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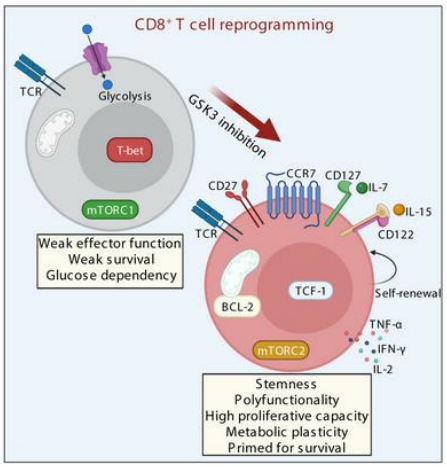
Graphical abstract



Reprogramming dysfunctional CD8$^+$ T cells to promote properties associated with natural HIV control

Federico Perdomo-Celis[1], Caroline Passaes[1], Valérie Monceaux[1], Stevenn Volant[2], Faroudy Boufassa[3], Pierre de Truchis[4], Morgane Marcou[4], Katia Bourdic[5], Laurence Weiss[6], Corinne Jung[6], Christine Bourgeois[5], Cécile Goujard[7], Laurence Meyer[3], Michaela Müller-Trutwin[1], Olivier Lambotte[5], and Asier Sáez-Cirión[1,*]

[1]Institut Pasteur, Université Paris Cité, Unité HIV Inflammation et Persistance, F-75015 Paris, France.

[2]Institut Pasteur, Université Paris Cité, Hub Bioinformatique et Biostatistique, F-75015 Paris, France.

[3]Université Paris Saclay; Inserm CESP U1018; APHP, Department of Public Health, Bicêtre Hospital, Paris Saclay, France.

[4]Université Paris-Saclay, AP-HP Hôpital Raymond Poincaré, Garches, France

[5]Université Paris-Saclay, AP-HP, Bicêtre Hospital, UMR1184 INSERM CEA, Le Kremlin Bicêtre, France.

[6]Université de Paris Cité, AP-HP, Paris Centre, Hôtel Dieu, Paris, France

[7]Université Paris-Saclay, AP-HP, Hôpital Bicêtre, DMU 7, Inserm U1018, CESP, 94290, Le Kremlin Bicêtre, France.

*Corresponding author:

Asier Sáez-Cirión, Institut Pasteur, HIV inflammation and persistance, 25 rue du Docteur Roux, 75015 Paris, France. Tel : +33145688944. E-mail : asier.saez-cirion@pasteur.fr

ABSTRACT

Virus-specific $CD8^+$ T cells play a central role in HIV-1 natural controllers to maintain suppressed viremia in the absence of antiretroviral therapy. These cells display a memory program that confers them stemness properties, high survival, polyfunctionality, proliferative capacity, metabolic plasticity, and antiviral potential. The development and maintenance of such qualities by memory $CD8^+$ T cells appear crucial to achieving natural HIV-1 control. Here we show that targeting the signaling pathways Wnt/TCF-1 and mTORC through GSK3 inhibition to reprogram HIV-specific $CD8^+$ T cells from non-controllers promoted functional capacities associated with natural control of infection. Features of such reprogrammed cells included the enrichment in TCF-1^+ less-differentiated subsets, superior response to antigen, enhanced survival, polyfunctionality, metabolic plasticity, less mTORC1-dependency, improved response to γ-chain cytokines and stronger HIV suppressive capacity. Thus, such $CD8^+$ T cell reprogramming, combined with other available immunomodulators, might represent a promising strategy for adoptive cell therapy in the search for an HIV-1 cure.

Keywords: $CD8^+$ T cell; reprogramming; stemness; HIV-1; mTORC; immunotherapy.

INTRODUCTION

For activation and differentiation, $CD8^+$ T cells require T cell receptor (TCR) signals provided by peptide/major histocompatibility complex class I, together with costimulation, and cytokines. The amount and duration of these signals influence the priming and fate of memory $CD8^+$ T cells (1). Human immunodeficiency virus type 1 (HIV-1) infection is an example of the different outcomes a $CD8^+$ T cell can reach according to the quality of the priming received early after infection. For instance, HIV-1 controllers (HICs), who represent less than 0.5% of people living with HIV-1, control viremia for long periods in the absence of antiretroviral therapy (ART), and such control is associated with strong HIV-specific $CD8^+$ T cell responses (2, 3). Compelling studies indicate that HIV-specific $CD8^+$ T cells from HICs exhibit stemness properties, high survival, proliferative potential, and polyfunctionality upon antigen stimulation (4–7). Recently, we demonstrated that HIV-specific central memory $CD8^+$ T cells from HICs have a stem-like transcriptional program, characterized by enhanced polyfunctionality and survival, compared with cells from non-controllers on ART (8). Moreover, we reported that early in primary simian immune deficiency virus (SIV) infection there is a divergence in the memory compartment of SIV-specific $CD8^+$ T cells of macaques that naturally controlled or did not control infection later in the chronic phase. In this animal model, SIV controllers developed stem-like SIV-specific $CD8^+$ T cells, which preceded the optimal maturation of the $CD8^+$ T cell response (i.e., acquisition of SIV-suppressive properties) during infection (9). Contrary to cells from HICs or SIV controllers, virus-specific memory $CD8^+$ T cells from non-controllers exhibit an effector-like and exhausted profile, limited survival capacity, and poor antiviral potential throughout infection (8–10). Overall, these observations are consistent with tuned priming of virus-specific $CD8^+$ T cells early after infection in natural controllers, promoting long-lived stem-like memory responses that mature throughout infection, whereas

there is a skewed or non-regulated CD8$^+$ T cell priming in non-controllers that promotes short-lived effector-like responses.

In part, the stem-like profile of memory CD8$^+$ T cells is mediated by the transcription factor TCF-1, a downstream component of the Wnt/β-catenin pathway (11). As such, TCF-1 regulates stemness properties of CD8$^+$ T cells, including longevity, self-renewal, and the potential to differentiate into multiple subsets (12). Evidence from chronic infection models indicates that stem-like memory TCF-1$^+$ CD8$^+$ T cells have an enhanced capacity to contain infection and respond to secondary viral challenges (13–15). Fittingly, we and others have demonstrated that virus-specific CD8$^+$ T cells from HIV-1 and SIV controllers exhibit higher levels of TCF-1 compared with cells from non-controllers (7, 9, 16), further suggesting that stem-like memory cells exert a protective role in HIV-1/SIV infections.

Several metabolic cues, such as the mammalian target of rapamycin complex (mTORC) 1 and 2, also regulate CD8$^+$ T cell fate, longevity, and effector functions (17). Constitutive activation of the mTORC1 pathway results in terminal differentiation of CD8$^+$ T cells and lack of long-term memory, whereas inhibition of mTORC2 leads to excessive activation and cell death (18). Accordingly, mTORC1 inhibition with rapamycin increases the number, quality, and survival of stem-like memory CD8$^+$ T cells (19, 20). In line with those data, we previously showed that HIV-specific memory CD8$^+$ T cells from HICs maintain metabolic plasticity and preferential activation of the mTORC2 pathway, whereas cells from non-controllers are more dependent on glycolysis and mTORC1 (8). Thus, the ability to use diverse metabolic resources, as well as the suitable engagement of mTORC1 and 2 pathways, contribute to the enhanced functionality and longevity of memory HIV-specific CD8$^+$ T cells from HICs.

Having identified some signaling pathways and metabolic cues that appear important for natural HIV-1/SIV control, we hypothesized that reprogramming of HIV-specific $CD8^+$ T cells from non-controllers to exhibit characteristics of natural controllers could boost their antiviral potential. We recently showed that metabolic reprogramming with IL-15 increases mitochondrial respiration in $CD8^+$ T cells from non-controllers, contributing to improve their viral-suppressive capacity ex vivo (8). In the present study, we evaluated the potential of $CD8^+$ T cell reprogramming for inducing stem-like properties in cells derived from non-controller individuals, via the manipulation of the TCF-1 and mTORC pathways. We found that, after reprogramming, virus-specific $CD8^+$ T cells from non-controllers acquire phenotypic, transcriptional, metabolic, and functional attributes associated with natural control of HIV-1 infection. Our results shed light on the potential of such $CD8^+$ T cell reprogramming as novel therapeutics in the search for an HIV-1 cure.

RESULTS

CD8$^+$ T cell reprogramming towards a stem-like memory profile

To test the hypothesis that CD8$^+$ T cell reprogramming can invigorate stem-like properties, we used the glycogen synthase kinase-3 (GSK3) inhibitor 6-Bromoindirubin-3'-oxime (BIO), which modulates pathways involved in the generation and maintenance of stem-like CD8$^+$ T cells, such as Wnt/β-catenin and mTORC2 (12), that we and others have found to be upregulated in CD8$^+$ T cells from natural controllers (7, 8, 16). We first characterized the effect of BIO-mediated reprogramming on bulk CD8$^+$ T cells from people without HIV in terms of the memory phenotype (Supplementary Fig. 1A). Of note, we performed dose-effect experiments to stablish the optimal dose and time for BIO treatment. We chose 3 μM and 12 hs incubation because under these conditions we observed the highest effects on the expression of representative phenotypic markers, and no drug-induced cellular toxicity (Supplementary Fig. 1B). Treatment with BIO (in the absence of further stimulation) was accompanied by upregulation of surface expression of CCR7 and CD27 (Figure 1A). Accordingly, we observed an enrichment of CD8$^+$ T cells with a less-differentiated stem cell memory (TSCM) and central memory (TCM) phenotype, and a decrease of more-differentiated effector memory (TEM), and terminal effector (TTE) cells (Figure 1A-C). We confirmed the effect of the GSK3 inhibitor by the enhanced expression of TCF-1 (Figure 1D). To confirm that these changes were related to the regulation of GSK3 activity, we also assessed the impact of TWS119, another GSK3 inhibitor previously used to promote stem-like memory CD8$^+$ T cells (21). The treatment with both GSK3 inhibitors under parallel experimental conditions induced CCR7, CD27, and TCF-1 (Supplementary Fig 1C), increase of TSCM and TCM cells, and decrease of TEM cells (Supplementary Fig. 1D). However, BIO

consistently showed a greater effect than TWS119 (Supplementary Fig. 1C and D) and was used throughout the study. These results confirm that transient exposure to a GSK3 inhibitor, hereinafter referred to as $CD8^+$ T cell reprogramming, promotes a memory-like phenotype in $CD8^+$ T cells.

We wondered whether reprogrammed $CD8^+$ T cells maintained their memory-like profile upon activation. Following anti-CD3/CD28 stimulation, reprogrammed $CD8^+$ T cells, when compared to cells in the control condition, exhibited lower expression of the activation markers HLA-DR and CD38, the inhibitory receptors PD-1, LAG-3, and TIM-3, and the transcription factors T-bet and BLIMP-1, which are associated with an effector $CD8^+$ T cell profile (Figure 1E and Supplementary Fig. 1E) (22). On the contrary, reprogrammed $CD8^+$ T cells maintained higher levels of the transcription factors BCL-6 and TCF-1, associated with a memory profile (23, 24), as well as CD127, critical for $CD8^+$ T cell survival (Figure 1E) (25). In addition, reprogrammed cells exhibited higher expression of TOX. While this transcription factor regulates the transcriptional and epigenetic signature of exhausted T cells (26), it also restricts terminal effector differentiation and allows the persistence of antiviral $CD8^+$ T cells (27, 28), and is found in polyfunctional memory $CD8^+$ T cells (16). Consistent with the higher expression of CD127 and restriction of T-bet, reprogrammed $CD8^+$ T cells showed lower levels of activation-induced cell death than cells in the control condition (Figure 1F). The effects of $CD8^+$ T cell reprogramming were also observed in the setting of a lower and higher TCR-mediated stimulation with anti-CD3/CD28 ± ICAM-1 (29) since reprogrammed $CD8^+$ T cells maintained lower frequencies of $T\text{-}bet^+$ cells than non-reprogrammed cells (Supplementary Fig. 1F and G).

Reprogrammed $CD8^+$ T cells show enhanced polyfunctionality

As reprogramming of $CD8^+$ T cells decreased the proportion of effector cells and restricted cell activation, we wondered whether reprogrammed $CD8^+$ T cells could acquire functional capacity. Consistent with the restriction of an effector-like transcriptional program, reprogrammed total $CD8^+$ T cells had lower expression of granzyme B and IL-2 than non-reprogrammed cells upon stimulation with anti-CD3/CD28 , although there was no change in IFN-γ production (Figure 1G). In contrast, there was a marked increase in the capacity of reprogrammed $CD8^+$ T cells to produce TNF-α and overall increased polyfunctionality (Figure 1G and H). We did not observe significant differences between non-reprogrammed vs reprogrammed cells in the expression of the effector molecules on cells responding to simulation on a per cell basis (e.g. fluorescence intensity of granzyme B in granzyme B^+ cells), (Supplementary Fig. 1H). Higher polyfunctionality was also observed after stimulation with anti-CD3/CD28 + ICAM-1 (Supplementary Fig. 1I).

We also assessed the impact of $CD8^+$ T cell reprogramming on the proliferative capacity and effector maturation upon sequential polyclonal re-stimulation. Interestingly, reprogramming did not affect $CD8^+$ T cell capacity to execute 1-3 division cycles but prevented extensive proliferation (≥4 cell divisions) and maintained a higher proportion of quiescent cells (Supplementary Fig. 1J). This pattern of regulated proliferation of reprogrammed cells was associated with a lower coexpression of PD-1, LAG-3, TIM3, and TIGIT on $CD8^+$ T cells after re-stimulation (Supplementary Fig. 1K). Notably, reprogramming did not irreversibly arrest effector maturation of less-differentiated TCM and transitional memory (TTM) cells since these subsets could readily upregulate T-bet under the re-stimulation setting (Supplementary Fig. 1L). Collectively, these results indicate that $CD8^+$ T

177 cell reprogramming induces TNF-α production and enhanced polyfunctionality, while
178 maintaining responsiveness to recall.
179
180 **Reprogramming modifies the functional properties and transcriptional signature of all**
181 **CD8$^+$ T cell memory subsets**
182 We asked if the changes induced by cell reprogramming were related to phenotype
183 conversion between CD8$^+$ T cell subpopulations or were intrinsic to each subset. To solve
184 this issue, we isolated TCM, TTM, TEM, and TTE CD8$^+$ T cells and treated them with the
185 GSK3 inhibitor, followed by resting or stimulation with anti-CD3/CD28. In basal conditions,
186 cell reprogramming caused the upregulation of CCR7 and CD27 in all cell subsets (Figure
187 2A). Thus, the enrichment in less-differentiated cells observed after CD8$^+$ T cell
188 reprogramming (Figure 1A-C) is most likely the result of the conversion of CD27$^-$ and CCR7$^-$
189 cells into CD27$^+$ and CCR7$^+$ subsets. In addition, reprogramming further enhanced the
190 expression of CD28 and TCF-1 in TCM and TTM cells (Figure 2A), while preventing the loss
191 of CD127 and limiting the upregulation of T-bet upon stimulation of CD8$^+$ T cell memory
192 subsets (Figure 2B). Notably, reprogramming of TCM and TTM cells restrained the
193 expression of granzyme B, IFN-γ, and IL-2, but TEM and TTE cells maintained these effector
194 properties (Figure 2C). As was observed with bulk CD8$^+$ T cells, all reprogrammed CD8$^+$ T
195 cell subsets exhibited an increased TNF-α production (Figure 2C).
196
197 To better characterize the changes induced in reprogrammed CD8$^+$ T cells, we
198 performed a gene expression analysis of sorted memory cells, analyzing 96 genes associated
199 with CD8$^+$ T cell function, differentiation, metabolism, and survival (see Supplementary Table
200 1). A principal component analysis (PCA) focused on TCM cells showed that reprogrammed

and non-reprogrammed cells had a distinct gene expression profile (Figure 2D). In basal conditions, compared with non-reprogrammed cells, reprogrammed TCM cells exhibited lower levels of *IFNG*, *BATF*, *IFNGR1*, and *GZMK* genes, which are associated with an effector lineage (30, 31), as well as *CD8A*, *CD244*, and *HAVCR2* (which codifies for TIM3), which are associated with $CD8^+$ T cell activation and exhaustion (32) (Figure 2E). Reprogrammed $CD8^+$ T cells exhibited in contrast a gene profile suggestive of metabolic quiescence (33), with lower levels of key metabolic regulators active during T cell activation, including *MLST8* (which codifies for MTOR Associated Protein, LST8 Homolog; required for mTORC pathway activation (34)), *ESRRA* (which codifies for Estrogen-related receptor α; metabolic regulator of effector T cells (35)), *GSL* and *GSL2* (which codify for glutaminase 1 and 2, respectively (36)), and *PRKAA1* (which codifies for AMP-activated protein kinase; important regulator of cell catabolic pathways (37)) (Figure 2E). Importantly, after anti-CD3/CD28 stimulation, reprogrammed TCM cells had higher levels of the antiapoptotic gene *BCL2* than non-reprogrammed cells, and lower levels of effector-associated genes *FASLG*, *HAVCR2*, and *GZMB*, as well as *PRKAA1* (Figure 2E). The gene expression profile in reprogrammed TTM, TEM, and TTE was also modified by reprogramming (Supplementary Fig. 2), and, among others, higher expression of *BCL2* and the mTORC2-related gene *CDC42* was observed in these memory subsets (Figure 2E, Supplementary Fig. 2, and Supplementary Table 2), explaining in part the improved survival capacity of reprogrammed $CD8^+$ T cells.

The stem-like profile and polyfunctionality of reprogrammed $CD8^+$ T cells is associated with the downregulation of anabolic metabolism and the mTORC1 pathway

Considering that regulation of cell metabolism and mTORC pathways is a characteristic of memory-like $CD8^+$ T cells (38), as we and others have reported for cells from HICs (8, 39), and that $CD8^+$ T cell reprogramming readily modulated metabolism-related

genes, we performed functional assays to evaluate the metabolic profile of reprogrammed cells. After anti-CD3/CD28 stimulation, non-reprogrammed cells increased their 2-NBDG and BODIPY labelling (commonly used as indicators of glucose and lipid uptake), augmented the mitochondrial mass, and the production of reactive oxygen species (ROS) (Figure 3A and B), a profile characteristic of recently activated T cells (33). However, reprogrammed $CD8^+$ T cells showed lower levels of each of these metabolic parameters (Figure 3A and B), consistent with reduced anabolic metabolism.

We next focused on the mTORC pathways and evaluated their activation by analyzing the phosphorylation of ribosomal S6 (pS6 Ser235/236) and AKT (pAKT Ser473) proteins, markers of activation of mTORC1 and mTORC2, respectively (40). Upon activation in control conditions, $CD8^+$ T cells upregulated pS6 in parallel with pAKT ($pS6^+$ $pAKT^+$ cells; Figure 3C). Consistent with the reported modulation of the mTORC1 pathway by GSK3 inhibitors (20), reprogrammed $CD8^+$ T cells had less upregulation of pS6 (Figure 3C). Notably, reprogrammed $CD8^+$ T cells had higher levels of $pS6^-$ $pAKT^+$ (Figure 3C), confirming that reprogrammed cells maintained a relative metabolic quiescence supported by preferential upregulation of the mTORC2 pathway. As shown above, reprogrammed $CD8^+$ T cells had increase capacity to produce TNF-α upon polyclonal stimulation. When analyzing IFN-γ and IL-2 production among TNF-α-producing $CD8^+$ T cells, two major subpopulations were identified: IFN-γ^+ IL-2^+ and IFN-γ^- IL-2^-, the latter corresponding to cells producing TNF-α only and being increased among reprogrammed cells (Figure 3D). In keeping with our previous studies on cells from HICs (8), the production of TNF-α by reprogrammed cells was less dependent on mTORC1. This was particularly the case for cells only producing TNF-α, since the proportion of $pS6^+$ cells was lower in this subset relative to cells co-producing IFN-γ and

IL-2 (Figure 3D). Altogether, these data indicate that the stem-like profile and polyfunctionality observed in reprogrammed CD8$^+$ T cells are linked to the active regulation of anabolic metabolism, mTORC1 inhibition, and preferential engagement of mTORC2. Our data also highlight the differential role of mTORC pathways on the regulation of CD8$^+$ T cell effector functions.

Reprogramming of HIV-specific CD8$^+$ T cells promotes polyfunctionality, survival, and expansion

Our results on polyclonally-stimulated cells from people without HIV indicated that reprogramming improves several functional capacities of CD8$^+$ T cells, without impairing others. However, HIV-specific CD8$^+$ T cells from non-controllers have a biased program characterized by exhaustion, low survival, and poor antiviral potential (8). Thus, we explored if HIV-specific CD8$^+$ T cells from non-controller individuals (people on ART to suppress viremia [Supplementary Table 3]) can also benefit from reprogramming and acquire properties found in HIV-specific CD8$^+$ T cells associated with natural control of infection. First, we evaluated the effect of reprogramming on the phenotype of CD8$^+$ T cells labeled with HIV-specific HLA dextramers. In line with the results on total CD8$^+$ T cells from people without HIV, a Uniform Manifold Approximation and Projection (UMAP) analysis focused on HIV dextramer$^+$ cells revealed enrichment of populations with a less-differentiated phenotype after reprogramming (Figure 4A), linked to the upregulation of CCR7, CD27, and TCF-1 (Figure 4B), increase in the proportions of naïve/TSCM and TCM cells, and decrease of TEM and TTE cells (Figure 4C).

Next, we evaluated the functionality of HIV-specific CD8$^+$ T cells, detected by the peptide-induced production of IFN-γ, IL-2, TNF-α, or by the expression of the degranulation

marker CD107a. Reprogrammed $CD8^+$ T cells contained a significantly higher frequency of total HIV-specific responses (Figure 4D), that were enriched in TCM cells (Figure 4E). Instead, non-reprogrammed cells were mostly TEM and TTE cells (Figure 4E). Notably, higher production of TNF-α by reprogrammed cells was observed in terms of both frequency (Supplementary Fig. 3A) and level of expression (Figure 4F) in HIV-specific $CD8^+$ T cells, whereas there were no differences in the levels of CD107a, IFN-γ, granzyme B, and IL-2 (Figure 4F). An overall increased polyfunctionality was observed in reprogrammed HIV-specific $CD8^+$ T cells relative to non-reprogrammed cells (Figure 4G). Of note, the survival capacity of reprogrammed HIV-specific cells was higher relative to non-reprogrammed cells (Figure 4H and Supplementary Fig. 3B), in agreement with our observation that reprogramming induced the upregulation of anti-apoptotic factors (Figure 2E). This resulted in enhanced survival of HIV-specific cells over 6 days culture in the setting of sequential peptide re-stimulation (Figure 4I).

We next wondered whether the effects of $CD8^+$ T cell reprogramming are restricted to HIV-specific cells or also impact other antigen specific cells, such as those specific of human cytomegalovirus (HCMV). When analyzing HCMV-specific $CD8^+$ T cells from people without HIV we did not observe an increase in the total frequency of HCMV-specific $CD8^+$ T cells after reprogramming (Supplementary Fig. 4A and B). Nonetheless, reprogrammed HCMV-specific were enriched in a TCM phenotype (Supplementary Fig. 4C) and had higher TNF-α expression relative to non-reprogrammed cells (Supplementary Fig. 4D). We next performed parallel stimulations with HCMV pp65 or HIV Gag peptides and evaluated the phenotype and function of antigen-specific cells from the same individuals with HIV. In line with our previous results (Figure 4F), we did not observe significant differences in the frequencies of $CD107a^+$

or IFN-γ^+ HCMV or HIV-specific $CD8^+$ T cells between reprogrammed versus non-reprogrammed cells (Supplementary Fig. 4E). Instead, we readily observed an increase of TNF-α^+ HCMV and HIV-specific $CD8^+$ T cells in reprogrammed cells relative to non-reprogrammed cells. The enhancement appeared stronger for HIV-specific cells, since the Δ change in TNF-α production by reprogrammed/non-reprogrammed cells was higher in HIV-specific versus HCMV-specific cells (Supplementary Fig. 4F). Overall, these data suggest that the effects of reprogramming might be stronger in HIV-specific cells than in HCMV-specific cells, which may be related to metabolic and functional differences in these antigen-specific populations in the same host (8, 41).

Collectively, these results indicate that reprogramming of HIV-specific $CD8^+$ T cells from non-controllers promotes a quantitatively and qualitatively superior response to antigen stimulation, reflected in higher functionality, survival, and expansion capacity, which are features observed in cells from natural HIV-1 controllers.

Metabolic plasticity restored in reprogrammed HIV-specific $CD8^+$ T cells

We next assessed whether reprogramming could improve the marked dependency on mTORC1 and glycolysis that characterizes HIV-specific $CD8^+$ T cells from HIV-1 non-controllers (8). Stimulation of non-reprogrammed cells from non-controllers with HIV-1 Gag peptides resulted in upregulation of pS6 (Supplementary Fig. 5A), both alone and together with pAKT (Figure 4J). In contrast, reprogrammed HIV-specific $CD8^+$ T cells had lower proportions of $pS6^+$ $pAKT^-$ cells (Figure 4K). A significantly lower expression of pS6 was also observed on a per-cell basis in reprogrammed HIV-specific $CD8^+$ T cells (Figure 4K and Supplementary Fig. 5A). In contrast, we did not observe significant differences in the intensity of pAKT in reprogrammed cells (Figure 4J and K). These data indicate that reprogrammed

HIV-specific CD8$^+$ T cells had a diminished dependency on mTORC1 for supporting a strong antigen-induced response, while preserving mTORC2 activation to exert their functions. We additionally evaluated glucose dependency of reprogrammed HIV-specific CD8$^+$ T cells in an assay where Gag peptide stimulation was performed in medium with versus without glucose (8). Glucose deprivation decreased the frequency of total HIV-specific cells at comparable levels in reprogrammed versus non-reprogrammed cells (Supplementary Fig. 5B and C). However, reprogrammed cells maintained higher production of TNF-α despite glucose deprivation (Supplementary Fig. 5D). Altogether, these data indicate that reprogramming of HIV-specific cells from non-controllers promotes metabolic plasticity and decreases metabolic restrictions.

Reprogramming reinvigorates CD8$^+$ T cells to suppress HIV-1 replication

To further elucidate the antiviral potential of reprogrammed CD8$^+$ T cells, we evaluated the capacity of non-stimulated cells from non-controller individuals under ART to suppress HIV-1 infection of autologous CD4$^+$ T cells (42). According to our previous studies (8, 10, 43), non-reprogrammed CD8$^+$ T cells from HIV-1 non-controllers under ART exhibited poor capacity to counteract HIV-1 infection when co-cultured with infected CD4$^+$ T cells; in contrast, reprogramming strongly improved the capacity of CD8$^+$ T cells to suppress HIV-1 infection (Figure 5A and B, and Supplementary Fig. 6A). It should be noted that we cannot exclude that, at the time of starting the coculture, the frequency of HIV-specific CD8$^+$ T cells was higher upon reprogramming, due to the promotion of the survival capacity of the cells. However, we did not observe differences in the frequency of IFN-γ^+ CD8$^+$ T cells (presumably HIV-specific) between reprogrammed and non-reprogrammed cells at the end of the coculture (day 7; Figure 5C), but reprogrammed IFN-γ^+ CD8$^+$ T cells maintained a memory-like profile,

with higher expression of CCR7 and decreased expression of the inhibitory receptors PD-1 and LAG-3 (Figure 5D and Supplementary Fig. 6B). Therefore, our data indicate that reprogramming of CD8$^+$ T cells from non-controllers strengthens their direct anti-HIV potential while restricting the acquisition of a terminally differentiated, exhausted program.

Reprogrammed CD8$^+$ T cells respond better to homeostatic γ-chain cytokines

The long-term maintenance of memory T cells requires an optimal response to the γ-chain cytokines IL-7 and IL-15 for self-renewal (44). Thus, we evaluated how reprogramming impacted the capacity of CD8$^+$ T cells to respond to these homeostatic cytokines. We have shown that reprogrammed bulk CD8$^+$ T cells were characterized by higher levels of CD127 (IL-7Rα chain; Figure 1E). We also found that reprogrammed CD8$^+$ T cells had higher expression of CD122 (IL-2Rβ chain, component of the IL-15 receptor) and the IL-15Rα chain CD215 (Supplementary Fig. 7A and B), as well as a higher proportion of cells co-expressing the transcription factor Eomesodermin (Eomes) and CD122 (Figure 6A). This may be related to previous observations showing that TCF-1 regulates the expression of Eomes, which in turn promotes CD122 (24).

The increased expression of IL-7 and IL-15 receptors in reprogrammed bulk CD8$^+$ T cells from people without HIV was reflected in augmented proliferation in response to both cytokines when compared to non-reprogrammed cells (Figure 6B). As expected, the proliferative potential was higher in naïve/TSCM and TCM cells than in more differentiated cells (Figure 6B). Reprogramming also improved the proliferative response to IL-15 of HIV dextramer$^+$ cells from HIV-1 non-controllers (Figure 6C and D). Finally, the maturation of the effector response induced by IL-15 in terms of upregulation of T-bet was comparable between

371 reprogrammed and non-reprogrammed cells (Supplementary Fig. 7C). Overall, our results
372 show that CD8$^+$ T cell reprogramming improves the response to homeostatic γ-chain
373 cytokines in cells with otherwise diminished capacity.
374
375 **DISCUSSION**
376
377 We report that reprogramming of HIV-specific CD8$^+$ T cells from HIV-1 non-controllers
378 via GSK3 inhibition confers them higher polyfunctionality, survival, expansion capacity,
379 diminished dependency on mTORC1 and glucose, better responsiveness to γ-chain
380 cytokines, and strong HIV-1 suppressive capacity. These characteristics have been
381 previously associated with efficient responses in natural HIV-1 controllers (4–8). Thus, such
382 CD8$^+$ T cell reprogramming represents a promising option to enhance the efficacy of cell-
383 based therapies for HIV-1 infection.
384
385 Our choice of targeting GSK3 was based on the implication of this molecule in the
386 modulation of several intracellular pathways that we have identified to be of potential
387 relevance in virus-specific CD8$^+$ T cells from HIV-1 (8) and SIV natural controllers (9). BIO
388 and TWS119, the two molecules that we used here, are highly selective inhibitors of GSK3
389 and induce the activation of the Wnt/β-catenin/TCF-1 pathway (45). Higher TCF-1 expression
390 is a characteristic of virus-specific CD8$^+$ T cells in HIV-1 and SIV controllers (7, 9, 16), and
391 the intensification of its function promotes CD8$^+$ T cell stemness, which associates with potent
392 viral and tumor control (7, 46). It is also well described that stem-like memory TCF-1$^+$ CD8$^+$
393 T cells have strong antiviral potential and therapeutic benefit (13–15, 47, 48), related to their
394 longevity and enhanced ability to expand and differentiate into effector subsets (48, 49). In

addition, GSK3 interacts with the mTOR signaling network (50), and its inhibition promotes the activation of the mTORC2 pathway (51). The mTORC2 pathway promotes the generation of memory $CD8^+$ T cells (52) and is preferentially upregulated in HIV-specific $CD8^+$ T cells from HICs (8).

According to the effects of GSK3 inhibition, in our study, we show that $CD8^+$ T cell reprogramming promoted the expression of TCF-1, a TCM and TSCM phenotype, as well as restrained effector differentiation. Reprogrammed $CD8^+$ T cells also regulated proliferation to maintain quiescence, and to limit the consequences of excessive activation (21, 53, 54). Correspondingly, reprogrammed $CD8^+$ T cells shifted their transcriptomic profile towards a program of quiescence and survival, and downregulated several genes associated with anabolic metabolism (55), reflected in lower glucose and lipid consumption, and mitochondrial activity. These data are in line with previous studies showing the critical role of metabolism on $CD8^+$ T cell effector versus memory fate (56, 57), and the quiescent profile of less differentiated subsets (49, 58). Remarkably, both polyclonal and HIV-specific reprogrammed $CD8^+$ T cells showed lower activation of mTORC1, and a predilection for mTORC2 pathway activation to sustain their activities. Moreover, reprogrammed HIV-specific $CD8^+$ T cells depended less on glucose. Such metabolic plasticity is also found in cells from HICs and may be particularly helpful in hostile metabolic environments such as tissues, with infected cells competing for nutrients (59) and high antigen load and inflammation impacting the survival and function of $CD8^+$ T cells (60). The modulation of metabolism and the induction of a stem-like profile are likely related and synergize to improve $CD8^+$ T cell antiviral potential, even in settings of persistent or strong antigen stimulation. Indeed, the maintenance of a stem-like profile and less effector differentiation and immune exhaustion were associated with a stronger HIV-1 suppression by reprogrammed $CD8^+$ T cells. These properties exhibited by

reprogrammed HIV-specific CD8$^+$ T cells might contribute to long-term viral control in vivo. Supporting this notion, stem-like SIV-specific CD8$^+$ T cells are long-term maintained and are linked to the natural control of infection (9).

We found that reprogramming did not affect all CD8$^+$ T cells uniformly. Although BIO induced very similar effects globally on CD8$^+$ T cells, reprogramming seemed to have the strongest enhancing effect of the activities of HIV-specific CD8$^+$ T cells from non-controllers when compared to cells responding to polyclonal stimulation or HCMV peptides. We have previously shown that HCMV and HIV-specific cells from HICs are relatively similar in terms of memory metabolic program (8). In contrast, HIV-specific cells from non-controllers have an effector-like and exhausted profile (8, 61, 62), and such a biased program can be found even among the TCM population (8). These divergent signatures in cells from controllers and non-controllers could be related to the TCR signaling and priming signals they received in vivo early after infection (9), which may be exacerbated by the persistence of high amounts of antigen and inflammation in non-controller individuals. Thus, it was expected that HIV-specific CD8$^+$ T cells derived from non-controllers benefited the most from the effects of reprogramming when compared to better-fitted HCMV-specific cells. Along these lines, our results show that the skewed program of HIV-specific CD8$^+$ T cells from non-controllers is at least partially reversible.

Our results also show that not all CD8$^+$ T cells activities are equally regulated by metabolic pathways. Observations in one of our previous studies suggested that polyfunctionality, and in particular TNF-α production, might depend on the mTORC2 pathway (8). This aspect was confirmed here. We observed a selective increase in the production of

444 TNF-α by reprogrammed CD8⁺ T cells, which was related to the inhibition of mTORC1,
445 preservation of the mTORC2 pathway, and less dependency on glycolysis. In contrast, IFN-γ
446 and IL-2 production was associated with mTORC1 activation. In keeping with our results,
447 previous studies have demonstrated that TNF-α production is associated with a less-
448 differentiated CD8⁺ T cell profile (63, 64), while the transcription factor NF-κB (regulator of
449 TNF-α expression) is active in stem-like CD8⁺ T cells (48, 65), suggesting a role of this
450 signaling pathway as a regulator of this subset. The underlying mechanism of greater TNF-α
451 production by reprogrammed CD8⁺ T cells might be related to epigenetic modifications and/or
452 posttranscriptional events (64, 66), that deserve further study. Moreover, although further
453 studies are necessary to fully elucidate this aspect, our data suggest that the production of
454 TNF-α in the absence of mTORC1 activity (pS6 expression) could serve as an additional
455 marker of stem-like memory CD8⁺ T cells. In the context of HIV-1 control, TNF-α might help
456 to inhibit HIV replication, as has previously been shown in monocytes and macrophages (67,
457 68), and its production, together with other soluble factors, may contribute to provide an
458 antiviral environment while CD8⁺ T cells are arrested to killed target cells (69). Interestingly,
459 a previous study showed that the cytotoxic capacity of HIV-specific CD8⁺ T cells is enhanced
460 in cells producing both IFN-γ and TNF-α (70). However, this did not appear to be related to a
461 direct cytotoxic effect of TNF-α. Simultaneous production of IFN-γ and TNF-α rather revealed
462 a subset of cells with qualitatively superior cytotoxic potential (70). Moreover, TNF-α has also
463 been shown to reverse latency of provirus in persistently infected cells (71, 72). This might
464 be relevant in vivo, where local production of TNF-α may reveal the presence of latently
465 infected cells, facilitating their elimination by the cytotoxic activity of the CD8⁺ T cells. Thus,
466 TNF-α production and polyfunctionality are a feature of the stem-like profile in HIV-specific
467 CD8⁺ T cells promoted by reprogramming, that, together with other functional attributes

(including higher survival, proliferative potential, and metabolic plasticity) may collectively contribute to the augmented antiviral potential observed.

We propose that reprogramming HIV-specific $CD8^+$ T cells may be of potential interest for interventions aiming at HIV-1 remission. Two beneficial effects could be expected: first, we have shown here that reprogramming reinvigorates the antiviral potential of HIV-specific $CD8^+$ T cells; secondly, the promotion of self-renewal and long-term survival of the cells may improve the therapeutic efficacy of adoptive immunotherapies. Indeed, one of the major caveats in adoptive immunotherapies is the low persistence of transferred cells, which impairs the long-term therapeutic efficacy, as has been observed in $HIV\text{-}1^+$ individuals receiving autologous, ex vivo-expanded HIV-specific $CD8^+$ T cells (73–75). In line with this notion, complete cancer regression and persistence of transferred tumor-reactive $CD8^+$ T cells have been associated with a stem-like population capable of self-renewal, expansion, and antitumor responses (76). Of note, $CD8^+$ T cell reprogramming could be combined with other interventions to further enhance their antiviral potential. We have previously shown that short IL-15 treatment of HIV-specific $CD8^+$ T cells from non-controllers increased their capacity to mobilize mitochondrial activities and their capacity to suppress HIV-1 infection of $CD4^+$ T cells (8). As reprogrammed $CD8^+$ T cells are more responsive to IL-15 in terms of proliferation and maturation of their effector profile, we hypothesize that a combined approach may have synergistic effects.

A limitation of our study is that all analyses were performed ex vivo. Therefore, it remains to elucidate the antiviral potential and lifespan of adoptively transferred, reprogrammed HIV-specific $CD8^+$ T cells in vivo, considering potential escape mutations, inflammatory conditions, and the nature of the HIV-1 reservoir. Indeed, a high proportion of

493 latent viruses exhibit escape mutations to evade the CD8$^+$ T cell response (77), which may
494 compromise the efficacy of CD8$^+$ T cell-based cure strategies. However, the superior in vivo
495 antitumor response of transferred stem-like CD8$^+$ T cells in human cancer (76), as well as the
496 enhanced anti-HIV potential exhibited by reprogrammed CD8$^+$ T cells ex vivo, support the
497 rationale of exploring this immunotherapy, in combination with other methodologies (such as
498 γ-chain cytokines, or chimeric antigen receptor cells), in the context of HIV-1 infection.
499 Another limitation in our study is that we only evaluated the impact of reprogramming in CD8$^+$
500 T cells from people with HIV receiving ART, and not from viremic untreated individuals, who
501 are expected to have more dysfunctional cells. However, individuals under ART would be the
502 main target population for these immunotherapies given the well-known benefits of treatment
503 initiation. Moreover, in this study we only analyzed cells derived from blood and not from
504 tissues. Thus, future studies could evaluate if GSK3 inhibitors also modify the program of
505 tissue resident memory cells. In addition, in our study we propose the use of reprogramming
506 with BIO for adoptive cell therapies. Given the multisystemic effects that GSK3-targeting
507 drugs could have in vivo (78), it remains to be explored the use of other GSK3 inhibitors with
508 proved clinical safety and tolerability (78, 79), that could be injected systemically to reprogram
509 CD8$^+$ T cells in vivo.
510
511 In summary, here we show that HIV-specific CD8$^+$ T cell pharmacologic
512 reprogramming into stem-like cells induces several properties associated with natural control
513 of infection and help to improve the response to immunomodulators. CD8$^+$ T cell
514 reprogramming could be used to potentiate the therapeutic efficacy of this cell population in
515 adoptive transfer strategies, as well as the effect of other immunotherapies, in the search for
516 an HIV-1 cure or remission or development of therapeutic vaccines. In addition, the benefits

517 of T cell reprogramming could be extended to the context of other chronic viral infections or
518 tumors where antigen-specific $CD8^+$ T cells have a skewed and dysfunctional profile.

MATERIALS AND METHODS

Participants

Samples from people without HIV were obtained from the Etablissement Français du Sang in the context of a collaboration agreement with Institut Pasteur. HIV-1 non-controller participants (on ART for at least 2 years and undetectable HIV-1 RNA) were recruited in the context of the ANRS EP36 XII mTOR study or ANRS CO6 PRIMO cohort. Clinical data for participants included are summarized in Supplementary Table 3. All analyses were done on cryopreserved PBMCs isolated from blood samples.

In vitro reprogramming of $CD8^+$ T cells

Frozen PBMC were thawed and rested overnight at 37°C, 5% CO_2, in RPMI 1640 GlutaMAX medium supplemented with 10% fetal calf serum and penicillin/streptomycin (complete medium). For reprogramming of $CD8^+$ T cells followed by polyclonal stimulation, cells were isolated by negative magnetic bead sorting (Stemcell Technologies). For reprogramming of $CD8^+$ T cells followed by antigen-specific stimulation, we used PBMC and magnetically separated $CD8^+$ T cells and non-$CD8^+$ T cells (REAlease® CD8 MicroBead Kit; Miltenyi Biotech). Then, $CD8^+$ T cells were treated with a GSK3 inhibitor (6-Bromoindirubin-3'-oxime; BIO; Sigma-Aldrich), at 3 μM, or an equivalent concentration of dimethyl sulfoxide (DMSO) vehicle control, in complete medium, for 12 hs at 37°C, 5% CO_2. Non-CD8 cells were left resting during this time in complete medium. $CD8^+$ T cells were washed with complete medium and stimulated with the peptide pool in presence of non-CD8 T cells. To confirm the effects of BIO, another GSK3 inhibitor, TWS119 (Sigma-Aldrich), was used under similar conditions (at 3 μM, 12 hs incubation).

543

544 **Polyclonal, antigen-specific, and cytokine stimulation of $CD8^+$ T cells**

545 After reprogramming, $CD8^+$ T cells were polyclonally stimulated with plate-bound anti-
546 CD3 and CD28 antibodies (both at 1 μg/mL; clones OKT3 and CD28.2, respectively; both
547 from Thermo Fisher), in the presence or absence of human recombinant ICAM-
548 1/CD54 Fc Chimera Protein (at 50 μg/mL; R&D) and cultured for 48 hs at 37°C, 5% CO_2,
549 accordingly. For intracellular cytokine analyses upon polyclonal stimulation, brefeldin A (at 10
550 μg/mL; Sigma-Aldrich) was added to the cells and incubated during the last 12 hs of culture.
551 Antigen-specific stimulation was performed by mixing $CD8^+$ and non-CD8 cells (maintaining
552 the original cell ratio) and incubating with overlapping peptide pools encompassing HIV-1
553 consensus subtype B Gag or HCMV pp65 (both at 2 μg/ml; obtained through the National
554 Institute of Health (NIH) AIDS Reagent Program, Division of AIDS, National Institute of Allergy
555 and Infectious Diseases, NIH, catalog numbers 12425 and 11549). For subsequent
556 intracellular cytokine analysis, cells were incubated for 6 hs in the presence of anti-
557 CD28+CD49d antibodies (clones L293 and L25, respectively; both at 1 μg/mL; BD
558 Biosciences), an anti-CD107a FITC antibody (BD Biosciences), plus brefeldin A (at 10 μg/mL)
559 and monensin (at 1 μg/mL; BD Biosciences), the two latter added 30 min after the start of all
560 incubations. In some experiments, cells were culture in RPMI medium without glucose (MP
561 Biomedicals). $CD8^+$ T cell proliferation was evaluated by CFSE dilution (at 1 μM, Thermo
562 Fisher Scientific). In additional experiments, reprogrammed $CD8^+$ T cells were stimulated with
563 IL-7 or IL-15 (both at 10 ng/mL; R&D) and cultured for 6 days. In all the cases, cells were
564 cultured at a density of $2x10^6$ cells/mL.

565

566 **Flow cytometry analysis**

After cultures, cells were stained with the LIVE/DEAD Fixable Aqua Dead Cell Stain kit (Thermo Fisher Scientific), with anti-CD3 Alexa Fluor 700 or APC efluor 780 and anti-CD8 PE Texas Red antibodies, accordingly. For phenotype analyses, cells were additionally stained with anti-CCR7 PE Cy7, anti-CD45RA APC H7, anti-CD27 PerCP Cy5.5, anti-CD95 APC, or anti-CD127 Alexa Fluor 488 antibodies, incubated for 15 minutes at room temperature. In some experiments, cells were stained with APC-conjugated dextramers (Immudex), incubated for 10 minutes at room temperature: HLA-A*0201 (SLYNTVATL) Gag, HLA-A*0301 (RLRPGGKKK) Gag, HLA-A*0301 (QVPLRPMTYK) Nef, and HLA-B*2705 (KRWIILGLNK) Gag-Pol. Additional surface staining panels included anti-HLA-DR Superbright 780, anti-CD38 Superbright 600, anti-PD-1 BV421, anti-LAG-3 APC efluor 780, anti-TIM3 PE Cy7, and anti-TIGIT BV786 antibodies, or anti-CD122 PE and anti-CD215 FITC antibodies. The BD Transcription Factor Buffer Set kit (BD Biosciences) was used for cell fixation and permeabilization. Intracellular staining panels for detection of transcription factors included anti-TCF-1 PE, anti-T-bet V450, anti-Eomes APC, anti-TOX efluor 660, anti-BLIMP1 CF594, or anti-BCL6 Alexa Fluor 488 accordingly. Intracellular cytokine staining panels included anti-IFN-γ PE Cy7 or V450, anti-IL-2 APC-R700, anti-TNF-α PerCP Cy5.5, or anti-granzyme B Alexa Fluor 647 antibodies, accordingly. In some experiments, cells were fixed and permeabilized with Phosflow fix/perm buffers (BD Biosciences) and cells were stained with anti-pS6 Ser235/236 Pacific blue and anti-pAKT Ser473 FITC antibodies (Cell Signaling). Cell acquisition was performed using LSR II, LSR Fortessa X-20, or ARIA III flow cytometers (both from BD Biosciences), and data were analyzed with FlowJo v.10 software (BD Biosciences). The list of flow cytometry antibodies used in this study is shown in Supplementary Table 4.

Measurement of metabolite uptake

592 CD8$^+$ T cells were split into four parts and put into contact with 2-NBDG (2-(N-(7-
593 Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose) (150 μM, 30 min) for glucose
594 uptake measurement, BODIPY 500/510 C_1, C_{12} (4,4-Difluoro-5-Methyl-4-Bora-3a,4a-Diaza-
595 s-Indacene-3-Dodecanoic acid) (5 μM, 5 min) for fatty acid uptake measurement, MitoTracker
596 Green FM (100 nM, 45 min) for mitochondrial mass measurement, and CellROX Deep Red
597 (5 μM, 30 min) for measurement of oxygen reactive species (all from Thermo Fisher
598 Scientific). After these incubations, cells were stained with the LIVE/DEAD Fixable Aqua
599 Dead Cell Stain kit, as well as with phenotype antibodies, as described above.
600
601 **Gene expression analysis in sorted memory cells**
602 Purified CD8$^+$ T cells were stained with the LIVEDEAD Fixable Aqua Dead Cell Stain
603 Kit (Thermo Fisher Scientific) and the following antibodies: anti-CD3 Alexa Fluor 700, anti-
604 CD8 APC Cy7, anti-CCR7 PE Cy7, anti-CD45RA BV421, and anti-CD27 PE (all from BD
605 Biosciences). Viable central memory, transitional memory, effector memory, and terminal
606 effector CD8$^+$ T cells were bulk sorted (BD FACS ARIA III, BD Biosciences), left rested for at
607 least 6 hs in complete medium before treatment with the GSK3 inhibitor or vehicle control.
608 Then, cells were washed and stimulated with plate-bound anti-CD3/CD28 (1 μg/mL) for 48
609 hs. After culture, cells were counted, and a total of 2,500 cells per condition were put in 96-
610 well plates containing the VILO Reaction Mix, SUPERase-In, and NP40 (all from Thermo
611 Fisher Scientific). Plates were snap-frozen and stored at −80°C. Analysis of gene expression
612 was performed as previously described (8), using Delta Gene primers, 96.96 Dynamic Array
613 chips, and a Biomark instrument for microfluidics-based qPCR (Fluidigm). Linear derivative
614 mode baseline correction was applied. Data were normalized using *GAPDH* as a

615 housekeeping gene and using the delta Ct method. Gene expression values are plotted as
616 log $2^{-\Delta Ct}$.
617
618 **Viral suppression assays**
619 PBMC from people with HIV were used to evaluate the capacity of reprogrammed
620 $CD8^+$ T cells to suppress HIV infection *ex vivo* (42). $CD4^+$ and $CD8^+$ T cells were separated
621 by, respectively, successive positive and negative magnetic bead sorting (Stemcell
622 Technologies). Human $CD4^+$ T cells were cultured for 3 days in complete medium
623 supplemented with IL-2 (100 IU/mL), in the presence of phytohemagglutinin (PHA; 2 μg/mL).
624 In parallel, purified $CD8^+$ T cells were treated with DMSO vehicle control or with the GSK3
625 inhibitor for 12 hs, followed by washing and incubation for 6 days in complete medium alone.
626 Then, activated $CD4^+$ T cells were superinfected with HIV-1 BaL, by spinoculation for 1 h at
627 2,000 *g* and 22°C, and cultured alone or with $CD8^+$ T cells at a 1:1 ratio for 7 days in complete
628 medium supplemented with IL-2. Viral replication was measured in terms of p24 production
629 in the culture supernatants using ELISA (XpressBio), or by flow cytometry by quantifying
630 intracellular HIV-1 Gag products (KC57-FITC antibody [Beckman Coulter]). The HIV-
631 suppressive capacity of $CD8^+$ T cells was calculated as the log10 fold decrease in the median
632 levels of p24 when $CD4^+$ T cells were incubated in the presence of $CD8^+$ T cells. For the
633 detection of HIV-specific $CD8^+$ T cells, at the end of the coculture and previous collection of
634 supernatants, cells were incubated with brefeldin A (10 μg/mL), monensin (2 μg/mL), and an
635 anti-CD107a BV786 antibody (BD Biosciences), followed by intracellular cytokine staining, as
636 described above.
637
638 **Statistical analyses**

GraphPad Prism software version 9.0 was used for statistical analysis. Data are presented as medians and ranges. The Wilcoxon test was used for the comparison of two paired data. The Friedman test, and the Dunn's multiple comparisons test, were used for the comparison of three or more paired groups. The Šidák method was applied to correct for multiple comparisons, where needed. All P values less than 0.05 were considered as significant. To determine differentially expressed genes for each $CD8^+$ T cell memory population, we defined a mixed effect model including treatment (vehicle control versus GSK3 inhibitor), condition (unstimulated or anti-CD3/CD28 stimulated), and their interaction as fixed effects, as well as considered participant and technical replicates as a random effect. The parameters have been estimated using the lme4 package (v1.1-23) in R.

Study approval

The study was approved by the ethics committee (Comité de Protection des Personnes) of Île-de-France XI. All participants gave their informed consent.

AUTHOR CONTRIBUTIONS

FP-C and AS-C conceived the study. FP-C, CP, and VM performed experiments and analyzed the data. SV assisted computational analyses. FB, PT, MM, KB, LW, CJ, CB, CG, LM, and OL participated in participants recruitment. FP-C and AS-C wrote the manuscript. All authors revised the manuscript. AS-C provided financial support and supervised the work.

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DECLARATION OF INTERESTS

Some authors have potential conflict of interest to declare: FP-C, CP, MM-T and AS-C are listed as inventors in a patent application by Institut Pasteur, partially based on the results presented here.

DATA AND MATERIALS AVAILABILITY

All data and methods reported in this paper are included in the manuscript or in the supplemental information.

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FIGURES

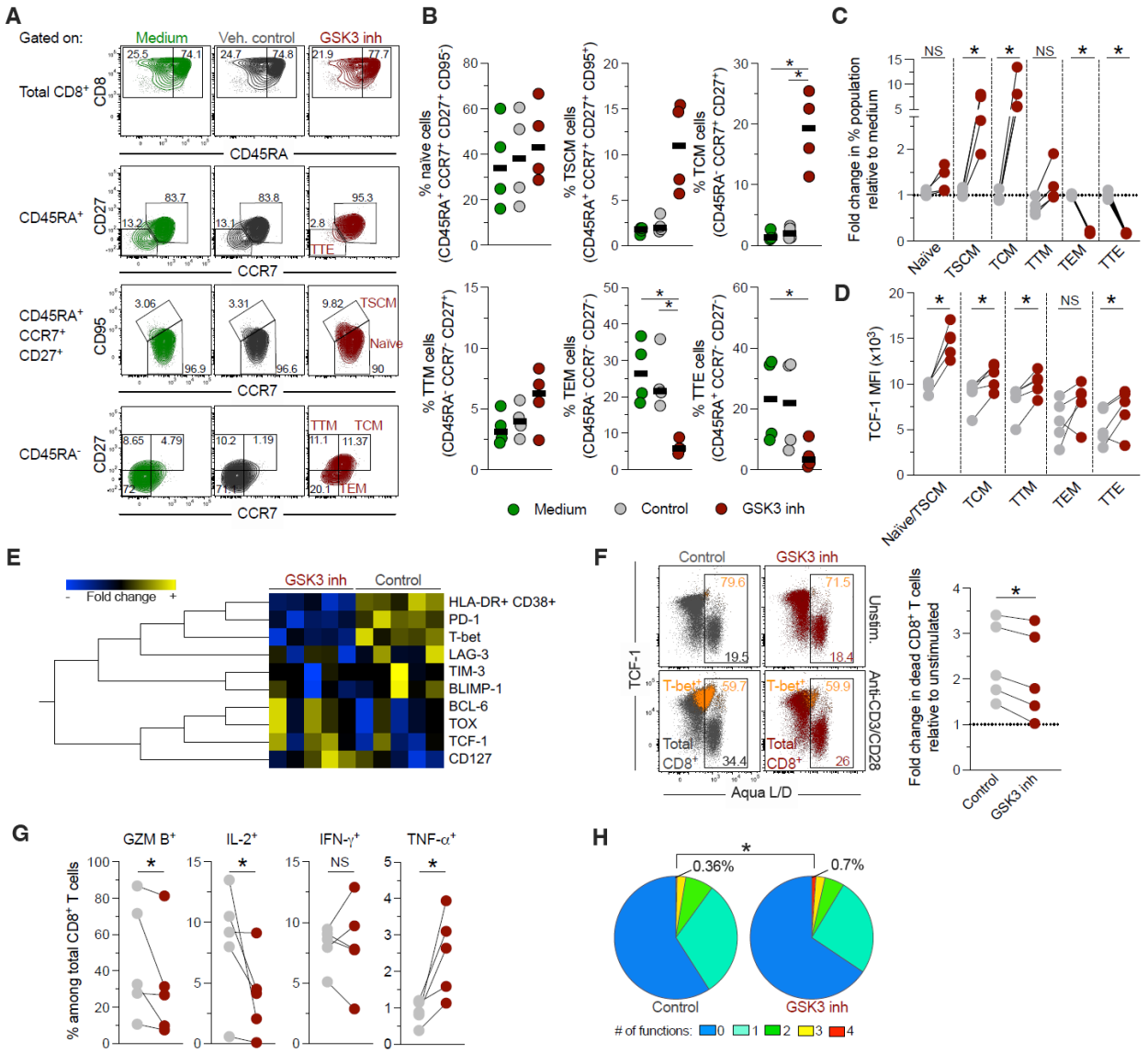

Figure 1. Induction of stem-like CD8⁺ T cells with high survival capacity and polyfunctionality by in vitro reprogramming. Total CD8⁺ T cells from people without HIV were treated with medium, vehicle control, or the GSK3 inhibitor, followed by incubation under basal conditions or anti-CD3/CD28 stimulation for 48 hs. **A-B.** Analysis of CD8⁺ T cell subpopulations in unstimulated cells (n=4; Dunn's test). **C**. Fold change of CD8⁺ T cell subpopulations upon vehicle control or a GSK3 inhibitor treatment, relative to medium alone

886 condition (n=4). **D.** Expression of TCF-1 in $CD8^+$ T cell subsets (n=4). **E.** Fold change in the
887 expression of the indicated markers induced by anti-CD3/CD28 stimulation, relative to
888 unstimulated cells (n=5). **F**. Analysis of dead cells (Aqua Live/$Dead^+$) among total and T-bet^+
889 $CD8^+$ T cells, and fold change in dead $CD8^+$ T cells induced by anti-CD3/CD28 stimulation,
890 relative to the unstimulated condition (n=5). **G**. Frequencies of granzyme B^+, IL-2^+, IFN-γ^+,
891 and TNF-α^+ $CD8^+$ T cells after anti-CD3/CD28 stimulation. **H**. Expression of 1 to 4 functions
892 in $CD8^+$ T cells (n=5). *$P<0.05$*; **$P\leq0.01$. *NS:* Not statistically significant. In C-D, and F-H,
893 the Wilcoxon test was used. The data obtained in two (A-F) or three (G,H) independent
894 experiments is shown.
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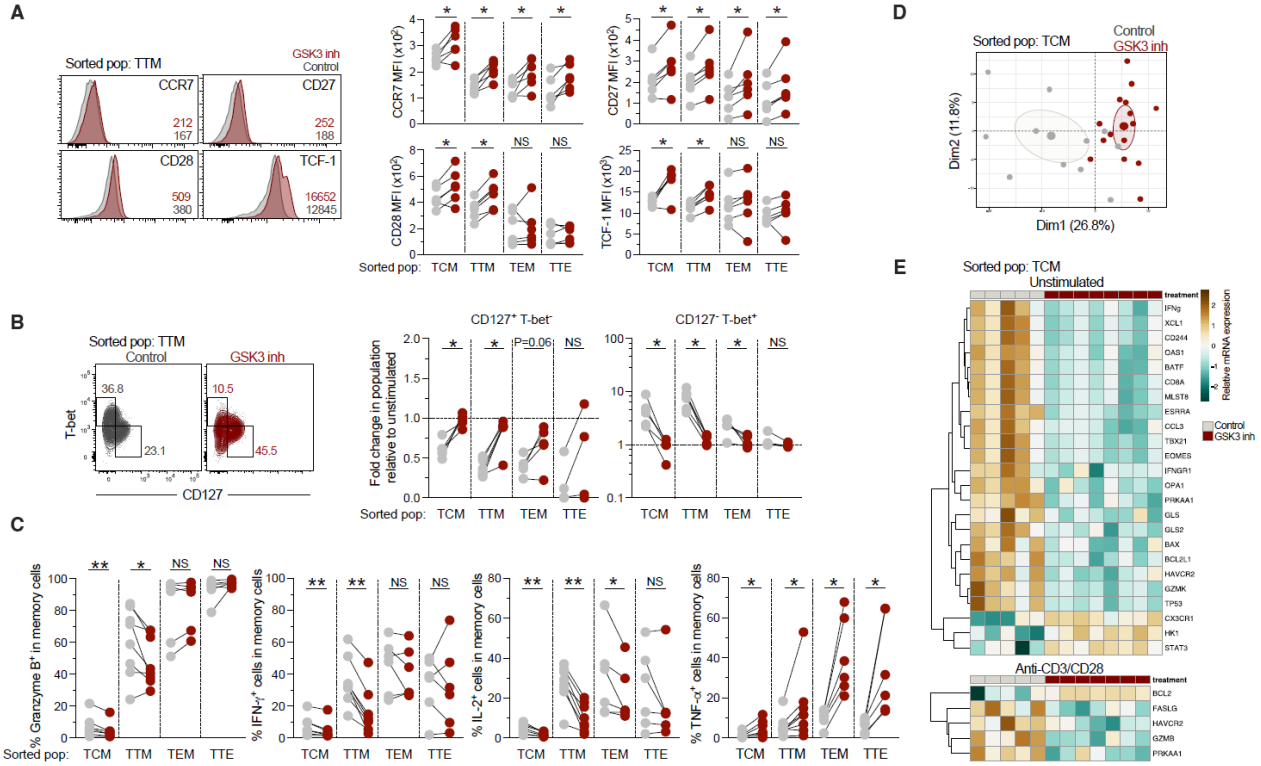

Figure 2. Intrinsic effects of reprogramming in CD8⁺ T cell memory subpopulations. Sorted TCM, TTM, TEM and TEM from people without HIV were treated with the GSK3 inhibitor or vehicle control, followed by incubation under basal conditions or anti-CD3/CD28 stimulation for 48 hs. **A.** Representative histograms showing the expression of CCR7, CD27, CD28, and TCF-1 in sorted TTM cells, in basal conditions. The median fluorescence intensity of each marker is depicted. The summary of the expression of each marker in memory cells after vehicle control or GSK3 inhibitor treatment, in the absence of stimulation, is shown. **B**. Flow cytometry analysis of CD127⁺ T-bet⁻ and CD127⁻ T-bet⁺ cells after anti-CD3/CD28 stimulation, and fold change in the indicated subsets among memory cells induced by anti-CD3/CD28 stimulation, relative to the unstimulated condition. **C.** Frequencies of granzyme B⁺, IFN-γ⁺, IL-2⁺, and TNF-α⁺ among memory cells after anti-CD3/CD28 stimulation. At least 5 donors were included for each comparison; data from 7 independent experiments. *$P<0.05$; **$P\leq0.01$; *NS:* Not statistically significant; Wilcoxon test. **D**. Principal Component Analysis

911 (PCA) of gene expression by TCM cells treated with vehicle control or GSK3 inhibitor. **E**. Heat
912 map of genes differentially expressed (P<0.05 according to a mixed effect model) in
913 reprogrammed versus non-reprogrammed TCM cells. The scale on the heatmap indicates
914 the mRNA levels relative to the housekeeping gene (*GAPDH*) and plotted as log $2^{-\Delta Ct}$, in
915 unstimulated and polyclonally-stimulated cells (At least 5 donors were included for each
916 comparison; data from two independent experiments).
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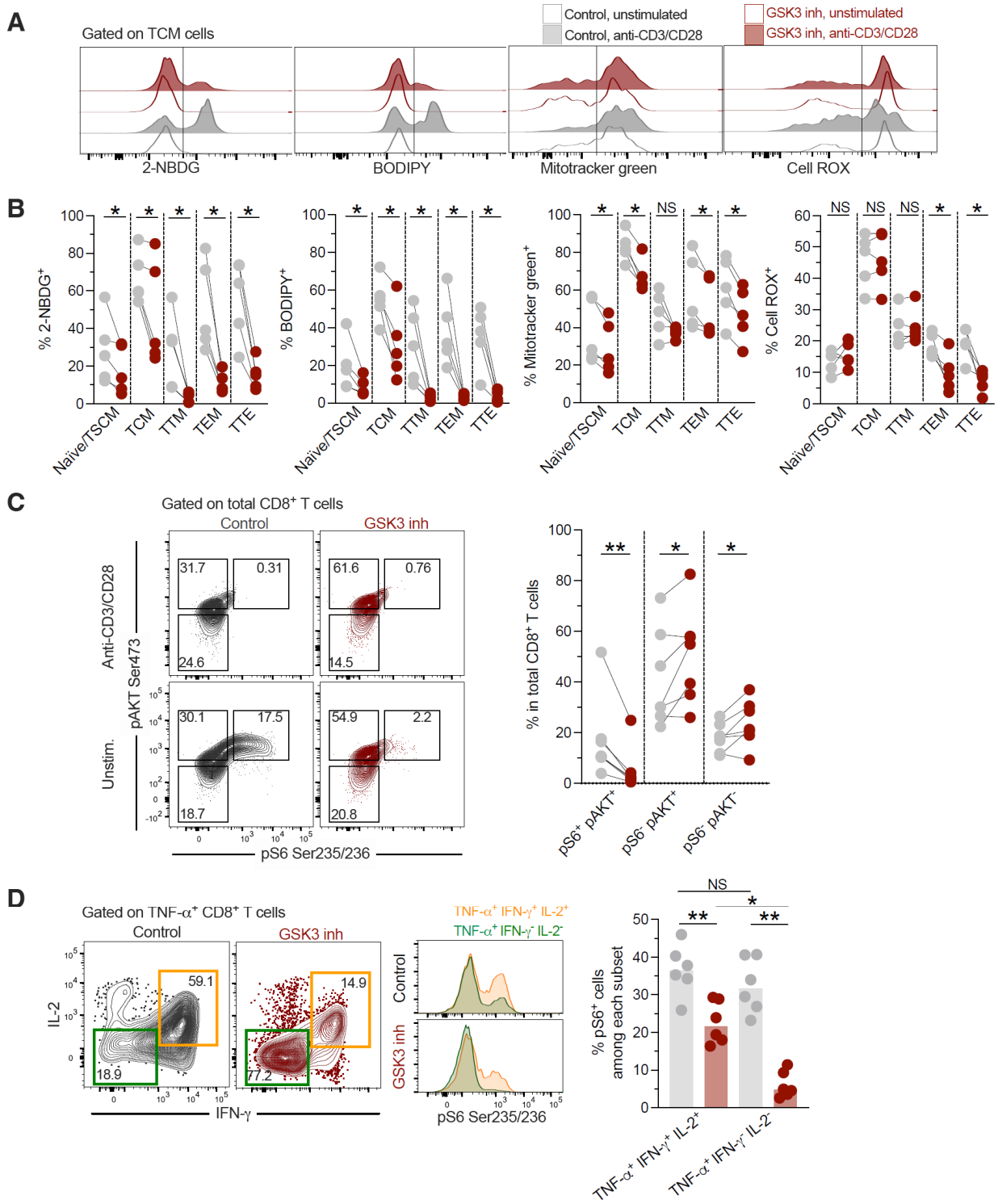

Figure 3. Modulation of anabolic metabolism in reprogrammed CD8⁺ T cells. A-B. Total CD8⁺ T cells from people without HIV were treated with vehicle control or the GSK3 inhibitor, followed by incubation under basal conditions or anti-CD3/CD28 stimulation for 48 hs. Then,

922 the expression of 2-NBDG, BODIPY, Mitotracker green, and Cell ROX among $CD8^+$ T cell
923 subsets was evaluated (**A**). In **B**, the frequencies of 2-$NBDG^+$, $BODIPY^+$, Mitotracker $green^+$,
924 and Cell ROX^+ in $CD8^+$ T cell subpopulations after stimulation are shown (n=5; Wilcoxon test).
925 **C-D**. Total $CD8^+$ T cells from people without HIV were treated with vehicle control or the GSK3
926 inhibitor, followed by incubation under basal conditions or anti-CD3/CD28 stimulation for 48
927 hs. **C.** Flow cytometry analysis of the expression of pS6 and pAKT in total $CD8^+$ T cells, and
928 frequencies of $pS6^+$ $pAKT^+$, $pS6^-$ $pAKT^+$ and $pS6^-$ $pAKT^-$ subsets in total $CD8^+$ T cells (n=7;
929 Wilcoxon test). **D.** Flow cytometry analysis of the expression of IL-2 and IFN-γ among TNF-
930 α^+ $CD8^+$ T cells, after anti-CD3/CD28 stimulation. The histograms show the expression of
931 pS6 in the indicated subsets, in reprogrammed and non-reprogrammed cells. The frequency
932 of $pS6^+$ cells among TNF-α^+ IFN-γ^+ IL-2^+ or TNF-α^+ IFN-γ^- IL-2^- subsets is shown (n=6 people
933 without HIV; Šidák multiple comparison test). *$P<0.05$; **$P\leq0.01$; *NS:* Not statistically
934 significant. Data were obtained in two independent experiments.
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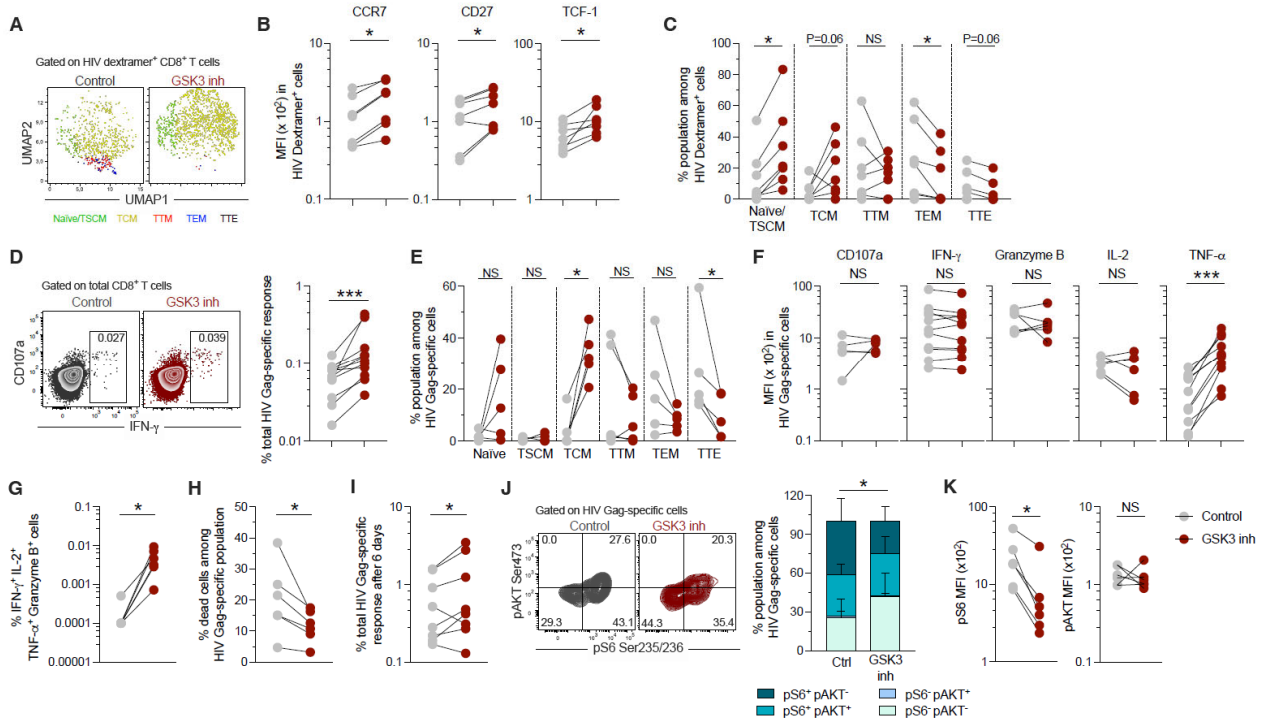

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937 **Figure 4. Enhanced functionality and survival of reprogrammed HIV-specific CD8⁺ T**
938 **cells.** After treatment with the GSK3 inhibitor, CD8⁺ T cells from people with HIV were stained
939 with HLA-matched HIV dextramers, for the analysis of the phenotype of HIV dextramer⁺ cells
940 **A.** UMAP plots generated from HIV dextramer⁺ CD8⁺ T cells after data concatenation (n=4).
941 Vehicle control and GSK3 inhibitor treatments, as well as CD8⁺ T cell subpopulations were
942 identified by manual gating and projected into the UMAP space. **B.** Summary of the
943 expression of CCR7, CD27 and TCF-1 in HIV dextramer⁺ CD8⁺ T cells (n=7). **C.** Frequencies
944 of CD8⁺ T cell subpopulations among HIV dextramer⁺ CD8⁺ T cells (n=7). **D-G.** After
945 reprogramming, cells from people with HIV were stimulated for 6 hs with Gag peptides, for
946 the analysis of the total frequency (IFN-γ⁺ or CD107a⁺ or IL-2⁺ or TNF-α⁺) of antigen-specific
947 CD8⁺ T cells (n=5) (**D**), the proportion of memory subpopulations (n=5) (**E**), the expression of
948 CD107a, IFN-γ, granzyme B, IL-2, and TNF-α on a per cell basis (n=5-11) (**F**), and the
949 frequency of polyfunctional cells (n=6) (**G**). **H-I.** Cells from people with HIV were stimulated
950 for 6 days with Gag peptides and re-stimulated with the same peptides for another 12 hs, for

951 the analysis of the viability of proliferating HIV Gag-specific $CD8^+$ T cells (n=6) (**H**), and the
952 frequency of the total (live IFN-γ^+ or IL-2^+ or TNF-α^+) HIV Gag-specific response (n=8) (**I**). **J-**
953 **K.** After reprogramming, cells from people with HIV (n=6) were stimulated for 6 hs with Gag
954 peptides, for the analysis of $pS6^+$ $pAKT^-$, $pS6^+$ $pAKT^+$, $pS6^-$ $pAKT^+$ and $pS6^-$ $pAKT^-$ subsets
955 (**J**) or the intensity of expression of pS6 and pAKT (**K**) in HIV Gag-specific (IFN-γ^+ and/or IL-
956 2^+) $CD8^+$ T cells. *$P<0.05$*; ***$P\leq0.001$; *NS:* Not statistically significant; Wilcoxon test. Data
957 were obtained in three independent experiments.
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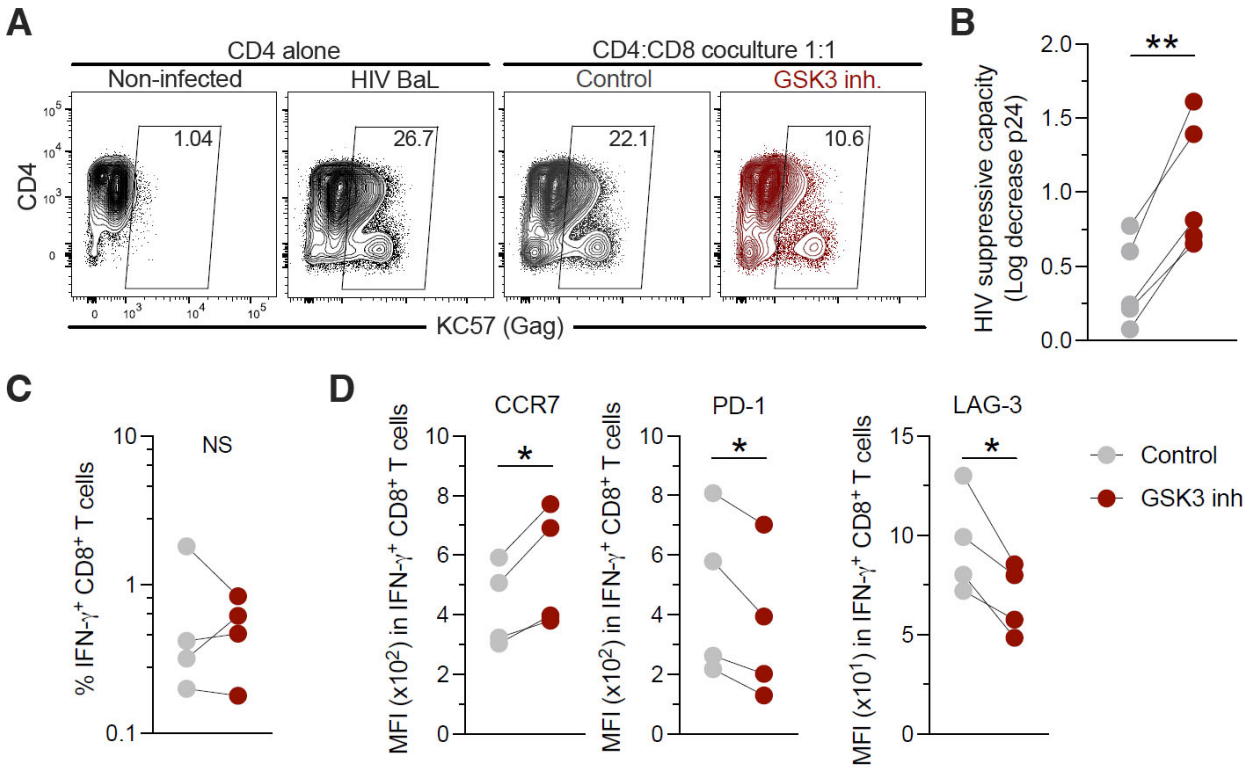

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960 **Figure 5. Reprogramming increases the HIV-1 suppressive capacity of CD8⁺ T cells.**
961 HIV-1 BaL-superinfected CD4⁺ T cells from people with HIV were cultured alone or in the
962 presence of autologous non-reprogrammed or reprogrammed CD8⁺ T cells. After 7 days, the
963 levels of infection were measured by flow cytometry (KC57 anti-Gag antibody) or ELISA (p24
964 in culture supernatant). **A.** Representative flow cytometry analysis of the frequency of infected
965 CD4⁺ T cells (from a total of 4 donors). **B.** HIV suppressive capacity of non-reprogrammed
966 and reprogrammed CD8⁺ T cells (Log10 decrease of p24 levels in culture supernatant; n=5
967 individuals, median of triplicates for each experiment). **C-D** The frequency of IFN-γ⁺ HIV-
968 specific CD8⁺ T cells (**C**) and the expression of CCR7, PD-1, and LAG-3 in HIV-specific CD8⁺
969 T cells (**D**) were measured after 7 days of coculture. **P<0.05; **P≤0.01; NS:* Not statistically
970 significant; Wilcoxon test.

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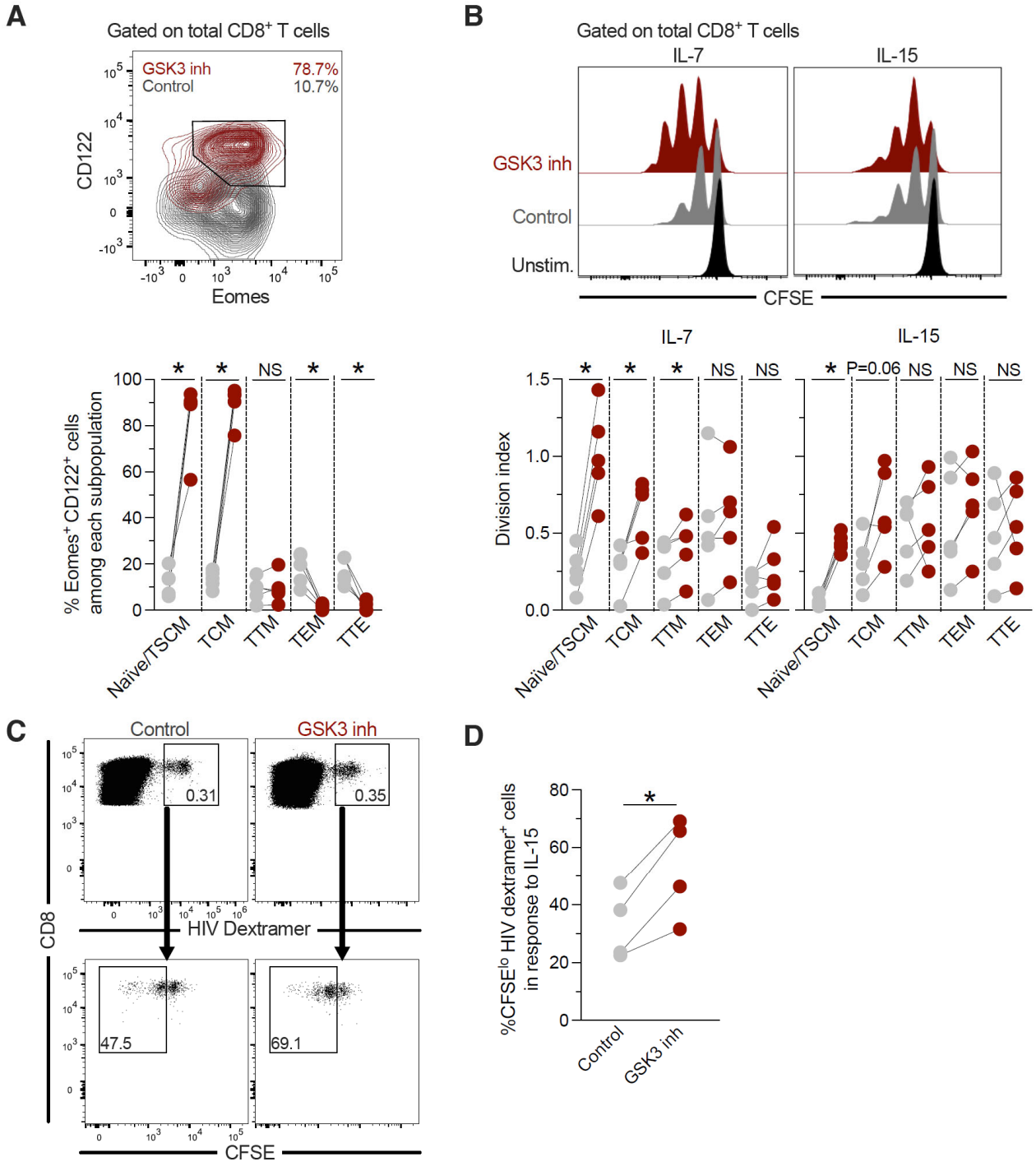

Figure 6. Superior response to γ-chain cytokines by reprogrammed HIV-specific CD8⁺ T cells. A. Total CD8⁺ T cells from people without HIV were treated with vehicle control or the GSK3 inhibitor, followed by evaluation of the expression of Eomes and CD122 in CD8⁺ T cell subsets (n=5). **B.** After vehicle control or GSK3 inhibitor treatment, CD8⁺ T cells from

977 people without HIV were left unstimulated or stimulated with IL-7 or IL-15 for 6 days, for the
978 analysis of cell proliferation (n=5, data from two independent experiments). **C-D**. $CD8^+$ T cells
979 from people with HIV were treated with vehicle control or the GSK3 inhibitor, followed by
980 stimulation with IL-15 for 6 days, for the analysis of the proliferation of HIV $dextramer^+$ $CD8^+$
981 T cells (n=4; data from three independent experiments). *$P<0.05$; *NS:* Not statistically
982 significant; Wilcoxon test.