer field is striding forward in achieving not only the reinvigoration of the patient's dysfunctional T cell subsets, but also the replacement of the patient's T cells with adoptive T cells engineered to be (relatively) resistant to exhaustion onset through CRISPR/Cas9 deletions of certain checkpoint inhibitors (Stadtmauer et al., 2020). Thus, work is urgently needed to bridge the findings and key strategies of these two fields. In this respect, an *in vitro* exhaustion system would provide not only a uniform, adaptable platform for the generation of HIV-specific or cancer-specific exhausted T cells, but also a shared steppingstone from where insights could be easily shared.

Interestingly, while here we set to identify promising T cell-based cure candidates using *in vitro* exhausted HIV-specific T cells, it is likely that the beneficial effects brought by such agents *in vivo* would highly overshoot their *in vitro* expectations. For instance, while checkpoint blockade will be examined here solely for its T cell exhaustion reversal capability, many other beneficial effects have been noted, such as 1) improving $CD4^+$ and B cell (dys)function, 2) improving *de novo* T cell priming, and 3) disrupting the latent HIV-1 reservoir (Fromentin et al., 2019; McLane et al., 2019). Thus, while *in vitro* work is vital to the discovery and testing of existing and novel therapy options tackling T cell exhaustion in HIV-1 infection, it is likely that a broader pattern of favorable outcomes will emerge once the strategies proposed here are pursued in animal or human studies. Another similar advantage of the *in vitro* experimental approach outlined here is that it allows the effect of novel treatment combinations to be thoroughly tested – such as combinations of transcriptome/ epigenome modifier drugs, checkpoint blockade, metabolic modulators, cART, and others (for a prime example, see Li et al., 2021). Importantly, many impeccable studies from the past year have revealed that despite the cessation of chronic antigenic stimulation or even the administration of PD-1 blockade, exhausted T cells only partially recover, due to what is termed 'epigenetic scarring' (Abdel-Hakeem et al., 2021; Abhimanyu et al., 2021; Martin et al., 2021; Yates et al., 2021). Our *in vitro* work will allow the thorough examination of the effects of multiple exhaustion reversal strategies, and thus our insights will be key to reaching a 'full' and durable reversal of T cell exhaustion, and perhaps the prevention of T cell exhaustion onset.

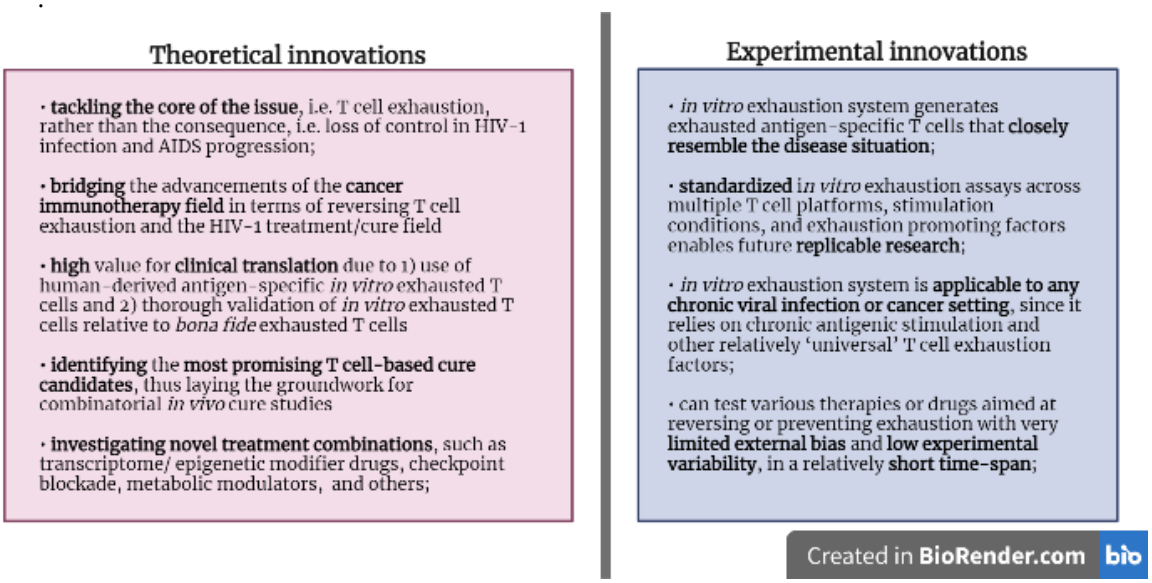

Figure 3: Graphical overview of theoretical and experimental innovations of the proposed project.

B.2.2 Methods

Objective 1

Since T cell exhaustion is the result of chronic antigenic stimulation via the TCR (Bucks et al., 2009; Wherry, 2011), the *in vitro* experimental set-up requires T cell specificity for a cognate HIV peptide. To achieve that, we will pursue and later compare two experimental options. In both cases we will use freshly isolated $CD8^+$ T cells from healthy donor (HD) peripheral blood (PB). First, we will perform a TCR knock-in by delivering a known HIV-specific recombinant TCR (rTCR) using adeno-associated virus (AAV) transduction while knocking out the native TCR using CRISPR/Cas9 (see **Box 1**). rTCR-positive T cells will then be sorted using fluorescence-activated cell sorting (FACS) to reach a pure, HIV-specific recombinant T cell population. Secondly, we aim to enrich for HIV-specific $CD8^+$ T cells from the normal TCR repertoire of HD PB T cells of numerous donors. This will be achieved by *in vitro* stimulation with autologous $CD8^+$-depleted PBMCs pulsed with HIV-1 peptide pools (see **Box 1**).

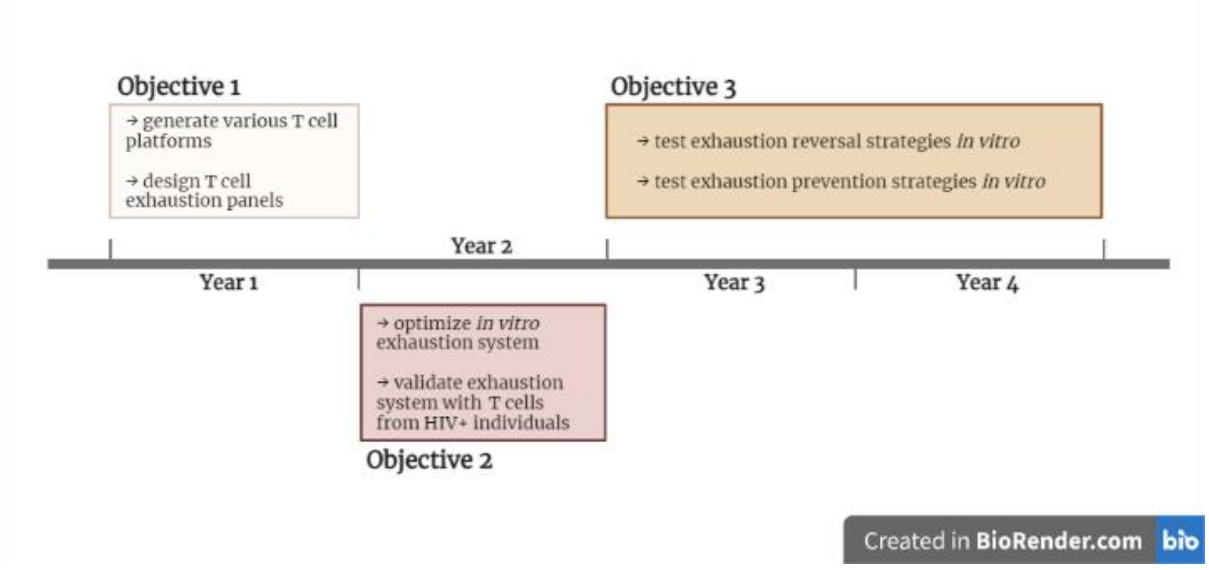

Figure 4: Graphical overview of the timeline of the proposed project.

To enable the in-depth characterization of *in vitro* exhausted T cells, we will next define and optimize various validation panels (see **Box 2**). First, we will design several flow cytometry-based staining panels to analyse the extent of T cell exhaustion achieved through the *in vitro* exhaustion assay: a surface staining panel for phenotypic analysis, an intracellular staining panel analysing cytotoxic cytokine release, and lastly another intracellular panel for

transcription factor analysis. Second, we will use bulk RNA sequencing to assess changes in cellular transcription patterns following *in vitro* T cell exhaustion. Importantly, to validate the *in vitro* exhaustion system in the HIV-1 infection context, we will characterize the *in vitro* exhausted T cells relative to *bona fide* exhausted T cells isolated from HIV-seropositive, ART-naïve patients, as well as to HIV-seropositive, long-term treated patients, and elite controllers. In this context, we will establish the extent of resemblance of *in vitro* exhausted HIV-specific T cells to $CD8^+$ T cells extracted from various types of HIV-positive patients; this will allow us to accurately determine the extent of inference that can be drawn based on our proposed *in vitro* work. Finally, we will test direct T cell cytotoxicity using viral inhibition assays (VIAs), which test the viral suppressive capacity (VSC) of $CD8^+$ T cells (see **Box 2**). Interestingly, *in vitro* VSC is indicative of reduced viral loads *in vivo* (Adams et al., 2021; Warren et al., 2019), which suggests a relatively high predicative ability of the *in vitro* assay for *in vivo* T cell function. While the VSC of different classes of bulk patient T cells are well documented (Warren et al., 2019), we will first replicate such findings with our spectrum of healthy and *bona fide* exhausted HIV-specific T cells. Subsequently, we will use the VIA to determine the viral inhibition capacity of *in vitro* exhausted T cells generated using various T cell exhaustion setups (see **Box 3**).

Thus, at the end of **Objective 1** we will have achieved 1) two distinct T cell platforms for the *in vitro* exhaustion system; 2) positive and negative controls for the *in vitro* exhaustion system in the form of healthy to *bona fide* exhausted patient T cells; 3) a thorough validation platform for *in vitro* exhausted T cells.

Box 1 | **T cell platform generation**

A good T cell platform for *in vitro* exhaustion systems requires 1) an HIV-specific TCR of 2) high affinity for its cognate peptide and 3) of various HLA subtypes to allow a larger array of downstream applications.

For the recombinant TCR (rTCR) choice, we will compare a rTCR isolated from an elite controller (EC) – thus of high TCR 'strength' for cognate peptide – restricted to HLA-B*57 (which is relatively rare in the general population), and a high-affinity rTCR restricted to HLA-A*2 (which is very prominent in the Caucasian population). While multiple peptide/epitope specificities of rTCRs restricted within these two HLA subtypes would be desirable, as proof of principle we will use an (enhanced) high-affinity TCR against p17 $Gag_{77\text{-}85}$ (SLYNTVATL) peptide that is HLA-A*02 restricted (Varela-Rohena et al., 2008) and a (naturally-occurring) high-affinity TCR against p24 $Gag_{30\text{-}40}$ (KAFSPEVIPMF) peptide that is HLA-B*57 restricted (Berger et al., 2011).

First, we will pursue a rTCR strategy of site-specific integration into the host cell genome by performing a simultaneous native TCR knock-out using CRISPR/Cas9 and delivering the rTCR using a suitable adeno-associated virus (AAV) vector (Chandran & Klebanoff, 2019; Eyquem et al., 2017; Hale et al., 2017). Not only will this approach allow the direct the integration of the rTCR into the native TCR locus, but the deletion of the endogenous TCR using CRISPR/Cas9 will increase the correct pairing of the rTCR α and β chains, thus increasing the overall transduction efficacy. Such advances (among many others) have recently emerged in the cancer immunotherapy field, allowing the engineering of 'genetically safe', powerful, and highly transduced rTCR-T cell populations (Presti et al., 2020).

For the peptide-enriched (gene-unmodified) $CD8^+$ T cells, we will first screen healthy donor (HD) peripheral blood mononuclear cell (PBMC) samples for HLA typing. To be able to best compare with HIV-specific rTCR T cells, we will select all PBMC samples with HLA-B*57 and HLA-A*2. We will isolate $CD8^+$ T cells using magnetic-activated cell sorting (MACS), and the negative fraction resulting from the isolation (i.e. $CD8^+$-depleted PBMCs) will be used as the source of autologous cells for peptide enrichment. With regards to HIV-1 peptides used for enrichment, all $CD8^+$ T cell epitopes restricted to HLA-A*2 and B*57 respectively will be selected from the Los Alamos "A-List" of CTL epitopes (Best-defined CTL/CD8+ Epitope Summary, https://www.hiv.lanl.gov/content/immunology/tables/optimal_ctl_summary.html). $CD8^+$-depleted PBMCs will be pulsed with the HLA-matched HIV-1 peptide pool, and autologous $CD8^+$ T cells will be co-cultured with the peptide-loaded PBMCs for 7-10 days. We expect this experimental setting to raise sufficient numbers of highly reactive HIV-specific $CD8^+$ T cells, given the presence of strong CTL epitopes, helper $CD4^+$ T cells, as well as professional antigen-presenting cells in the co-culture system. At the end of the enrichment period, activated $CD8^+$ T cells will be selected using the Activation-induced Marker Assay (AIM assay, (Reiss et al., 2017)) and sorted using FACS. Lastly, we will use the fluorescently labelled tetramers to determine the breadth and magnitude of the $CD8^+$ T cell response generated following HIV-1 peptide pool stimulation.

Box 2 | **T cell exhaustion validation**

Concerning the flow cytometry-based staining panels (both extracellular and intracellular), fluorescently labeled antibodies will be used that recognize key T cell markers, such as CD4/CD8, CD45RA, CCR7 and CD62L for effector/memory differentiation. For the T cell exhaustion surface staining panel, markers such as PD-1, TIM-3, LAG3, TIGIT, CTLA-4, 2B4, CD160, BTLA, and CD39 will be used, among others (Chen et al., 2020). For the intracellular cytokine staining panel, antibodies recognizing TNFα, IL-2, and IFNγ will be included, since these cytokines are involved the stepwise loss of T cell functionality during exhaustion onset (Wherry, 2011). For the intracellular transcription factor staining panel, the following transcription factors (TFs) that have been associated with T cell dysfunction will be included: Tcf-1, TOX, T-bet, Eomes, NFAT, IRF4, Blimp-1, BAFT (McLane et al., 2019).

For the RNA sequencing analysis, we will first identify differentially expressed genes (DEGs) between T cells from HIV-uninfected individuals, HIV-infected cART-naïve and ART-treated individuals, as well as from elite controllers. Interestingly, while most RNA transcriptomics studies have used bulk $CD8^+$ T cells across these patient groups, here we will use HIV-specific T cells, which will allow 1) the direct probing of the transcriptional profile of antigen-specific T cells involved in HIV control and dysfunction, and 2) the comparison with bulk T cells of previous studies using publicly available data. The data will be obtained from the Gene Expression Omnibus (GEO) (Home - GEO - NCBI (nih.gov)), and the analysis pipeline detailed in Ivanov et al. (2021) will be used. This analysis will permit the identification of DEGs across T cell groups involved in T cell activation, signaling, survival, proliferation, cell cycle progression, metabolism, dysfunction, and others. Second, the mRNA sequencing data will be invaluable in validating the profile of the *in vitro* exhausted HIV-specific T cells generated by our experimental approach, relative to different classes of patient T cells. While we expect *in vitro* exhausted T cells to closely resemble HIV-infected cART-naïve patient T cells as a successful measure of T cell dysfunction, it is likely that T cell exhaustion cannot be completely achieved *in vitro*. Thus, the direct comparison of *in vitro* and *in vivo* exhausted T cells will allow us to make correct inferences of the applicability of findings based on *in vitro* exhausted T cells to patient T cells. Ultimately, the RNA transcriptomics will be used to undoubtedly demonstrate the contribution of the therapeutic approaches that will later be tested to prevent or reverse T cell exhaustion in *in vitro* exhausted T cells.

Regarding the *in vitro* viral inhibition assay (VIA), we will follow the methodological approach perfected by Adams et al. (2021). $CD4^+$ and $CD8^+$ T cells following HIV-1 peptide pool enrichment (see **Box 1**) will be extracted using positive selection from the co-culture. The $CD4^+$ T cell fraction will be infected by spinoculation with a HIV-1 lab strain at a constant multiplicity of infection (MOI), and the $CD8^+$ T cell fraction will be co-cultured with the infected $CD4^+$ T cells at various effector:target (E:T) ratios for 14 days. Importantly, for various classes of patient T cells – where cART-naïve progressors are expected to harbour significantly more HIV-1 DNA compared to individuals on long-term cART or ECs, the HIV-1 DNA in $CD4^+$ T cells will be quantified prior to the VIA and the MOI will be adjusted accordingly.

Objective 2

Based on previous findings (see Zhao et al., 2020 and **Figure 1**), we will perform initial *in vitro* exhaustion systems using a 5-day 10 ng/ml cognate peptide stimulation setup. Therefore, the HLA-A*02 and HLA-B*57-restricted rTCR T cells will be stimulated with p17 $Gag_{77\text{-}85}$ and p24 $Gag_{30\text{-}40}$ peptides respectively, whereas the peptide-enriched gene-unmodified $CD8^+$ T cells will be stimulated with their corresponding peptide pools (10 ng/ml per peptide). Following the 5-day stimulation, the extent of exhaustion will be thoroughly validated with respect to phenotype, transcriptome, and function (refer to **Box 2**). Additionally, the findings of these initial exhaustion systems will be compared and placed in the context of HIV-seropositive T cell data acquired during ***Objective 1***. While we do not expect an extensive T cell exhaustion profile following the 5-day 10 ng/ml experimental system (see **Figure 1**), there are several experimental options worth pursuing. First, given that T cell exhaustion arises in a chronic infection/ cancer setting within two to four weeks (Wherry, 2011), it is likely that the length of *in vitro* T cell stimulation needs to be considerable. While we will test assay durations between 5 and 28 days, given the relative low lifespan of primary T cells in *in vitro* culture, we expect that exhaustion systems with longer durations will be skewed by T cell unresponsiveness and death. As such, we aim to create a relatively short-term *in vitro* system that recapitulates the *in vivo* early exhaustion onset. In this respect, there is increasing understanding in the HIV-1 cure field that an early intervention (3-14 days post infection preferably) – be it cART or broadly neutralizing antibody administration –

could 'save' the $CD8^+$ T cells from dysfunction (Cockerham et al., 2016; Niessl et al., 2020; Nishimura et al., 2021; Sáez-Cirión et al., 2013; Shingai et al., 2014; Veenhuis et al., 2021). Inversely, this implies that the patient $CD8^+$ T cells are quickly engaged on a 'path' to exhaustion in a relatively short timeframe. We hypothesize that by replicating more of the *in vivo* factors involved in chronic viral infections and/or cancers, we could programme T cell exhaustion *in vitro* as well. To achieve this, we will create a T cell exhaustion medium (refer to **Box 3**) that incorporates multiple factors that have been identified to contribute to the dysfunctional state of T cells infiltrating the tumour microenvironment (TME) in cancers and of virus-specific T cells in chronic infections (McLane et al., 2019; Wherry, 2011; Wherry & Kurachi, 2015). We will then define the effect of different T cell exhaustion media on the outcome of the *in vitro* exhaustion system; importantly, we will need to strike a balance between T cell survival and T cell exhaustion. Ultimately, we will validate the final exhaustion setup for both rTCR T cells and peptide-enriched gene-unmodified T cells using the validation panels, and we will situate these results within the framework of T cells from elite controllers and *bona fide* exhausted T cells from cART-naïve HIV-1 progressors.

Thus, at the end of **Objective 2** we will have achieved a highly efficient *in vitro* exhaustion system that generates phenotypically and functionally exhausted T cells.

Box 3 | **T cell exhaustion medium generation**

Few factors beyond chronic antigenic stimulation have been definitively linked to T cell exhaustion maintenance, if not onset; this is in great part due to the complexity of interrelating immune factors involved in chronic viral infections and cancers. Here, we will first explore the impact of soluble exhaustion mediators (i.e. immune-suppressive or immune-potentiating cytokines), such as IL-10, TGFβ, and IFNα/β, among others (McLane et al., 2019; Saeidi et al., 2018). Then, we will study the role of certain tumour metabolites, such as adenosine, nitric oxide, and lactic acid, on the outcome of the *in vitro* exhaustion system (Singer et al., 2011). Importantly, adenosine is a by-product of the oxygen deprivation typical of the TME, and upon binding to cellular receptors can suppress the function of immune cells (Ohta, 2016); similarly, the low glucose availability of the TME impairs T cell glycolysis, and associated metabolic changes lead to T cell dysfunction (Chang et al., 2015). To add to this, due to the high proportion of dying cells within the TME, the local extracellular concentration of potassium greatly exceed 'normal' concentrations, which was found to inhibit T cell nutrient uptake from the extracellular space thereby potentiating T cell "starvation", and to also inhibit TCR-mediated T cell signaling, leading to a state of T cell dysfunction (Eil et al., 2016; Vodnala et al., 2019). Thus, we hypothesize that administering exogenous adenosine and potassium (among others) would help better 'programme' T cell exhaustion *in vitro* by mimicking resource competition within the TME.

However, to protect T cell viability while increasing dysfunction, we assume a cytokine-rich medium will be needed. Thus, we aim to concurrently test the impact of T cell-supportive cytokines such as IL-2, IL-7, IL-15, and IL-21 on T cell (dys)function during the *in vitro* system and compare to the T cell dysfunction achieved

Objective 3

The most well-studied exhaustion reversal strategy across the chronic infection and cancer fields has undoutedly been checkpoint blockade immunotherapy. Numerous studies have shown that 1) PD-1 is a very strong predictor of T cell dysfunction, high viral loads, and negative clinical outcomes during chronic HIV-1 infection, and that 2) *ex vivo* treatment with checkpoint blockade can alleviate the dysfunction of HIV-specific T cells from chronically infected $HIV\text{-}1^+$ individuals (Barber et al., 2006; Mylvaganam et al., 2018; Saeidi et al., 2018; Trautmann et al., 2006; Velu et al., 2009, 2015). To extend current knowledge, we will start by treating *in vitro* exhausted T cells with anti-PD-1 monoclonal antibodies *in vitro*, after which the same validation methods described in **Box 2** will be applied to assess the extent of exhaustion reversal across T cell phenotype and function. Since 1) exhausted immune cells in chronic HIV-1 infection co-express multiple inhibitory receptors and 2) there appears to be relatively little redundancy in these pathways, combinatorial checkpoint blockade therapies are being explored (Chen et al., 2020; Saeidi et al., 2018). As such, the *in vitro* exhausted T cells constitute an optimal platform for the testing of the possible synergistic effect of multiple checkpoint blockade therapies. We will begin testing (combinations of) anti-PD-1, anti-CTLA-4, anti-TIM3, and anti-TIGIT monoclonal antibody therapies, since all four therapies have been examined in previous *in vitro* work (Chen et al., 2020). Of note, to save substantial 'time' from the lifespan of *in vitro* exhausted T cells, we will assess the effect of different monoclonal antibodies *in vitro* by incubating various antibody doses during the VIA, and all other validation methods will be performed at the end (see **Box 2**). A similar experimental approach will be

undertaken to investigate the potential benefit of enhancing T cell costimulation in order to reverse the T cell exhaustion phenotype and function. Briefly, various antibodies targeting co-stimulatory receptors (such as 4-1BB, CD28, ICOS, OX-40, etc.) or stimulatory cytokine receptors (such as IL-15R) will be tested. While theoretically the administration of stronger costimulation signals could serve to amplify T cell exhaustion (Wijewarnasuriya et al., 2020), others have noted a beneficial effect when administering agonistic IL-15, 4-1BB, ICOS, or OX-40 and checkpoint blockade immunotherapy in different HIV-1 and cancer settings (Chen et al., 2020; McLane et al., 2019; Wherry & Kurachi, 2015). Thus, it remains to be seen whether a combinatorial approach will benefit the reversal of T cell exhaustion in our *in vitro* setting.

Recent years have brought the emerging understanding that despite the cessation of antigenic stimulation that normally fuels T cell exhaustion, a fixed state of 'epigenetic scarring' occurs, especially in intronic gene regions that control the expression of key transcription factors and other exhaustion-associated genes (Martin et al., 2021; Yates et al., 2021; Youngblood et al., 2013). This epigenetic 'identity' largely prevents the full reversal of T cell exhaustion despite additional therapeutics (such as checkpoint blockade or early ART initiation), and leads to an impaired memory response with inadequate recall capacities upon antigen rechallenge (Abdel-Hakeem et al., 2021). Importantly, it appears that the lack of epigenetic plasticity of exhausted T cells largely reinforces the expression of the exhaustion-associated transcriptome (Yates et al., 2021), suggesting that a therapeutic strategy aiming to increase the epigenetic remodelling of exhausted T cells would also act at the level of the transcriptome. Recent efforts aiming to reverse the exhaustion-associated epigenetic state include: inhibiting repressive T cell signaling molecules, blocking various transcription factors pathways, reducing exhaustion-promoting metabolic processes, and directly using epigenetic modifier drugs (for a complete overview, see Abhimanyu et al. (2021)). Here we will focus on the latter, specifically on the effect of various histone deacetylase (HDAC) inhibitors, DNA methyltransferase (DNMT) inhibitors, DNA hypomethylating agents, and lysine methyltransferase (KMT) inhibitors, which all have demonstrated some degree of effectiveness in relieving T cell exhaustion across several mouse and human chronic viral infection and cancer models (Abhimanyu et al., 2021; McLane et al., 2019; Vigano et al., 2020). Thus, we will test the effect of individual and combinations of different classes of epigenetic compounds, and assess the extent of epigenetic remodelling of *in vitro* exhausted T cells by changes in T cell phenotype, function, and transcriptional profile. Ideally, this work will highlight the potential of epigenetic modifier drugs to lead to a durable 'remission' of T cell exhaustion. With regards to preventing the very onset of T cell dysfunction, it is likely that a combination of transcriptional and epigenetic modifier drugs administered early during HIV-1 infection could prevent or delay certain exhaustion-associated states from becoming 'fixed'; therefore, we will investigate whether the compounds most effective at reversing T cell exhaustion could also help stave off its onset.

Lastly, a novel research topic is the reinvigoration of $CD8^+$ T cell responses by way of $CD4^+$ T cell help. The importance of $CD4^+$ help for $CD8^+$ T cell function has been established in two distinct immunological settings – either during the priming of $CD8^+$ T cells, or during secondary $CD8^+$ T cell responses. During the former, it is widely agreed that $CD4^+$ help is vital for the establishment of a good antigen-specific $CD8^+$ response; during the latter setting, there is increasing understanding of the importance of $CD4^+$ help for the quality of the $CD8^+$ recall response (Ahrends et al., 2019; Borst et al., 2018; Wiesel & Oxenius, 2012). Importantly, an emerging hypothesis proposes a key role for $CD4^+$ T cell help in protecting $CD8^+$ T cells from differentiating into exhausted progenitor cells (Busselaar et al., 2020). In the context of chronic viral infections, T cell exhaustion is suggested to occur due to the ongoing depletion of $CD4^+$ T cells that often accompanies viral pathogenesis, which in turn impairs the effector differentiation of naïve Ag-specific $CD8^+$ T cells, leading to the formation of a predysfunctional $CD8^+$ T cell subset that can be easily exhausted in the setting of chronic antigenic stimulation. To support this, Wu et al. (2021) showed that $CD4^+$-helped $CD8^+$ T cells demonstrate enhanced functional capacities compared to helpless $CD8^+$ T cells in a mycobacterium tuberculosis infection model, and moreover, helpless $CD8^+$ T cells resemble exhausted T cells from a phenotypic, functional, and transcriptomic perspective. Several manuscripts have now proposed to alleviate the dysfunctional state of cytotoxic T cells by integrating $CD4^+$ T cell help signals (Ahrends et al., 2017; Busselaar et al., 2020; Lichterfeld et al., 2004; Wu et al., 2021); however, whether the addition of $CD4^+$ help can stave off T cell exhaustion beyond the priming of novel $CD8^+$ T cell responses remains to be seen. As proof of concept, here we propose two *in vitro* experimental settings of rescuing T cell exhaustion. First, autologous HIV-specific healthy $CD4^+$ T cells (enriched by peptide pool stimulation as described in **Box 1**) will be incubated with *in vitro* exhausted $CD8^+$ T cells in a viral inhibition assay (VIA), and the extent of T cell exhaustion 'rescue' will be assessed. This setup will determine the ability of $CD4^+$ T cells to improve the *de novo* priming of $CD8^+$ T cells from a pool of precursor and terminally exhausted $CD8^+$ T cells. Additionally, we will also incubate autologous HIV-specific healthy $CD4^+$ T cells during the generation of *in vitro* exhausted $CD8^+$

T cells, to assess whether the presence of $CD4^+$ T cell help can (somewhat) prevent the very onset of T cell exhaustion. Ultimately, should either strategy be successful, future studies could further identify the specific molecular mechanisms of $CD4^+$ help that are largely responsible for the improvement of the dysfunctional $CD8^+$ T cell subsets (Borst et al., 2018; Zhang et al., 2009), and the $CD4^+$ help mediators could be provided exogenously alongside current combinatorial HIV-1 cure strategies. Alternatively, allogeneic healthy HIV-specific $CD4^+$ T cells engineered to resist HIV-1 entry (see Liu et al., 2017)) could be given therapeutically to chronically infected HIV-1 patients in an effort to boost the patients' own HIV-specific $CD8^+$ T cell responses. Altogether, this novel research avenue has the potential to not only generate *de novo* functional $CD8^+$ T cells in chronically infected individuals, but to also prevent the formation of precusor exhausted cytotoxic T cells.

B.2.3 Feasibility & Risk Assessment

One of the possible experimental risks is the generation of (in)sufficient numbers of HIV-specific $CD8^+$ T cells from healthy donor peripheral blood. While it is well known that HIV-specific CTLs are enriched during HIV infection, making it a large source of antigen-specific T cells for the exhaustion system, we would not like to 'taint' the results of the exhaustion assay by using patient T cells (neither from an ART-supressed, aviremic, nor early diagnosed patient). As such, should we not be able to raise sufficient HIV-specific T cell numbers by the method described above, another option would be to use blood from healthy donors who received various experimental HIV-1 vaccinations (Lévy et al., 2021). Indeed, the enrichment of HIV-specific T cells *in situ* by different existing vaccine candidates could provide the necessary threshold of antigen-specific cells for our experimental setting.

Another likely risk of this project concerns the generation of dysfunctional T cells from healthy human T cells *in vitro*. To our knowledge, different research groups have undertaken very diverse experimental approaches in terms of the T cell platforms and stimulation conditions used to generate exhausted T cells (Balkhi et al., 2018; 'Immuno-Oncology. Cellular and Translational Approaches', 2020, Chapter 6; Zhao et al., 2020). Importantly, while these models have been relatively internally validated, there is a lack of replicability and standardization across existing models. To amend these issues, here we aim to 1) compare two distinct T cell platforms; 2) validate *in vitro* exhausted T cells both intrinsically (i.e., phenotypically, functionally, and transcriptomically), but also in comparison to *bona fide* HIV-specific exhausted T cells; 3) optimize the strength and length of peptide stimulation; 4) incorporate other exhaustion-contributing factors. Indeed, other contributors, such as chronic inflammation, high antigenic load, tumour metabolites, inflammatory and immunosuppressive cytokines, cell debris, etc. are a unique selling point of this project as these exhaustion-promoting factors have been linked to exhaustion onset and/or maintenance *in vivo*, but few have been explored *in vitro* (Chang et al., 2015; Eil et al., 2016; Ohta, 2016; Saeidi et al., 2018; Singer et al., 2011; Vodnala et al., 2019). In short, we seek to best mimic the complex *in vivo* situation that results in T cell exhaustion rather than relying on repeated antigenic stimulation via the TCR alone (Wherry & Kurachi, 2015).

B.2.4 Scientific & Societal Impact

Two findings are well-established in the HIV-1 field: first, cytotoxic T cells (CTLs) are crucial in affording long-term protection in HIV-1 infection, and secondly, a failure in CTLs to control HIV infection is linked to chronic HIV-1 infection and AIDS progression. In this context, while it is likely that monotherapy will not be sufficient for a functional HIV-1 cure, improving the function of the T cell compartment is expected to be part of any combinatorial cure strategy. Thus, by aiming to invigorate the HIV-1 specific T cell response, the proposed project will be integral to any functional HIV-1 cure. To this end, the *in vitro* exhaustion system delineated here has the unique benefit of identifying promising HIV-1 T cell-based cure candidates. Indeed, by testing the potential of T cell exhaustion reversal agents to reinvigorate exhausted T cells as well as of exhaustion prevention therapies to stave off the onset of T cell exhaustion, our simple *in vitro* approach could profit both those suffering from the chronic stages of HIV-1 infection as well as those newly diagnosed with HIV-1. Additionally, we expect that the translational benefits of the *in vitro* insights of this project to be immense: by standardizing and thoroughly validating the treatment strategies proposed here, a solid basis for future *in vivo* work will be laid.

While the need to improve the function of patients' cytotoxic T cells is well understood, a popular approach is to replace the 'defective' T cells (such as by adoptive T cell therapy). However, even if successful, this strategy could never benefit the millions of HIV-positive (and at risk!) individuals living in less affluent countries. Their current lack of access to lifelong cART and pre-exposure prophylaxis (among others), together with the fact that developing countries are those under the highest (current and projected) HIV-1 burden, should play a deciding role in the direction of future HIV-1 cure research. We strongly believe that helping improve patients' own T cells (i.e., pursuing

T cell invigoration rather than replacement) is the only *equitable* HIV-1 cure option worth pursuing. Thus, we hope that the research objectives highlighted here will one day serve as an integral part of synergistic treatment options for *all* HIV-infected individuals, such as in combination with latency reversing agents, therapeutic vaccines, or alongside cART.

In the end, our novel approach of tackling T cell exhaustion directly (rather than aiming to eliminate HIV-infected cells) will likely constitute the 'missing link' in the current landscape of HIV-1 cure approaches. Ultimately, given that T cell exhaustion is a shared problem in many chronic viral infections and cancers, the experimental platform proposed here could serve as a common steppingstone for interdisciplinary cure research and insights.

B.2.5 Ethical considerations

For this project, blood samples will be obtained from healthy donors and HIV-positive individuals following written informed consent in accordance with the Declaration of Helsinki.

B.2.6 Works cited

Abdel-Hakeem, M. S., Manne, S., Beltra, J. C., Stelekati, E., Chen, Z., Nzingha, K., Ali, M. A., Johnson, J. L., Giles, J. R., Mathew, D., Greenplate, A. R., Vahedi, G., & Wherry, E. J. (2021). Epigenetic scarring of exhausted T cells hinders memory differentiation upon eliminating chronic antigenic stimulation. *Nature Immunology*, *22*(8), 1008–1019. https://doi.org/10.1038/s41590-021-00975-5

Abhimanyu, Ontiveros, C. O., Guerra-Resendez, R. S., Nishiguchi, T., Ladki, M., Hilton, I. B., Schlesinger, L. S., & DiNardo, A. R. (2021). Reversing Post-Infectious Epigenetic-Mediated Immune Suppression. *Frontiers in Immunology*, *12*(June), 1–19. https://doi.org/10.3389/fimmu.2021.688132

Adams, P., Iserentant, G., Servais, J. Y., Vandekerckhove, L., Vanham, G., & Seguin-Devaux, C. (2021). Cytotoxic CD8+ T Cells Expressing CXCR5 Are Detectable in HIV-1 Elite Controllers After Prolonged In Vitro Peptide Stimulation. *Frontiers in Immunology*, *11*(February), 1–14. https://doi.org/10.3389/fimmu.2020.622343

Ahrends, T., Busselaar, J., Severson, T. M., Bąbała, N., de Vries, E., Bovens, A., Wessels, L., van Leeuwen, F., & Borst, J. (2019). CD4+ T cell help creates memory CD8+ T cells with innate and help-independent recall capacities. *Nature Communications*, *10*(1), 1–13. https://doi.org/10.1038/s41467-019-13438-1

Ahrends, T., Spanjaard, A., Pilzecker, B., Bąbała, N., Bovens, A., Xiao, Y., Jacobs, H., & Borst, J. (2017). CD4+ T Cell Help Confers a Cytotoxic T Cell Effector Program Including Coinhibitory Receptor Downregulation and Increased Tissue Invasiveness. *Immunity*, *47*(5), 848-861.e5. https://doi.org/10.1016/j.immuni.2017.10.009

Balkhi, M. Y., Wittmann, G., Xiong, F., & Junghans, R. P. (2018). YY1 Upregulates Checkpoint Receptors and Downregulates Type I Cytokines in Exhausted, Chronically Stimulated Human T Cells. *IScience*, *2*, 105–122. https://doi.org/10.1016/j.isci.2018.03.009

Barber, D. L., Wherry, E. J., Masopust, D., Zhu, B., Allison, J. P., Sharpe, A. H., Freeman, G. J., & Ahmed, R. (2006). Restoring function in exhausted CD8 T cells during chronic viral infection. *Nature*, *439*(7077), 682–687. https://doi.org/10.1038/nature04444

Berger, C. T., Frahm, N., Price, D. A., Mothe, B., Ghebremichael, M., Hartman, K. L., Henry, L. M., Brenchley, J. M., Ruff, L. E., Venturi, V., Pereyra, F., Sidney, J., Sette, A., Douek, D. C., Walker, B. D., Kaufmann, D. E., & Brander, C. (2011). High-Functional-Avidity Cytotoxic T Lymphocyte Responses to HLA-B-Restricted Gag-Derived Epitopes Associated with Relative HIV Control. *Journal of Virology*, *85*(18), 9334–9345. https://doi.org/10.1128/jvi.00460-11

Borst, J., Ahrends, T., Bąbała, N., Melief, C. J. M., & Kastenmüller, W. (2018). CD4+ T cell help in cancer immunology and immunotherapy. *Nature Reviews Immunology*, *18*(10), 635–647. https://doi.org/10.1038/s41577-018-0044-0

Bucks, C. M., Norton, J. A., Boesteanu, A. C., Mueller, Y. M., & Katsikis, P. D. (2009). Chronic Antigen Stimulation Alone Is Sufficient to Drive CD8 + T Cell Exhaustion . *The Journal of Immunology*, *182*(11), 6697–6708. https://doi.org/10.4049/jimmunol.0800997

Busselaar, J., Tian, S., van Eenennaam, H., & Borst, J. (2020). Helpless Priming Sends CD8+ T Cells on the Road to Exhaustion. *Frontiers in Immunology*, *11*(October), 1–12. https://doi.org/10.3389/fimmu.2020.592569

Castelli, V., Lombardi, A., Palomba, E., Bozzi, G., Ungaro, R., Alagna, L., Mangioni, D., Muscatello, A., Bandera, A., & Gori, A. (2021). Immune Checkpoint Inhibitors in People Living with HIV/AIDS: Facts and Controversies. *Cells*, *10*(9), 2227. https://doi.org/10.3390/cells10092227

Chandran, S. S., & Klebanoff, C. A. (2019). T cell receptor-based cancer immunotherapy: Emerging efficacy

and pathways of resistance. *Immunological Reviews*, *290*(1), 127–147. https://doi.org/10.1111/imr.12772

Chang, C. H., Qiu, J., O'Sullivan, D., Buck, M. D., Noguchi, T., Curtis, J. D., Chen, Q., Gindin, M., Gubin, M. M., Van Der Windt, G. J. W., Tonc, E., Schreiber, R. D., Pearce, E. J., & Pearce, E. L. (2015). Metabolic Competition in the Tumor Microenvironment Is a Driver of Cancer Progression. *Cell*, *162*(6), 1229–1241. https://doi.org/10.1016/j.cell.2015.08.016

Chen, H., Moussa, M., & Catalfamo, M. (2020). The Role of Immunomodulatory Receptors in the Pathogenesis of HIV Infection: A Therapeutic Opportunity for HIV Cure? *Frontiers in Immunology*, *11*(July). https://doi.org/10.3389/fimmu.2020.01223

Cockerham, L. R., Hatano, H., & Deeks, S. G. (2016). Post-Treatment Controllers: Role in HIV "Cure" Research. *Current HIV/AIDS Reports*, *13*(1), 1–9. https://doi.org/10.1007/s11904-016-0296-x

Eil, R., Vodnala, S. K., Clever, D., Klebanoff, C. A., Sukumar, M., Pan, J. H., Palmer, D. C., Gros, A., Yamamoto, T. N., Patel, S. J., Guittard, G. C., Yu, Z., Carbonaro, V., Okkenhaug, K., Schrump, D. S., Linehan, W. M., Roychoudhuri, R., & Restifo, N. P. (2016). Ionic immune suppression within the tumour microenvironment limits T cell effector function. *Nature*, *537*(7621), 539–543. https://doi.org/10.1038/nature19364

Eyquem, J., Mansilla-Soto, J., Giavridis, T., Van Der Stegen, S. J. C., Hamieh, M., Cunanan, K. M., Odak, A., Gönen, M., & Sadelain, M. (2017). Targeting a CAR to the TRAC locus with CRISPR/Cas9 enhances tumour rejection. *Nature*, *543*(7643), 113–117. https://doi.org/10.1038/nature21405

Fenwick, C., Joo, V., Jacquier, P., Noto, A., Banga, R., Perreau, M., & Pantaleo, G. (2019). T-cell exhaustion in HIV infection. *Immunological Reviews*, *292*(1), 149–163. https://doi.org/10.1111/imr.12823

Fromentin, R., DaFonseca, S., Costiniuk, C. T., El-Far, M., Procopio, F. A., Hecht, F. M., Hoh, R., Deeks, S. G., Hazuda, D. J., Lewin, S. R., Routy, J. P., Sékaly, R. P., & Chomont, N. (2019). PD-1 blockade potentiates HIV latency reversal ex vivo in CD4 + T cells from ART-suppressed individuals. *Nature Communications*, *10*(1), 1–7. https://doi.org/10.1038/s41467-019-08798-7

Gallimore, B. A., Glithero, A., Godkin, A., Tissot, A. C., Plückthun, A., Elliott, T., Hengartner, H., & Zinkernagel, R. (1998). Induction and Exhaustion of Lymphocytic Choriomeningitis Virus – specific Cytotoxic T Lymphocytes Visualized Using Class I – Peptide Complexes. *Journal of Experimental Medicine*, *187*(9), 1383–1393. http://jem.rupress.org/content/187/9/1383.full.pdf+html

Hale, M., Lee, B., Honaker, Y., Leung, W. H., Grier, A. E., Jacobs, H. M., Sommer, K., Sahni, J., Jackson, S. W., Scharenberg, A. M., Astrakhan, A., & Rawlings, D. J. (2017). Homology-Directed Recombination for Enhanced Engineering of Chimeric Antigen Receptor T Cells. *Molecular Therapy - Methods and Clinical Development*, *4*(March), 192–203. https://doi.org/10.1016/j.omtm.2016.12.008

Hoffmann, M., Pantazis, N., Martin, G. E., Hickling, S., Hurst, J., Meyerowitz, J., Willberg, C. B., Robinson, N., Brown, H., Fisher, M., Kinloch, S., Babiker, A., Weber, J., Nwokolo, N., Fox, J., Fidler, S., Phillips, R., & Frater, J. (2016). Exhaustion of Activated CD8 T Cells Predicts Disease Progression in Primary HIV-1 Infection. *PLoS Pathogens*, *12*(7), 1–19. https://doi.org/10.1371/journal.ppat.1005661

Immuno-Oncology. Cellular and Translational Approaches. (2020). In S.-L. Tan (Ed.), *Anticancer research* (Vol. 40, Issue 4). Humana Press.

Ivanov, S., Filimonov, D., & Tarasova, O. (2021). A computational analysis of transcriptional profiles from CD8(+) T lymphocytes reveals potential mechanisms of HIV/AIDS control and progression. *Computational and Structural Biotechnology Journal*, *19*, 2447–2459. https://doi.org/10.1016/j.csbj.2021.04.056

Lévy, Y., Lacabaratz, C., Lhomme, E., Wiedemann, A., Bauduin, C., Fenwick, C., Foucat, E., Surenaud, M., Guillaumat, L., Boilet, V., Rieux, V., Bouchaud, O., Girard, P.-M., Molina, J.-M., Morlat, P., Hocqueloux, L., Richert, L., Pantaleo, G., Lelièvre, J. D., & Thiébaut, R. (2021). A Randomized Placebo-Controlled Efficacy Study of a Prime Boost Therapeutic Vaccination Strategy in HIV-1-Infected Individuals: VRI02 ANRS 149 LIGHT Phase II Trial. *Journal of Virology*, *95*(9). https://doi.org/10.1128/JVI.02165-20

Li, J., Huang, H. H., Tu, B., Zhou, M. J., Hu, W., Fu, Y. L., Li, X. Y., Yang, T., Song, J. W., Fan, X., Jiao, Y. M., Xu, R. N., Zhang, J. Y., Zhou, C. B., Yuan, J. H., Zhen, C., Shi, M., Wang, F. S., & Zhang, C. (2021). Reversal of the CD8+ T-Cell Exhaustion Induced by Chronic HIV-1 Infection Through Combined Blockade of the Adenosine and PD-1 Pathways. *Frontiers in Immunology*, *12*(June), 1–11. https://doi.org/10.3389/fimmu.2021.687296

Lichterfeld, M., Kaufmann, D. E., Yu, X. G., Mui, S. K., Addo, M. M., Johnston, M. N., Cohen, D., Robbins, G. K., Pae, E., Alter, G., Wurcel, A., Stone, D., Rosenberg, E. S., Walker, B. D., & Altfeld, M. (2004). Loss of HIV-1-specific CD8+ T cell proliferation after acute HIV-1 infection and restoration by vaccine-induced HIV-1-specific CD4 + T cells. *Journal of Experimental Medicine*, *200*(6), 701–712. https://doi.org/10.1084/jem.20041270

Liu, Z., Chen, S., Jin, X., Wang, Q., Yang, K., Li, C., Xiao, Q., Hou, P., Liu, S., Wu, S., Hou, W., Xiong, Y., Kong, C., Zhao, X., Wu, L., Li, C., Sun, G., & Guo, D. (2017). Genome editing of the HIV co-receptors CCR5 and CXCR4 by CRISPR-Cas9 protects CD4+ T cells from HIV-1 infection. *Cell and Bioscience*, *7*(1), 1–15. https://doi.org/10.1186/s13578-017-0174-2

Martin, G. E., Sen, D. R., Pace, M., Robinson, N., Meyerowitz, J., Adland, E., Thornhill, J. P., Jones, M., Ogbe, A., Parolini, L., Olejniczak, N., Zacharopoulou, P., Brown, H., Willberg, C. B., Nwokolo, N., Fox, J., Fidler, S., Haining, W. N., & Frater, J. (2021). Epigenetic Features of HIV-Induced T-Cell Exhaustion Persist Despite Early Antiretroviral Therapy. *Frontiers in Immunology*, *12*(June), 1–16. https://doi.org/10.3389/fimmu.2021.647688

McBrien, J. B., Kumar, N. A., & Silvestri, G. (2018). Mechanisms of CD8+ T cell-mediated suppression of HIV/SIV replication. *European Journal of Immunology*, *48*(6), 898–914. https://doi.org/10.1002/eji.201747172

McLane, L. M., Abdel-Hakeem, M. S., & Wherry, E. J. (2019). CD8 T Cell Exhaustion During Chronic Viral Infection and Cancer. *Annual Review of Immunology*, *37*, 457–495. https://doi.org/10.1146/annurev-immunol-041015-055318

Mylvaganam, G. H., Chea, L. S., Tharp, G. K., Hicks, S., Velu, V., Iyer, S. S., Deleage, C., Estes, J. D., Bosinger, S. E., Freeman, G. J., Ahmed, R., & Amara, R. R. (2018). Combination anti-PD-1 and antiretroviral therapy provides therapeutic benefit against SIV. *JCI Insight*, *3*(18). https://doi.org/10.1172/jci.insight.122940

Niessl, J., Baxter, A. E., Mendoza, P., Jankovic, M., Cohen, Y. Z., Butler, A. L., Lu, C. L., Dubé, M., Shimeliovich, I., Gruell, H., Klein, F., Caskey, M., Nussenzweig, M. C., & Kaufmann, D. E. (2020). Combination anti-HIV-1 antibody therapy is associated with increased virus-specific T cell immunity. *Nature Medicine*, *26*(2), 222–227. https://doi.org/10.1038/s41591-019-0747-1

Nishimura, Y., Donau, O. K., Dias, J., Ferrando-Martinez, S., Jesteadt, E., Sadjadpour, R., Gautam, R., Buckler-White, A., Geleziunas, R., Koup, R. A., Nussenzweig, M. C., & Martin, M. A. (2021). Immunotherapy during the acute SHIV infection of macaques confers long-term suppression of viremia. *Journal of Experimental Medicine*, *218*(1). https://doi.org/10.1084/JEM.20201214

Ohta, A. (2016). A Metabolic Immune Checkpoint: Adenosine in Tumor Microenvironment. *Frontiers in Immunology*, *7*(MAR), 1–11. https://doi.org/10.3389/fimmu.2016.00109

Perdomo-Celis, F., Taborda, N. A., & Rugeles, M. T. (2019). CD8+ T-cell response to HIV infection in the era of antiretroviral therapy. *Frontiers in Immunology*, *10*(AUG). https://doi.org/10.3389/fimmu.2019.01896

Presti, V. Lo, Buitenwerf, F., van Til, N. P., & Nierkens, S. (2020). Gene augmentation and editing to improve tcr engineered t cell therapy against solid tumors. *Vaccines*, *8*(4), 1–22. https://doi.org/10.3390/vaccines8040733

Reiss, S., Baxter, A. E., Cirelli, K. M., Dan, J. M., Morou, A., Daigneault, A., Brassard, N., Silvestri, G., Routy, J. P., Havenar-Daughton, C., Crotty, S., & Kaufmann, D. E. (2017). Comparative analysis of activation induced marker (AIM) assays for sensitive identification of antigen-specific CD4 T cells. *PLoS ONE*, *12*(10). https://doi.org/10.1371/journal.pone.0186998

Rogan, D. C., & Connors, M. (2021). Immunologic Control of HIV-1: What Have We Learned and Can We Induce It? *Current HIV/AIDS Reports*, *18*(3), 211–220. https://doi.org/10.1007/s11904-021-00545-2

Saeidi, A., Zandi, K., Cheok, Y. Y., Saeidi, H., Wong, W. F., Lee, C. Y. Q., Cheong, H. C., Yong, Y. K., Larsson, M., & Shankar, E. M. (2018). T-cell exhaustion in chronic infections: Reversing the state of exhaustion and reinvigorating optimal protective immune responses. *Frontiers in Immunology*, *9*(NOV), 1–12. https://doi.org/10.3389/fimmu.2018.02569

Sáez-Cirión, A., Bacchus, C., Hocqueloux, L., Avettand-Fenoel, V., Girault, I., Lecuroux, C., Potard, V., Versmisse, P., Melard, A., Prazuck, T., Descours, B., Guergnon, J., Viard, J. P., Boufassa, F., Lambotte, O., Goujard, C., Meyer, L., Costagliola, D., Venet, A., … Rouzioux, C. (2013). Post-Treatment HIV-1 Controllers with a Long-Term Virological Remission after the Interruption of Early Initiated Antiretroviral Therapy ANRS VISCONTI Study. *PLoS Pathogens*, *9*(3). https://doi.org/10.1371/journal.ppat.1003211

Salek-Ardakani, S., & Schoenberger, S. P. (2013). T cell exhaustion: A means or an end? *Nature Immunology*, *14*(6), 531–533. https://doi.org/10.1038/ni.2619

Shingai, M., Donau, O. K., Plishka, R. J., Buckler-White, A., Mascola, J. R., Nabel, G. J., Nason, M. C., Montefiori, D., Moldt, B., Poignard, P., Diskin, R., Bjorkman, P. J., Eckhaus, M. A., Klein, F., Mouquet, H., Lorenzi, J. C. C., Gazumyan, A., Burton, D. R., Nussenzweig, M. C., … Nishimura, Y. (2014). Passive transfer of modest titers of potent and broadly neutralizing anti-HIV monoclonal antibodies block SHIV infection in macaques. *Journal of Experimental Medicine*, *211*(10), 2061–2074.

https://doi.org/10.1084/jem.20132494
Singer, K., Gottfried, E., Kreutz, M., & Mackensen, A. (2011). Suppression of T-cell responses by tumor metabolites. *Cancer Immunology, Immunotherapy*, *60*(3), 425–431. https://doi.org/10.1007/s00262-010-0967-1
Stadtmauer, E. A., Fraietta, J. A., Davis, M. M., Cohen, A. D., Weber, K. L., Lancaster, E., Mangan, P. A., Kulikovskaya, I., Gupta, M., Chen, F., Tian, L., Gonzalez, V. E., Xu, J., Jung, I. young, Joseph Melenhorst, J., Plesa, G., Shea, J., Matlawski, T., Cervini, A., … June, C. H. (2020). CRISPR-engineered T cells in patients with refractory cancer. *Science*, *367*(6481). https://doi.org/10.1126/science.aba7365
Stephenson, K. E. (2018). Therapeutic vaccination for HIV: hopes and challenges. *Current Opinion in HIV and AIDS*, *13*(5), 408–415. https://doi.org/10.1097/COH.0000000000000491
Thorlund, K., Horwitz, M. S., Fife, B. T., Lester, R., & Cameron, D. W. (2017). Landscape review of current HIV 'kick and kill' cure research - some kicking, not enough killing. *BMC Infectious Diseases*, *17*(1), 1–12. https://doi.org/10.1186/s12879-017-2683-3
Trautmann, L., Janbazian, L., Chomont, N., Said, E. A., Gimmig, S., Bessette, B., Boulassel, M. R., Delwart, E., Sepulveda, H., Balderas, R. S., Routy, J. P., Haddad, E. K., & Sekaly, R. P. (2006). Upregulation of PD-1 expression on HIV-specific CD8+ T cells leads to reversible immune dysfunction. *Nature Medicine*, *12*(10), 1198–1202. https://doi.org/10.1038/nm1482
UNAIDS. (2021). *Global HIV-1 statistics fact sheet 2021*. 1–5. https://embargo.unaids.org/static/files/uploaded_files/UNAIDS_2021_FactSheet_en_em.pdf
Varela-Rohena, A., Molloy, P. E., Dunn, S. M., Li, Y., Suhoski, M. M., Carroll, R. G., Milicic, A., Mahon, T., Sutton, D. H., Laugel, B., Moysey, R., Cameron, B. J., Vuidepot, A., Purbhoo, M. A., Cole, D. K., Phillips, R. E., June, C. H., Jakobsen, B. K., Sewell, A. K., & Riley, J. L. (2008). Control of HIV-1 immune escape by CD8 T cells expressing enhanced T-cell receptor. *Nature Medicine*, *14*(12), 1390–1395. https://doi.org/10.1038/nm.1779
Veenhuis, R. T., Garliss, C. C., Bailey, J. R., & Blankson, J. N. (2021). CD8 Effector T Cells Function Synergistically With Broadly Neutralizing Antibodies to Enhance Suppression of HIV Infection. *Frontiers in Immunology*, *12*(July), 1–10. https://doi.org/10.3389/fimmu.2021.708355
Velu, V., Shetty, D. D., Larsson, M., & Shankar, E. M. (2015). Role of PD-1 co-inhibitory pathway in HIV infection and potential therapeutic options. *Retrovirology*, *12*(1), 1–17. https://doi.org/10.1186/s12977-015-0144-x
Velu, V., Titanji, K., Zhu, B., Husain, S., Pladevega, A., Lai, L., Vanderford, T. H., Chennareddi, L., Silvestri, G., Freeman, G. J., Ahmed, R., & Amara, R. R. (2009). Enhancing SIV-specific immunity in vivo by PD-1 blockade. *Nature*, *458*(7235), 206–210. https://doi.org/10.1038/nature07662
Vigano, S., Bobisse, S., Coukos, G., Perreau, M., & Harari, A. (2020). Cancer and HIV-1 Infection: Patterns of Chronic Antigen Exposure. *Frontiers in Immunology*, *11*(June), 1–18. https://doi.org/10.3389/fimmu.2020.01350
Vodnala, S. K., Eil, R., Kishton, R. J., Sukumar, M., Yamamoto, T. N., Ha, N. H., Lee, P. H., Shin, M. H., Patel, S. J., Yu, Z., Palmer, D. C., Kruhlak, M. J., Liu, X., Locasale, J. W., Huang, J., Roychoudhuri, R., Finkel, T., Klebanoff, C. A., & Restifo, N. P. (2019). T cell stemness and dysfunction in tumors are triggered by a common mechanism. *Science*, *363*(6434). https://doi.org/10.1126/science.aau0135
Warren, J. A., Clutton, G., & Goonetilleke, N. (2019). Harnessing CD8+ T cells under HIV antiretroviral therapy. *Frontiers in Immunology*, *10*(FEB), 1–14. https://doi.org/10.3389/fimmu.2019.00291
Wherry, E. J. (2011). T cell exhaustion. *Nature Immunology*, *12*(6), 492–499. https://doi.org/10.1038/ni.2035
Wherry, E. J., & Kurachi, M. (2015). Molecular and cellular insights into T cell exhaustion. *Nature Reviews Immunology*, *15*(8), 486–499. https://doi.org/10.1038/nri3862
Wiesel, M., & Oxenius, A. (2012). From crucial to negligible: Functional CD8 + T-cell responses and their dependence on CD4 + T-cell help. *European Journal of Immunology*, *42*(5), 1080–1088. https://doi.org/10.1002/eji.201142205
Wijewarnasuriya, D., Bebernitz, C., Lopez, A. V., Rafiq, S., & Brentjens, R. J. (2020). Excessive costimulation leads to dysfunction of adoptively transferred T cells. *Cancer Immunology Research*, *18*(6), 732–742. https://doi.org/10.1158/2326-6066.CIR-19-0908
Wu, Y.-J., Barreira-Silva, P., Boyce, S., Powers, J., Kelly, C., & Behar, S. M. (2021). CD4 T Cell Help Prevents CD8 T Cell Exhaustion and Promotes Control of *Mycobacterium tuberculosis* Infection. *SSRN Electronic Journal*. https://doi.org/10.2139/ssrn.3802857
Yates, K. B., Tonnerre, P., Martin, G. E., Gerdemann, U., Al Abosy, R., Comstock, D. E., Weiss, S. A., Wolski, D., Tully, D. C., Chung, R. T., Allen, T. M., Kim, A. Y., Fidler, S., Fox, J., Frater, J., Lauer, G. M., Haining, W. N., & Sen, D. R. (2021). Epigenetic scars of CD8+ T cell exhaustion persist after cure of

chronic infection in humans. *Nature Immunology*, *22*(8), 1020–1029. https://doi.org/10.1038/s41590-021-00979-1

Youngblood, B., Noto, A., Porichis, F., Akondy, R. S., Ndhlovu, Z. M., Austin, J. W., Bordi, R., Procopio, F. A., Miura, T., Allen, T. M., Sidney, J., Sette, A., Walker, B. D., Ahmed, R., Boss, J. M., Sékaly, R.-P., & Kaufmann, D. E. (2013). Cutting Edge: Prolonged Exposure to HIV Reinforces a Poised Epigenetic Program for PD-1 Expression in Virus-Specific CD8 T Cells. *The Journal of Immunology*, *191*(2), 540–544. https://doi.org/10.4049/jimmunol.1203161

Zajac, A. J., Blattman, J. N., Murali-Krishna, K., Sourdive, D. J. D., Suresh, M., Altman, J. D., & Ahmed, R. (1998). Viral immune evasion due to persistence of activated T cells without effector function. *Journal of Experimental Medicine*, *188*(12), 2205–2213. https://doi.org/10.1084/jem.188.12.2205

Zhang, S., Zhang, H., & Zhao, J. (2009). The role of CD4 T cell help for CD8 CTL activation. *Biochemical and Biophysical Research Communications*, *384*(4), 405–408. https://doi.org/10.1016/j.bbrc.2009.04.134

Zhao, M., Kiernan, C. H., Stairiker, C. J., Hope, J. L., Leon, L. G., van Meurs, M., Brouwers-Haspels, I., Boers, R., Boers, J., Gribnau, J., van IJcken, W. F. J., Bindels, E. M., Hoogenboezem, R. M., Erkeland, S. J., Mueller, Y. M., & Katsikis, P. D. (2020). Rapid in vitro generation of bona fide exhausted CD8+ T cells is accompanied by Tcf7 promotor methylation. *PLoS Pathogens*, *16*(6 June), 1–27. https://doi.org/10.1371/journal.ppat.1008555