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## Metabolic plasticity of HIV-specific CD8+ T cells is associated with enhanced antiviral potential and natural control of HIV-1 infection

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1 **Metabolic plasticity of HIV-specific CD8+ T-cells is associated with enhanced antiviral**  
2 **potential and natural control of HIV-1 infection.**

3

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25

26 **ABSTRACT**

27 Spontaneous control of HIV is generally associated with an enhanced capacity of CD8+ T-cells to  
28 eliminate infected CD4+ T-cells, but the molecular characteristics of these highly functional CD8+ T-  
29 cells are largely unknown. Here, using single-cell analysis, we show that HIV-specific central memory  
30 CD8+ T-cells from spontaneous HIV controllers (HIC) and antiretroviral-treated non-controllers have  
31 opposing transcriptomic profiles. Genes linked to effector functions and survival are upregulated in  
32 cells from HIC. In contrast, genes associated with activation, exhaustion and glycolysis are  
33 upregulated in cells from non-controllers. We show that HIV-specific CD8+ T-cells from non-  
34 controllers are largely glucose-dependent, while those from HIC have more diverse metabolic  
35 resources that enhance both their survival potential and their capacity to develop anti-HIV effector  
36 functions. The functional efficiency of the HIV-specific CD8+ T-cell response in HIC is thus engraved in  
37 their memory population and related to their metabolic program. Metabolic reprogramming in vitro  
38 through IL-15 treatment abrogated the glucose dependency and enhanced the antiviral potency of  
39 HIV-specific CD8+ T-cells from non-controllers.

40

41

42 **INTRODUCTION**

43 HIV-1-specific CD8+ T-cells develop promptly following HIV-1 infection and contribute to limiting  
44 viremia. However, they do not fully control the virus and their functions weaken further during  
45 chronic infection<sup>1</sup>. When compared to CD8+ T-cells elicited by other pathogens, HIV-specific CD8+ T-  
46 cells show delayed maturation and limited effector potential<sup>2,3</sup>. However, HIV-specific CD8+ T-cells  
47 endowed with enhanced proliferative capacity<sup>4</sup>, polyfunctionality<sup>5</sup> and cytotoxicity<sup>6</sup> have been  
48 described in rare individuals (HIV controllers, HIC) who maintain the virus below the detection limit  
49 without antiretroviral therapy<sup>7</sup>. HLA B\*27 and B\*57 are over-represented among HIC, further  
50 supporting a role of CD8+ T-cells in HIV control. Natural control of HIV infection is also usually  
51 associated with an ability of HIV-specific CD8+ T-cells to efficiently eliminate HIV-infected autologous  
52 CD4+ T-cells *ex vivo*<sup>8</sup>. Similar activity can also be found in HIV-2-controllers<sup>9</sup>, but not during the acute  
53 or chronic stage of HIV-1 infection in individuals who do not control infection<sup>10,11</sup>. Superior virus-  
54 inhibiting activity has been attributed to all memory CD8+ T-cell subsets from HIC<sup>12</sup>, pointing to  
55 intrinsic differences between cells from controllers and non-controllers.

56

57 Here we aimed to identify inherent differences between HIV-specific CD8 from HIC and those from  
58 HIV non-controllers with suppressed viremia on antiretroviral treatment (cART). We focused the  
59 analysis on central memory CD8+ T (CD8+ TCM)-cells, which represent an intermediate state  
60 between naïve and effector CD8+ T-cells and are responsible for long-term immune memory  
61 responses<sup>13</sup>. We postulated that any intrinsic differences between cells from HIC and non-controllers  
62 should be already imprinted in the CD8+ TCM subset. HIV-infected individuals with low virus set-  
63 points have significantly larger numbers of CD8+ TCM<sup>14</sup>, while HIV disease progression is associated  
64 with shorter CD8+ TCM lifespans and numbers<sup>15</sup>, highlighting their role in HIV control. However,  
65 CD8+ TCM cells are heterogeneous and have variable potential for proliferation and differentiation<sup>16</sup>.

66

67 We conducted single-cell gene expression analyses, as this approach is well-adapted to identify  
68 differences between cells that have the same phenotype in methods such as conventional flow  
69 cytometry<sup>17</sup>. We found that HIV-specific CD8+ TCM cells from HIC and non-controllers had distinct  
70 transcriptional signatures. Our findings reveal that metabolic plasticity is an important characteristic  
71 of efficient HIV-specific CD8+ T-cells that better equipped them to survive and to counteract the  
72 virus. We demonstrate that metabolic reprogramming in vitro of HIV-specific CD8+ T-cells from non-  
73 controllers enhanced their antiviral potency.

74

75

76

## 77 **RESULTS**

### 78 **Strong anti-HIV potential of HIC central memory CD8+ T-cells**

79 CD8+ T-cells with a strong capacity to suppress HIV infection of autologous CD4+ T-cells *ex vivo* can  
80 be isolated from HIC but not from cART individuals<sup>8,10,18</sup> (Figure 1A). Here, we first investigated  
81 whether this capacity of HIC CD8+ T-cells could be found among naïve, central memory, transitional  
82 memory and effector memory CD8+ T-cell subsets (Supplementary Figure 1). As expected, naïve  
83 CD8+ T-cells from HIC had no HIV-suppressive capacity. In contrast, all sorted memory CD8+ T-cell  
84 subsets from HIC, but not from cART individuals, suppressed HIV infection as efficiently as bulk CD8+  
85 T-cells (Figure 1B). Therefore, although effector memory cells likely had more immediate anti-viral  
86 capacity<sup>12</sup>, HIV-specific CD8+ TCM-cells from HIC can efficiently react to counteract HIV-1 infection  
87 during the co-culture. These results support the view that the strong antiviral potential of HIC is  
88 already embedded in the program of their HIV-specific CD8+ TCM-cells.

89

### 90 **Distinct gene expression profile in HIV-specific CD8+ TCM-cells from HIC**

91 We then compared the single-cell gene expression profiles (96 genes associated with CD8+ T-cell  
92 function, differentiation, metabolism and survival (Supplementary Figure 2, Supplementary Data 1))  
93 of HIV-specific CD8+ TCM cells from five HIC whose CD8+ T-cells had strong HIV-suppressive capacity  
94 and from five cART individuals (Supplementary Table 1). Cells from cART and HIC analyzed here  
95 shared not only the same differentiation status but also the same activation phenotype, as defined  
96 by flow cytometry (Figure 1C, Supplementary Table 2). Despite their similar phenotype, t-sne (t-  
97 distributed stochastic neighbor embedding algorithm) based analysis showed that HIC and cART cells  
98 had distinct gene expression profiles (Figure 1D), with stronger group differences between cells from  
99 HIC and cART than differences among cells from individuals in each group ( $p=0.003$  for inter-group vs  
100 intra-group variance).

101

## 102 **Upregulation of antiviral genes in HIV-specific CD8+ TCM-cells from HIC**

103 Next, we analyzed the genes differently expressed in HIV-specific CD8+ TCM-cells from HIC and cART  
104 individuals. At the single-cell level, gene expression is not continuous but occurs in pulses, with  
105 periods of inactivity. We therefore analyzed our data using MAST (Model-based Analysis of Single  
106 Cell Transcriptomics), which identified differently expressed genes (i) considering only the expressed  
107 genes (continuous, e.g. differences in mRNA expression levels); (ii) considering expressed vs non-  
108 expressed genes (discrete, i.e. differences in the frequency of cell expressing or not that gene); (iii)  
109 combining the discrete and continuous parts of the model (hurdle).

110

111 Overall, differences between cells from HIC and cART individuals were found for 38 genes (23 genes  
112 in the continuous analysis, 21 in the discrete analyses; 33 genes were found in the hurdle analysis;  
113 Supplementary Figures 3 and 4, Supplementary table 3). Ten genes relating to CD8+ T-cell effector  
114 activities were found to be more frequently active and/or expressed at higher levels in HIV-specific  
115 CD8+ TCM-cells from HIC than in those from cART (Figure 2). These genes included beta-chemokines  
116 (*CCL3*, *CCL3L1* and *XCL1*), which interfere with HIV-1 infection<sup>19</sup>; granzymes (*GZMB*, *GZMK*), which are  
117 major mediators of CD8+ T-cell antiviral cytotoxic activities<sup>6</sup>; and other molecules involved in CD8+ T-  
118 cell-induced apoptosis, such as Fas ligand (*FASL*), tumor necrosis factor (*TNF*) and TNF-related  
119 apoptosis-inducing ligand (*TRAIL/TNFSF10*). Intriguingly, although CD8+ T-cells are not considered to  
120 be major producers of type-I interferon, we found that cells from HIC expressed *IFNB* transcripts at  
121 higher levels than cells from cART. Only *PRF1* showed higher expression in cells from cART  
122 individuals. These results show that HIV-specific CD8+ TCM-cells are endowed with massive antiviral  
123 potential, in keeping with our demonstration that CD8+ TCM-cells from HIC efficiently suppress HIV  
124 infection *ex vivo*.

125

## 126 **Upregulation of cell survival genes in HIV-specific CD8+ TCM-cells from HIC**

127 We then analyzed the nature of the other 28 genes differently expressed in cells from HIC and cART  
128 (see Supplementary figure 5 for not differently expressed genes). Cells from cART more frequently  
129 expressed genes linked to activation (*CD69*, *KLRD1*) than cells from HIC (Figure 3A), despite similar  
130 surface expression of HLA-DR and CD38 (Figure 1C, Supplementary Table 2). Cells from cART also  
131 expressed *CD8A* mRNA and the inhibitory regulators *LAG-3* and *CDKN1B*, more frequently and/or at  
132 higher levels (Figure 3A), in line with their more highly activated profile. Cells from cART also more  
133 frequently expressed interferon-stimulated genes (*MX1*, *OAS1*) and *HIF1A*, a gene linked to the mTOR  
134 pathway and glycolysis<sup>20</sup> (Figure 3A). Overall, the profile of cells from cART individuals was  
135 compatible with activation of mTORC1<sup>20,21</sup>, which regulates cellular metabolism, and particularly  
136 anaerobic glycolysis, to keep up with energy requirements upon activation<sup>22</sup>.

137

138 In contrast, cells from HIC expressed more frequently or at higher levels several key genes of the  
139 mTORC2 pathway (*RICTOR*, *CDC42*, *RHOA*) (Figure 3B), which has been linked to cell survival<sup>23</sup>.  
140 Indeed, cells from HIC also upregulated genes directly associated with cell survival and resistance to  
141 apoptosis (*IL7R*, *BCL2*, *EOMES*, *ID2*) (Figure 3B). Interestingly, HIC cells expressed higher levels of *VHL*  
142 (Figure 3B), which targets HIF-1 for proteasomal degradation<sup>24</sup> and counteracts HIF-1-mediated  
143 upregulation of glycolysis in CD8+ T-cells<sup>25</sup>. Thus, HIV-specific CD8+ TCM-cells from HIC not only  
144 expressed lower levels of *HIF1A* when compared to cART cells, but were also set to actively counter  
145 this factor. Cells from HIC also expressed higher levels of *REPIN1*, which is involved in metabolic  
146 regulation and downregulation of glucose transport<sup>26</sup>. Overall, these results suggest that HIV-specific  
147 CD8 TCM-cells from cART individuals are primed for activation and glycolysis, while cells from HIC are  
148 rather primed for survival through the mTORC2 pathway.

149

#### 150 **Effector function and survival genes are co-expressed in HIC cells**

151 We explored whether differently expressed genes between HIV-specific CD8+ TCM-cells from HIC  
152 and cART individuals were inter-related, i.e. whether their respective expression profiles coincided in



153 individual cells. Although heterogeneity was observed, cells from HIC and cART segregated with  
154 opposing profiles (Figure 4): cells expressing the highest levels of effector function-related genes (e.g.  
155 *GZMB, CCL3, FASL, TNF, IFNB1*) also expressed high levels of genes associated with cell survival (*IL7R,*  
156 *BCL-2, ID2*) and were almost exclusively found in HIC. Moreover, these cells expressed relatively low  
157 levels of *CD8A, CD69, LAG3, KLRD1* and *HIF1A*, which were strongly expressed by cells from cART  
158 individuals (Figure 4A,B). These profiles support that HIC are characterized by the presence of HIV-  
159 specific CD8+ TCM-cells with high antiviral potential concomitantly primed for survival, while cART's  
160 cells have limited antiviral potential and exhibit upregulation of genes linked to glycolysis,  
161 senescence and exhaustion.

162

### 163 **Shared gene expression profile in cells from HIC with strong and weak responses**

164 Although most HIC have HIV-specific CD8+ T-cells that efficiently suppress the virus (strong  
165 responders, SR), some have weak CD8+ T-cell responses that fail to suppress HIV *ex vivo* (weak  
166 responders, WR)<sup>18</sup>. The differences between SR and WR cannot be explained by different HLA  
167 backgrounds, because the protective HLA B alleles are equally overrepresented in the two  
168 subgroups<sup>27</sup>. CD8+ T-cells from WR can recover their virus-suppressing capacity upon stimulated  
169 expansion<sup>28</sup>. We thus wondered whether HIV-specific CD8+ TCM-cells from WR and SR have the  
170 same transcription profile. We studied HIV-specific CD8+ TCM-cells from five WR (Supplementary  
171 Table 1) and found that their gene expression profile was intermediate between the SR and cART  
172 cells analyzed before (Figure 5A, Supplementary Table 4), although closer to that of SR. In particular,  
173 HIV-specific CD8+ TCM-cells from both WR and SR expressed higher levels of genes related to  
174 effector function (*CCL3L1, GZMB, IFNB, TNF*) (Figure 5B) (although less broadly for WR  
175 (Supplementary Figure 6)), and survival (*IL7R, PRKCA, ID2*) (Figure 5C) compared to cells from cART.  
176 As previously observed, cells from cART showed upregulation of genes linked to  
177 mTORC1/activation/glycolysis (*CD8A, HIF1A, KLRD1, CD69, IRF1*) and *LAG3* inhibitory receptor (Figure

178 5D) than both SR and WR. These data imply that HIV-specific CD8+ TCM-cells from HIC SR and WR  
179 share a close transcriptome program despite their different functional activities *ex vivo*.

180

#### 181 **Different metabolite uptake by CD8+ TCM cells from HIC and non-controllers**

182 We then explored if the changes in the transcriptional program of HIV-specific CD8+ TCM cells from  
183 HIC and cART individuals translated in differences at the protein and functional level. We observed  
184 that, although not statistically significant, HIC tended to carry more frequently cells with high levels  
185 of GRZB than cART individuals (Figure 6A). HIF1 was more strongly expressed in HIV-specific CD8+  
186 TCM-cells from cART individuals than in cells from HIC (figure 6A), strengthening our observations  
187 that mTORC1 and glycolysis were particularly upregulated in the cells from non-controllers<sup>20</sup>. As  
188 discussed above, cells from HIC showed upregulation of *VHL* and *REPIN1* (Figures 3B, 4) which may  
189 downplay glycolysis by counteracting HIF1A and decreasing glucose transport<sup>24,26</sup>. This implies  
190 different regulation of CD8+ T-cell metabolism in the two groups of individuals. The metabolic  
191 activity of cells is tightly linked to their function. Activated effector cells preferentially upregulate  
192 glycolysis to meet the energy demands imposed by T-cell proliferation, while memory cells rely on  
193 fatty acid oxidation to fuel mitochondrial oxidative phosphorylation and persist<sup>29</sup>. CD8+ TCM-cells  
194 from HIC exhibited *ex vivo* significantly higher fatty acid uptake than those from cART, whereas there  
195 was no significant difference in glucose uptake (Figure 6B). In consequence, CD8+ TCM-cells from HIC  
196 had a higher fatty acid/glucose uptake ratio than cells from cART individuals (Figure 6B).

197

#### 198 **Glucose dependency of HIV-specific CD8+ T-cells from non-controllers.**

199 The above results suggest that HIV-specific CD8+ T-cells from cART individuals might be more  
200 glucose-dependent than cells from HIC. We tested the capacity of CD8+ T-cells from HIC and cART  
201 (Supplementary Table 5) to react against a pool of HIV-1 Gag peptides in the presence and absence of  
202 glucose. We found that both TNF- $\alpha$  secretion and the overall magnitude of the HIV-specific CD8+ T-  
203 cell response (Figure 7A, 7C) from both the HIC and cART groups were significantly diminished in the

204 absence of glucose. However, the HIV-specific CD8+ T-cell response from cART was more strongly  
205 impacted and barely detectable or undetectable without glucose (Figure 7A, 7C). In contrast, CMV-  
206 specific CD8+ T-cell responses from cART were not significantly impacted by glucose deprivation  
207 (Supplementary Figure 8). The viability of CD8+ T-cells from HIC and cART individuals also decreased  
208 in the absence of glucose, but cells from cART individuals again tended to be more strongly affected  
209 (Supplementary Figure 9). These functional tests support our gene expression results demonstrating  
210 that HIV-specific CD8+ T-cells from cART individuals are dependent predominantly on glucose in  
211 contrast to HIC.

212

213 We wondered if the detected profiles on blood cells were representative of tissue cells. We therefore  
214 analyzed the impact of glucose deprivation on SIV-specific CD8+ T-cell responses from lymphoid  
215 tissues of macaques spontaneously controlling SIVmac251 infection (SIV controllers, SIC) or not  
216 controlling (viremic, VIR) (Supplementary Figure 10A). In line with what we described for HIV-  
217 infected individuals, blood and splenic CD8+ T-cells from SIC, but not from viremic animals, had a  
218 strong capacity *ex vivo* to suppress SIV infection of autologous CD4+ T-cells (Supplementary Figure  
219 10B). The splenic SIV-specific CD8+ T-cell response from SIC was not significantly affected by the  
220 absence of glucose, while it strongly decreased in conditions of glucose deprivation for viremic  
221 animals (Supplementary Figure 10C). These results confirm that strong glucose dependency is an  
222 inherent characteristic of the less efficient CD8+ T-cells found in non-controller subjects.

223

#### 224 **Metabolic plasticity of HIV-specific CD8+ T-cells from HIC**

225 Since HIV-specific CD8+ T-cells from HIC still responded despite glucose deprivation, our data suggest  
226 that they mobilize additional energetic resources. We also noted a trend toward larger mitochondrial  
227 mass in cells from cART individuals (Supplementary Figure 11), a feature that has been linked to  
228 mitochondrial dysfunction in senescent CD8+ T-cells<sup>30</sup> and exhausted HBV-specific CD8+ T-cells<sup>31</sup>. We  
229 wondered if cells from HIC and cART might differ with respect to their mitochondrial function. We

230 found that menadione, a drug that alters mitochondrial membrane polarization<sup>32</sup>, significantly  
231 reduced TNF- $\alpha$  and IFN- $\gamma$  secretion and diminished the overall response of CD8+ T-cells from HIC but  
232 not cART individuals (Figure 7A, C, Supplementary Figure 12). Altogether, cells from HIC, in contrast  
233 to cells from cART, appear to rely on mitochondrial function to fuel their anti-HIV CD8+ T-cell  
234 responses in addition to glycolysis.

235

236 We found that metabolic plasticity was important for sustaining qualitative differences between  
237 CD8+ T-cells from HIC and non controllers. The polyfunctionality of HIV-specific CD8+ T-cells from HIC  
238 was higher than that of cells from cART individuals in control conditions, in accordance with previous  
239 reports<sup>5</sup>. The percentage of highly polyfunctional cells in HIC (3 or 4 functions) was not significantly  
240 altered in the absence of glucose (Figure 7B, 7C), but decreased significantly in the presence of  
241 menadione (Figure 7B, 7C). No significant changes were observed in the polyfunctionality of HIV-  
242 specific CD8+ T-cells from cART individuals in the absence of glucose or the presence of menadione.  
243 Highly polyfunctional cells were only observed in two cART individuals at very low frequencies, and  
244 they were still detectable in the absence of glucose but could not be detected in the presence of  
245 menadione. Thus, the polyfunctionality of CD8+ T-cells appeared to depend on mitochondrial  
246 function rather than on glycolysis, which suggests that not all CD8+ T-cell functions are equally  
247 controlled by the same metabolic pathways.

248

249 To further explore this possibility, we analyzed phosphorylation of ribosomal S6 (pS6) and AKT (pAKT)  
250 proteins, as markers, respectively, of engagement of mTORC1 and mTORC2 pathways, in CD8+ T-cells  
251 from HIC producing IFN $\gamma$  or TNF $\alpha$  upon stimulation with HIV-1 Gag peptides. Interestingly, while  
252 IFN $\gamma$ + HIV-specific CD8+ T-cells were characterized by high levels of pS6 and low levels of pAKT  
253 (Supplementary Figure 13), the cells producing TNF $\alpha$  showed both high levels of pAKT and pS6,  
254 although not necessarily simultaneously (Supplementary Figure 13). Therefore, our results indicate  
255 that while IFN $\gamma$  production, one of the last function to be lost in exhausted HIV-specific CD8+ T-

256 cells<sup>33</sup>, is regulated by mTORC1, polyfunctionality might depend on mTORC2. Moreover, mTORC1 did  
257 not appear critical for CD8+ T-cells from HIC during our HIV/SIV-suppression assay. Rapamycin  
258 treatment during the suppression assay decreased the infection levels of isolated CD4+ T-cells  
259 (Supplementary Figure 14A), further showing that metabolic activities (and the mTORC1 pathway in  
260 particular) are critical for the establishment of HIV/SIV infection in CD4+ T-cells<sup>34,35</sup>. However,  
261 rapamycin did not abolish the capacity of CD8+ T-cells from HIV or SIV controllers to suppress  
262 infection (Supplementary Figure 14A). We could analyze oxidative phosphorylation and glycolysis in  
263 purified CD8+ T-cells from a limited number of HIC (n=3) and cART individuals (n=2) after co-culture  
264 with non-infected or HIV pre-infected autologous CD4+ T-cells. No differences in cell metabolism  
265 were observed between the CD8+ T-cells from cART individuals whether they had been cultured with  
266 infected or non-infected cells. In contrast, CD8+ T-cells from HIC cultured in presence of infected CD4  
267 T-cells showed enhanced metabolic activities, and in particular oxidative phosphorylation when  
268 compared to CD8+ T-cells that had been cultured in presence of non-infected cells (Supplementary  
269 Figure 14B), suggesting that CD8+ T-cells from HIC mobilized different metabolic resources in  
270 presence of HIV infected CD4+ T-cells.

271

272 Our results globally indicate that the functions more commonly associated with efficient HIV-specific  
273 CD8+ T-cell responses in controllers (polyfunctionality, viral suppression capacity) are sustained by  
274 the mTORC2 pathway and rely on both glucose and oxidative phosphorylation, while cells from cART  
275 rely mostly on glucose metabolism. They strongly suggest that metabolic plasticity is a critical  
276 characteristic of highly efficient CD8+ T-cells.

277

### 278 **IL-15 reinvigorates HIV-specific CD8+ T-cells from non-controllers**

279 IL-15 promotes fatty acid oxidation and mitochondrial biogenesis in CD8+ T-cells<sup>29</sup>. Accordingly, we  
280 found that IL-15 pre-treatment enhanced the rates of fatty acid uptake and the basal metabolic  
281 activities and maximal mitochondrial respiratory capacity of bulk CD8+ T-cells from cART individuals

282 (Figure 8A and B). IL-15 increased the frequency of HIV-specific CD8+ T-cell responses in cART but it  
283 did not alter the frequency of CMV-specific CD8+ T-cells in the same individuals (Figure 8C).  
284 Importantly, IL-15 pre-treatment also strongly enhanced the capacity of CD8+ T-cells from cART  
285 individuals to suppress HIV infection (Figure 8D). IL-15 pre-treatment was also able to increase the  
286 SIV-CD8+ T-cell response in lymphoid tissues of SIV viremic animals, abrogated their glucose  
287 dependency (Supplementary Figure 10C) and strongly increased their capacity to suppress SIV  
288 infection of CD4+ T-cells (Supplementary Figure 10D). These results show that metabolic  
289 reprogramming rescues HIV/SIV-specific CD8+ T-cell responses in non-controller individuals and  
290 further confirm that metabolic plasticity is a critical element in the efficacy of the HIV-specific CD8+  
291 T-cell response.  
292

293

294 **DISCUSSION**

295 Durable natural HIV control has been linked to the presence of HIV-specific CD8+ T-cells with  
296 enhanced capacities in functional assays *in vitro*<sup>8</sup>. We show here that the superior antiviral potential  
297 of CD8+ T-cells from HIC is engraved in the transcriptional program of the central memory  
298 population. When compared to cells from non-controllers, CD8+ TCM-cells from HIC exhibited  
299 upregulation of a broad range of genes linked to effector functions, indicating that differences  
300 between HIC and cART HIV-specific CD8+ T-cell responses are related to different memory-cell  
301 programs rather than to the accumulation of flaws in non-controllers' effector cells. Cells from HIC  
302 upregulated genes linked to survival, while cells from non-controllers upregulated genes associated  
303 with mTORC1 activation and glycolysis. Functional analyses showed that HIV-specific CD8+ T-cells  
304 from HIC had metabolic plasticity while cells from non-controllers had a strong dependency on  
305 glycolysis, and similar results were obtained in tissues from a non-human primate model of durable  
306 SIVmac251 control. Pre-treatment with IL-15 reinvigorated HIV/SIV-specific CD8+ T-cell responses in  
307 non-controllers, ablating their dependency on glucose and enhancing their capacity to suppress  
308 infection.

309

310 Our results show that HIV-specific CD8+ T-cells from controllers are equipped with a large panel of  
311 mechanisms to counteract HIV infection, including soluble antiviral factors and direct cytotoxic  
312 activities. The different expression of T-cell "function" genes between HIC and cART was  
313 accompanied by different expression of other sets of genes indicating the preferential activation of  
314 mTORC1 in cART individuals and mTORC2 in HIC. mTORC1 and mTORC2 play distinct roles in CD8+ T-  
315 cell differentiation. mTORC1 is generally associated with the induction of glycolysis and the  
316 generation of effector cells, while mTORC2 is linked to the regulation of T-cell memory functions<sup>22</sup>.  
317 Expression of genes associated with mTORC1 in HIV-specific CD8+ TCM-cells from cART individuals  
318 points to a skewed priming of these cells. On the other hand, strong expression of effector-linked

319 genes in cells from HIC was accompanied by very weak expression of mTORC1-linked genes. We  
320 show here that production of IFN- $\gamma$  and TNF $\alpha$  by HIV-specific CD8+ T-cells from HIC were associated  
321 with different engagement of mTORC1 and mTORC2. Moreover, mTORC1 blocking with rapamycin  
322 did not diminish the capacity of CD8+ T-cells from controllers to suppress infection. It is still unclear  
323 which processes are controlled by mTORC1 during effector functioning and, although mTORC1  
324 activation appears necessary for IFN- $\gamma$  production, our results are in agreement with previous reports  
325 indicating that this pathway may not have a global impact on cytokine production<sup>36</sup>.

326

327 HIV-specific CD8+ TCM-cells from HIC also exhibited upregulation of genes linked to survival, while  
328 cells from cART rather had a pro-apoptotic signature. Susceptibility of HIV-specific CD8+ T-cells to  
329 apoptosis is well documented in non-controllers<sup>2,37</sup>. Effector memory CD8+ T-cells isolated from HIC  
330 are preserved after prolonged culture, while HIV-specific CD8+ T-cells from non-controllers are  
331 gradually lost in the same conditions<sup>38</sup>. Indeed, it is possible that the enhanced capacity of CD8+ T-  
332 cells from HIC to suppress HIV infection results from the combination of higher cytotoxic potential on  
333 a per-cell basis<sup>6</sup> and enhanced survival of the cells in absence of cytokines as required in the  
334 suppression assay prior to the co-culture<sup>8</sup>. Overall, our results indicate that HIV-specific CD8+ T-cells  
335 from HIC are better equipped to survive, including in conditions of metabolic stress, which may be an  
336 indication of enhanced stemness of these cells<sup>39</sup>.

337

338 Our results support the notion that differences in the functionality of HIV-specific CD8+ T-cells from  
339 HIC and non-controllers are imprinted in the program of their memory cells. While exhaustion is  
340 typically associated with effector T-cells having a very limited capacity to establish persistent  
341 memory<sup>40</sup>, HIV-specific CD8+ T-cells from the cART individuals studied here were characterized by a  
342 profile reminiscent of exhausted cells, including the concomitant upregulation of genes encoding  
343 several inhibitory receptors (e.g. *LAG3*, *KLRD1*), despite their central memory phenotype. This



344 supports the notion that exhausted CD8+ T-cells may represent a distinct subset of cells that have  
345 received specific differentiation priming<sup>41</sup>.

346

347 HIV-specific CD8+ CM T-cells from HIC patients classified as strong or weak responders in terms of  
348 the direct antiviral activity of their CD8+ T-cells *ex vivo*<sup>18</sup> shared many features and similarly differ of  
349 cells from non-controllers. When compared to SR HIC, WR HIC generally had lower ultrasensitive viral  
350 load<sup>27</sup> and a lower frequency of infected cells with reactivable virus<sup>42</sup>, and likely constitute a  
351 subgroup of HIC who have achieved tighter control of the infection. Accordingly, our results show  
352 that the responses of HIV-specific CD8+ T-cells from SR and WR receive similar priming, although the  
353 profile of cells from WR HIC reflects a longer period of quiescence<sup>18</sup>.

354

355 One possible limitation to our single cell analyses is that, due to sample availability, not all sorted CM  
356 cells had the same HIV antigen specificity, which might have some impact on their molecular  
357 program. However single cells from HIC and ART individuals showed a compelling segregation.  
358 Moreover, the direct comparison of cells from subjects HIC 01001 and ART 630104, which were  
359 sorted with the same dextramer (A03 Gag 020-028), recapitulated many of the differences that were  
360 found in the global analysis (Supplementary Figure 15), confirming that main differences between  
361 cells from HIC and ART were not related to epitope specificity.

362

363 Our findings suggest that the survival and functional advantages of HIV-specific CD8+ T-cells from HIC  
364 might be related to their greater metabolic plasticity. Indeed, CD8+ T-cells from HIC were only  
365 partially affected by glucose deprivation, while cells from cART were highly glucose-dependent.  
366 Importantly, the same difference in glucose dependency was found with SIV-specific CD8+ T-cells  
367 from spleen samples of SIV controllers and viremic macaques that strongly differed in their capacity  
368 to suppress SIV infection *ex vivo*. This further supports that strict glucose dependency is associated  
369 with inefficient HIV/SIV-specific CD8+ T-cells in blood and tissues. Glucose-dependency was observed

370 for HIV-specific CD8+ T responses from cART individuals but not for CMV-specific CD8+ T-cells, which  
371 is in agreement with skewed priming exclusively of HIV-specific CD8+ T-cells rather than a general  
372 characteristic of the CD8+ T-cells in these individuals. The glucose dependency of HIV-specific CD8+  
373 T-cells from non-controllers appeared to be associated with dysfunctional (or uncommitted)  
374 mitochondria, which could deprive these cells of important metabolic resources. Increased  
375 mitochondrial mass has been linked to a survival defect of HIV-specific CD8+ T-cells in non  
376 controllers<sup>43</sup>, in line with our results. Low mitochondrial membrane potential and high oxidative  
377 stress in lymphocytes have also been positively correlated with viral load in untreated HIV-1  
378 infection<sup>44</sup>, further supporting the idea that mitochondria dysfunction can negatively impact viral  
379 control. Although mitochondrial metabolism is important for the persistence of CD8+ T-cell memory,  
380 recent reports indicate that it is not indispensable for memory development<sup>45</sup>. Interestingly, VHL  
381 deficiency in CD8+ T-cells increases constitutive levels of HIF1 and glycolytic activity favoring the  
382 development of effector memory responses<sup>46</sup>. Although these cells were able to counteract LCMV  
383 infection in mice<sup>25</sup>, VHL deficiency impaired the formation of terminally differentiated cells. In  
384 contrast, VHL-sufficiency in the same model was characterized by the development of central  
385 memory responses with increased mitochondrial respiratory capacity<sup>46</sup>, which may be critical in the  
386 long-term. These results are coherent with our observation that HIV-specific CD8+ T CM cells from  
387 HIC and cART individuals were characterized by contrasting differential expression of *VHL*  
388 (upregulated in HIC) and *HIF1* (upregulated in cART individuals). The magnitude and polyfunctionality  
389 of HIV-specific CD8+ T-cells from HIC was reduced in the presence of menadione, a drug that alters  
390 mitochondrial membrane polarization<sup>32</sup>. Menadione can also perturb mitochondrial calcium  
391 homeostasis<sup>32</sup>, which also affects T-cell activation<sup>47</sup>. In any case, although the number of experiments  
392 was limited, CD8+ T-cells from HIC increased oxidative phosphorylation upon contact with infected  
393 CD4+ T-cells, further supporting that HIV-specific CD8+ T-cells from HIC had preserved mitochondrial  
394 metabolism.

395

396 This could be related to the *ID2* upregulation observed in HIC cells. *ID2* has been shown to promote  
397 effector differentiation and T-cell survival<sup>48</sup>, and to reduce mitochondrial damage induced by  
398 oxidative stress and to increase survival in cancer cell lines deprived of glucose<sup>49</sup>. CD8+ CM T-cells  
399 from HIC showed increased fatty acid uptake. This could indicate that CD8+ CM T-cells from HIC may  
400 also rely on fatty acid oxidation, which has been shown to enhance CD8+ T-cell memory generation<sup>29</sup>,  
401 facilitate the secretion of several effector cytokines<sup>50</sup> and provide superior protection against  
402 influenza virus infection<sup>51</sup>. Overall, our results imply that metabolic plasticity, as opposed to strict  
403 glucose-dependency, may contribute to the enhanced antiviral capacity of natural HIV controllers'  
404 CD8+ T-cells.

405

406 To avoid the impact of ongoing viral replication on our analysis, we compared cells from HIC to those  
407 from HIV-1-infected individuals on cART. Some nucleoside reverse transcriptase inhibitors (NRTI) can  
408 block mitochondrial DNA polymerase gamma function and induce mitochondrial dysfunction<sup>52</sup>.  
409 However, not all NRTIs have the same impact on mitochondrial function and the drugs used to treat  
410 the individuals whose cells were used in our functional assays are reported to have limited  
411 mitochondrial toxicity<sup>52,53</sup> (Supplementary Table 6). The increase in mitochondrial mass is a selective  
412 characteristic of HIV-specific CD8+ T-cell responses associated with uncontrolled infection<sup>43</sup>.  
413 Moreover, CMV-specific CD8+ T-cell responses from cART individuals did not show glucose  
414 dependency. Therefore, impact of antiretroviral treatment does not explain the metabolic defects  
415 observed in HIV-specific CD8+ T-cells from cART individuals. In addition, glucose dependency was also  
416 found in SIV-specific CD8+ T-cells from ART-naïve SIVmac251 infected macaques with high viremia,  
417 showing that this glucose dependency was associated with inefficient HIV/SIV specific CD8+ T-cell  
418 responses in subjects with active viral replication or ART-suppressed viremia.

419

420

421 Although glycolysis is a pivotal energy resource for CD8+ T-cell function, tight glucose dependency  
422 could impose serious limitations *in vivo*, as already shown in cancer. Tumor cell growth and the  
423 tumor microenvironment provoke nutrient restrictions and impair anti-tumoral T-cell effector  
424 functions<sup>54</sup>. Similarly, in the context of HIV infection, T-cell activation and homeostasis are  
425 accompanied by an increase in T-cell glucose uptake in lymph nodes<sup>55</sup>. HIV-1-infected CD4 T-cells  
426 have enhanced glucose uptake and glycolytic activity<sup>34</sup>. Lactic acid, the product of glycolysis, can  
427 suppress CD8+ T-cell cytotoxic functions<sup>56</sup>. The glucose dependency of CD8+ T-cells from HIV non-  
428 controllers could lead to functional impairment in this glucose-restricted microenvironment. In  
429 contrast, the greater metabolic plasticity of cells from HIC would allow them to continue to exert  
430 their functions, at least partially, in this setting.

431

432 Although, due to the heterogeneity of the data, the interpretation of some differences observed in  
433 our study between cells from HIC and cART individuals should be considered with caution, it is worth  
434 noting that another recent study showed that dysfunctional HBV-specific CD8+ T-cells are  
435 characterized by GLUT1 upregulation and glucose dependency, while functional CMV-specific CD8+  
436 T-cells are able to use oxidative phosphorylation in the absence of glucose<sup>31</sup>. In addition,  
437 mitochondria-targeting antioxidant treatment corrected mitochondrial dysfunction and restored  
438 HBV-specific T-cell functions<sup>57</sup>. This suggests that limited metabolic plasticity and glucose  
439 dependency is a hallmark of dysfunctional antigen-specific CD8+ T-cells.

440

441 We show here that HIV/SIV-specific CD8+ T-cell responses from cART individuals or viremic macaques  
442 can be reinvigorated by IL-15 pre-treatment. IL-15 induces mitochondrial biogenesis in CD8+ T-cells,  
443 to promote the use of fatty acids for energy<sup>29</sup>, and enhances the survival and function of HIV-specific  
444 CD8+ T-cells<sup>58</sup>. Our results are in agreement with these reports and indicate that these beneficial  
445 effects are interrelated. Moreover, our data show that antigen-specific CD8+ T-cells more dependent  
446 on glucose, such as HIV-specific CD8+ T-cells, benefited most of the IL-15 mediated enhancement of

447 oxidative metabolism, while the effect was mitigated in cells that were already using mitochondrial  
448 respiration to sustain their activity in the absence of glucose, e.g. CMV-specific CD8+ T-cell  
449 responses. Importantly, pre-incubation with IL-15 restored non-controllers' CD8+ T-cells' HIV-  
450 suppressive capacity *ex vivo*, as previously shown<sup>38</sup>. IL-15 immunotherapy is showing promising  
451 results *in vivo* in non-human primate models<sup>59</sup>, and this may be related in part to its direct effect on  
452 CD8+ T-cell function, as shown here.

453

454 Overall, our findings show that the enhanced functionality of HIV-specific CD8+ T-cells from HIC is  
455 imprinted in their memory program and is associated with their ability to use diverse metabolic  
456 resources. In contrast, poor functionality of cells from non-controllers is associated with dependency  
457 on glycolysis as primary energy source. Our data thus highlight the molecular singularity of HIV-  
458 specific CD8+ T-cells from HIC, and point to a metabolic profile compatible with better cell survival  
459 and a preserved potential to develop a higher quality anti-HIV effector functions in stressful  
460 conditions. Our results indicating that HIV-specific CD8+ T-cell metabolism is linked to control of HIV  
461 infection suggest that new pharmacological tools regulating metabolic activity, as explored in the  
462 cancer field<sup>60</sup>, could help to induce HIV remission and to develop therapeutic vaccines.

463

464

465 **MATERIALS AND METHODS**

466

467 **Subjects**

468 The HIV controllers (HIC, individuals naïve of antiretroviral treatment and whose last 5 consecutive  
469 plasma HIV RNA values were below 400 copies/ml) included in this study belonged to the ANRS CO21  
470 Codex cohort. Strong responders (SR) and weak responders (WR) were defined as HIV controllers  
471 whose CD8+ T-cells were able to reduce p24 production by autologous CD4+ T-cells by more and less  
472 than 2 logs, respectively (see *Viral Suppression Assay*)<sup>27</sup>. The antiretroviral-treated HIV-infected  
473 individuals (cART, antiretroviral treatment for at least 2 years, and undetectable HIV RNA) were part  
474 of the ANRS PRIMO cohort or recruited from the outpatients at CHU Kremlin-Bicêtre and CHU  
475 Georges Pompidou in Paris. Clinical data for the individuals studied in the single-cell gene expression  
476 assays are listed in Supplementary Table 1. Clinical data for the other study participants are  
477 summarized in Supplementary Table 5. All participants gave their informed consent and the study  
478 was approved by ethics committee (Comité de Protection des Personnes) of Île-de-France XI.

479

480 **Viral Suppression Assay**

481 The capacity of CD8+ T-cell to suppress HIV-1 infection was analyzed on co-cultures with autologous  
482 CD4+ T-cells<sup>61</sup>. After PBMC isolation from peripheral blood by density gradient centrifugation, CD4+  
483 and CD8+ T-cells were separated by, respectively, successive positive and negative magnetic bead  
484 sorting (STEMCELL Technologies). CD4+ T-cells were cultured for 3 days in complete RPMI medium in  
485 the presence of phytohemagglutinin (1 µg/ml) and interleukin-2 (100 IU/ml). In parallel, CD8+ T-cells  
486 were cultured in complete RPMI medium in the absence of stimuli. Activated CD4+ T-cells were  
487 infected with HIV-1 Bal by spinoculation for 2 h at 2,000 × g and 22°C<sup>62</sup> and cultured alone or with  
488 CD8+ T-cells at a 1:1 ratio for 14 days in interleukin-2 (100 IU/ml)-supplemented complete RPMI.  
489 Viral replication was measured in terms of p24 production in the culture supernatants by means of

490 enzyme-linked immunosorbent assay (XpressBio). In some experiments co-cultures were done in the  
491 presence of rapamycin (1 nM, Sigma Aldrich ).

492

493 For viral suppression assays with CD8+ T-cell subsets, CD4+ and CD8+ T-cells were isolated and  
494 cultured as described above. The day of infection, CD8+ T-cells were stained with anti-CD4 FITC (BD),  
495 anti-CCR7 PE-Cy7 (eBioscience), anti-CD27-PE (BD) and anti-CD45RA v421 (BD) antibodies. CD8+ T-  
496 cell subsets were isolated with a FACSAria III cell sorter (BD). CD8+ T-cell subsets were defined as  
497 Naïve (CD4-CD45RA+,CCR7+,CD27+), Central Memory (CD4-CD45RA-,CCR7+,CD27+), Transitional  
498 Memory (CD4-CD45RA-,CCR7-,CD27+) or Effector Memory (CD4-CD45RA-,CCR7-,CD27-). After  
499 sorting, CD4+ T-cells were challenged with HIV-1 Bal and cultured alone or with the autologous CD8+  
500 T-cell subsets as described above.

501

#### 502 **Single-Cell Flow Cytometry-assisted Cell Sorting**

503 For each individual twenty million PBMC were thawed and cultured overnight in complete RPMI with  
504 20% FBS. CD8+ T-cells were then separated by magnetic bead sorting (negative selection, STEMCELL  
505 Technologies) and stained with the LIVEDEAD Fixable Aqua Dead Cell Stain Kit (Thermo Ficher  
506 Scientific), APC-conjugated dextramers (Immudex) and the following antibodies: anti-CD3 AF-700  
507 (BD), CD8 APC-Cy7 (BD), CD45RA v421, CCR7 PE-Cy7 and CD27 PE. For each individual, viable Central  
508 Memory CD8+ T-cells were single-cell-sorted (BD FACS ARIA SORP, Becton-Dickinson) in 96-well  
509 plates containing the VILO Reaction Mix, SUPERase-In and NP40 (all from Thermo Ficher Scientific).  
510 480 cells were sorted for each group of patients (1440 cells in total). Plates were snap-frozen and  
511 stored at -80°C.

512

#### 513 **Single-Cell Gene Expression**

514 Analysis of gene expression in single cells from the different individuals, in no particular order, was  
515 performed as follows. Plates containing sorted single cells were thawed on ice. RNA was denatured

516 at 65°C for 90 seconds and then snap-chilled on ice. An RT mix containing SuperScript Enzyme and T4  
517 Gene 32 Protein was then added, followed by RT cycling (25°C: 5 min, 50°C: 30 min, 55°C: 25 min,  
518 60°C: 5 min, 70°C: 10 min). Specific target amplification (STA) was performed by adding TaqMan  
519 PreAmp Master Mix, 96 Primers mix and EDTA to cDNA, followed by STA cycling (95°C: 10 min, 20  
520 cycles of [96°C: 5 s, 60°C 4 min]). The sample was then treated with exonuclease I (New England  
521 Biolabs) (37°C: 30 min, 80°C: 15 min).

522

523 Sample pre-mix (SsoFast EvaGreen Supermix with Low ROX (Biorad), DNA Binding Dye (Fluidigm),  
524 preamplified Exo 1-treated sample) and Assay mix (Assay Loading Reagent (Fluidigm), Delta Gene  
525 primers (Fluidigm)) were loaded on a primed 96.96 Dynamic Array chip (Fluidigm). The chip was  
526 transferred into a Biomark (Fluidigm) for thermocycling, and fluorescence was acquired with the GE  
527 96x96 PCR+Melt v2 program. For quality control, amplification curves were validated with cDNA  
528 from bulk CD8+ T-cells. Linear derivative mode baseline correction was applied. Gene expression  
529 values are plotted as Log2Ex, where  $\text{Log2Ex} = \text{LoD} - \text{Ct}$  if  $\text{Ct} \leq \text{LoD}$  ( $\text{Log2Ex} = 0$  if  $\text{Ct} > \text{LoD}$ ), with LoD =  
530 limit of detection (24) and Ct = threshold cycle. A list of genes with raw Ct values is shown in  
531 Supplementary Data 1. Cells negative for *CD8*, *GAPDH* and *ACTB* gene expression were excluded from  
532 the analysis.

533

#### 534 **Statistical analyses**

535 The differential gene expression analysis was performed using MAST (Model-based Analysis of Single  
536 Cell Transcriptomics) (v1.8.2) packages from R software (v3.5.1). This package is based on the hurdle  
537 model, a two-part generalized model for bimodal and/or zero-inflated single cell gene expression  
538 data, simultaneously modeling the rate of expression over the background of various transcripts and  
539 the positive expression mean<sup>63</sup>. Cells are considered as outliers and removed from the analysis if  
540 their total expression (in log2EX) or the number of genes they express are less than two scaled  
541 median absolute deviations (MAD) away from the median. The total numbers of cells after data



542 filtration were as follows: 329 (HIV-controllers, strong responders), 348 (HIV-controllers, weak  
543 responders) and 320 (cART-treated individuals).

544

545 The hurdle model was applied to each gene in order to detect differentially expressed genes  
546 between the groups of interest (HIC [WR and SR], cART,). This model included the group and subject  
547 variables as fixed effects, as well as the interaction between group and subject. The last two terms  
548 were used to model the pairing between cells from the same subject. To test the difference in  
549 expression for each gene, we defined vectors of contrasts and then applied likelihood ratio tests. We  
550 obtained p-values that were adjusted according to the Benjamini and Hochberg procedure to control  
551 the false discovery rate at 5%<sup>64</sup>. To compare the *between inter-goup* and *intra-group* variance we  
552 calculated the F-statistic using one-way ANOVA with Welch correction (v2.4-2).

553

554 For t-sne analyses<sup>65</sup> gene expression values (Log2EX, SR and cART groups) of all genes and samples  
555 were used.

556

557 For additional statistical tests, Graphpad Prism software (GraphPad Software, Inc.) was used. Non  
558 parametric Mann-Whitney test was used to compare metabolite uptake and mitochondria mass and  
559 to compare the ratio between HIV-specific CD8+ T-cell TNF $\alpha$  responses in medium without and with  
560 glucose, between HIV controllers and cART individuals. A non-parametric Wilcoxon paired test was  
561 used to compare HIV-specific CD8+ T-cell responses in different conditions.

562

### 563 **Metabolite Uptake**

564 Fresh PBMC were stained with anti-CD3 AF 700, anti-CD8 APC-Cy7, CD45RA BV421, CD27 PE, and  
565 CCR7 PE-Cy7 (all from BD). Stained samples were then split into three parts and put into contact with  
566 2-NBDG (2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose) (150  $\mu$ M, 30 min) for  
567 glucose uptake measurement, BODIPY<sup>®</sup> 500/510 C1, C12 (4,4-Difluoro-5-Methyl-4-Bora-3a,4a-Diaza-

568 s-Indacene-3-Dodecanoic Acid) (5  $\mu$ M, 5 min) for fatty acid uptake measurement, and MitoTracker  
569 Green FM (100 nM, 45 min) (all from Thermo Fisher Scientific) for mitochondrial mass measurement  
570 (see Supplementary figure 16 for gating strategy). To minimize technical variability, metabolite  
571 uptake was compared to the uptake by cells from a reference non-infected individual always  
572 analyzed in parallel in each experiment. Metabolite uptake was thus expressed as the relative MFI for  
573 cells from each individual when compared to the MFI observed in cells from the reference donor.

574

### 575 **Phenotyping**

576 We analyzed by flow cytometry the expression of CCL3, FASL and GrzB as markers of antiviral  
577 potential, pS6 and pAKT as markers of mTORC1 and mTORC2 activation respectively, and HIF1,  
578 (Supplementary Figure 7). Purified PBMC were thawed and rested overnight at 37°C in RPMI medium  
579 (RPMI 1640 supplemented with L-glutamine and antibiotics) with 20% heat-inactivated FCS. For cell  
580 phenotyping, CD8<sup>+</sup> T-cells were separated by magnetic bead sorting (negative selection, STEMCELL  
581 Technologies) and stained with the LIVEDEAD Fixable Aqua Dead Cell Stain Kit (Thermo Fischer  
582 Scientific), APC-conjugated dextramers (Immudex) and the following antibodies: anti-PD1 BV785,  
583 CD45RA BV655, CCR7 Qdot605, CD8 BUV496, CD3 BUV395, CD27 PerCP-Cy5.5 (all from BD  
584 Bioscience) and FASL PE-Cy7 (Miltenyi Biotech). Cells were then incubated with PhosFlow Fix buffer,  
585 washed with Phosflow Perm-Wash Buffer and stained with anti- CCL3 APC-Cy7 (Miltenyi Biotech),  
586 Granzyme B PE-TR BD Bioscience, HIF1a PE (Biolegend), Phospho AKT FITC and pS6 PacBlue/BV421  
587 (both from Cell Signaling). Due to the rarity of HIV-specific CD8<sup>+</sup> TCM-cells, the number of events  
588 that we could analyze was limited and we could not find significant levels in the expression of CCL3,  
589 FASL, pAKT or pS6 in HIV-specific CD8<sup>+</sup> TCM-cells ex vivo to allow comparison between cells from HIC  
590 and cART individuals. Results for GrzB and HIF1 are depicted in Figure 6.

591

### 592 **Intracellular Cytokine Assay**

593 Purified PBMC were thawed and rested overnight at 37°C in RPMI medium (RPMI 1640  
594 supplemented with L-glutamine and antibiotics) with 20% heat-inactivated FCS. For the “No Glucose”  
595 condition, cells were rested and stimulated in RPMI without glucose. Cells were then incubated with  
596 overlapping peptide pools encompassing HIV-1 consensus subtype B Gag or HCMV pp65 (both  
597 obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH, cat #12425 and  
598 #11549) (2 µg/ml). Anti-CD28/anti-CD49d co-stimulation (1 µL/mL) and the anti-CD107α marker  
599 were used in each condition. Phorbol myristate acetate (80 ng/mL) together with ionomycin (1  
600 µg/mL; Sigma-Aldrich) was used as positive control. Golgi stop (1 µg/mL; BD Biosciences) and  
601 Brefeldin A (10 µg/mL; Sigma-Aldrich) were added 30 min after the start of all incubations. Cells were  
602 then stained with the LIVE/DEAD Fixable Aqua Dead Cell Stain kit (Thermo Fisher Scientific), and with  
603 anti-CD3 and anti-CD8 antibodies. BD Cytofix/Cytoperm™ (BD Biosciences) was used for cell  
604 permeabilization prior to staining for intracellular markers. Intracellular staining used anti-IFNγ, anti-  
605 IL-2 and anti-TNFα (see Supplementary Figure 17 for gating strategy). In some experiments cells were  
606 fixed with permeabilized with Phosflow fix/perm buffers (BD Bioscience) and cells were stained for  
607 intracellular IFNγ, TNFα, pS6 and pAKT as above. Menadione sodium bisulfite (Sigma-Aldrich) was  
608 used at 5 µM.

609

#### 610 **Measurement of oxygen consumption rate**

611 Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using a  
612 Seahorse XF96 metabolic analyser as indicated by the manufacturer (Seahorse XF Technology,  
613 Agilent). CD8+ T-cells ( $2 \times 10^5$  cells per well) were seeded on XF96 plates (Agilent Seahorse) pre-  
614 coated with 0.5 mg/ml Cell Tack (Corning). Cells were incubated for 50 min in a CO<sub>2</sub>-free incubator at  
615 37°C before loading the plate in the Seahorse analyser. Oligomycin (2.5 µM), FCCP (0.9 µM) 4) and  
616 Rotenone/antimycin A (1 µM each) were sequentially added to the cells and OCR was monitored  
617 overtime. The maximal respiratory capacity is calculated as the OCR after FCCP addition subtracted  
618 the OCR after oligomycin. For glycolysis (ECAR), cells were cultured in the absence of glucose and

619 then XFmedia, glucose (10 mM), oligomycin (2.65  $\mu$ M), and 2-DG (100 mM)) were sequentially added  
620 to the cells.

621

622 In some experiments OCR and ECAR were analyzed in CD8+ T-cells from HIC after co-culture with  
623 HIV-infected or non-infected autologous CD4+ T-cells. Briefly, CD4+ T-cells from HIC were or not  
624 infected in vitro with HIV-1 BaL as described above. After three days, cells were washed and  
625 autologous CD8+ T-cells were added to the cultures of non-infected and infected CD4+ T-cells at a 1:1  
626 ratio. Four days later, CD8+ T-cells were isolated again from the co-culture (flow sorted based on  
627 viability and expression of CD8) and their metabolic activity analyzed with the Seahorse XF96  
628 analyser.

629

### 630 **Studies with cells from SIVmac251 infected Cynomolgus macaques**

631 Adult male cynomolgus macaques (*Macaca fascicularis*) were imported from Mauritius and housed  
632 in the facilities of the “Commissariat à l'Energie Atomique et aux Energies Alternatives” (CEA,  
633 Fontenay-aux-Roses, France). All animals studied here were part of the ANRS SIC and ANRS  
634 pVISCNTI studies and were inoculated (viral doses ranging from 5 to 1000 AID<sub>50</sub>) with the same  
635 batch of uncloned SIVmac251 (provided by A. M. Aubertin, Université Louis Pasteur, Strasbourg,  
636 France). Cells used were obtained during necropsies of animals at the chronic phase of infection  
637 (between 8 and 18 months post-infection). Peripheral blood was collected by venous puncture into  
638 BD Vacutainer Plus Plastic K3EDTA tubes or BD Vacutainer CPT Mononuclear Cell Preparation Tube -  
639 Sodium Heparin (BD Biosciences). Spleen and lymph nodes were processed by mechanical disruption  
640 in RPMI media using a GentleMACS dissociator (Miltenyi Biotech), followed by gradient in  
641 Lymphocyte separation medium and red cell lysis.

642

643 *Viral load*

644 Plasma viremia was determined using quantitative real time PCR with sensitivity of 12.3 copies/mL.  
645 Viral RNA was prepared from 100 µl of cell-free plasma, using the NucleoSpin 96 Virus core kit  
646 (Macherey-Nagel SARL, Hoerdt, France) under vacuum, according to the manufacturer's instructions.  
647 RNA was eluted in 100 µl of nuclease-free water and frozen immediately at -80°C until analysis.  
648 SIVmac251 virus diluted in and EDTA-treated plasma samples from macaques not infected with SIV  
649 was used to generate a standard curve (serial 10-fold dilutions). Three titrated SIVmac251-infected  
650 EDTA-treated plasma samples and two EDTA-treated plasma samples from SIV-negative macaques  
651 were used as positive and negative reverse transcription-PCR controls, respectively. Standards,  
652 controls, and viral RNA samples were extracted and tested in parallel under the same conditions. The  
653 SIVmac251 gag cDNA sequence, ligated into the plasmid pCR2-TOPO (Invitrogen), was used as a  
654 positive control for PCR.

655

656 Quantitative RT-PCR was performed with a SuperScript III Platinum One-Step qRT-PCR Kit  
657 (Thermofisher) in a CFX96 Touch Real-Time PCR Detection System (BioRad) under the following  
658 conditions: 12.5 µl of 2X reaction mixture, 0.5 µl of RNaseOUT (40U/µl), 0.5 µl of Superscript III  
659 reverse transcriptase/Platinum,Taq DNA Polymerase, 1 µl of each primer (125 µM), 0.5 µl of the  
660 fluorogenic probe (135 µM), and 10-µl of RNA elution samples. The probe and primers were designed  
661 to amplify a region of SIVmac251 gag<sup>66</sup>. Forward primer was 5'-GCAGAGGAGGAAATTACCCAGTAC-3'  
662 (24 bp) and reverse primer was 5'-CAATTTTACCCAGGCATTTAATGTT-3' (25 bp). The TaqMan probe  
663 sequence was 5'-FAM-TGTCCACCTGCCATTAAGCCCGA-BHQ1-3' (23 bp). This probe had a fluorescent  
664 reporter dye, FAM (6-carboxyfluorescein), attached to its 5' end and the quencher BHQ1 (Black Hole  
665 Quencher 1) attached to its 3' end. Samples were heated for 30 min at 56°C and 5 min at 95°C,  
666 followed by 50 cycles of 95°C for 15 s and 60°C for 1 min.

667

668 *Intracellular cytokine staining*

669 Splenocytes were thawed, resuspended at  $1 \times 10^6$ /mL in R20 media and kept at 37°C overnight. Cells  
670 were then stimulated with 2 µg/mL of SIV peptide pools in the presence of costimulatory monoclonal  
671 antibodies anti-CD28 (clone L293) and anti-CD49d (clone 9F10) at 1 µg/mL and stained with anti-  
672 CD107a for 30 minutes. Golgi Stop (BD Biosciences) and brefeldin A (BFA, 5 µg/mL; Sigma) were then  
673 added. A pool containing 24 optimal peptides (ProImmune) was used to assess SIV-specific CD8+ T  
674 cell responses. Co-stimulatory antibodies + Golgi Stop + BFA alone were used as a negative control.  
675 Cells were incubated for a total of 6 hours and stained to assess cytokine production by SIV-specific  
676 cells. After washing, cells were surface stained with anti-CD3, CD4 and CD8 antibodies. Cells were  
677 then permeabilized (Cytofix/CytoPerm kit; BD Biosciences) and stained for IFN $\gamma$ , TNF $\alpha$ , IL-2 and  
678 Perforin. The following antibodies were used: CD107a – V450 (clone H4A3, BD), CD3 – AF700 (clone  
679 SP34-2, BD), CD4 – PerCP Cy5.5 (clone L200, BD), CD8 $\alpha$  – APC-Cy7 (clone RPA-T8, BD), IFN $\gamma$  – PE-Cy7  
680 (clone B27, BD), IL-2 – PE (clone MQ1-17H12, BD), TNF $\alpha$  – PE-CF594 (clone Mab11, BD) and Perforin –  
681 FITC (clone Pf-344, Mabtech). Flow cytometry was performed with an LSRII instrument (BD  
682 Biosciences), and data were analyzed with FlowJo V.10 software (TreeStar Inc.).

683

#### 684 *CD8 T cell suppression assay*

685 CD4+ and CD8+ T cells were purified from PBMCs or tissues cell suspensions with antibody-coated  
686 magnetic beads in a Robosep instrument (Stemcell Technologies). Custom non-human primate  
687 selection kits (Stemcell Technologies) were used to purify CD4+ T-cells (positive selection) and CD8+ T  
688 cells (negative selection). The capacity of CD8+ T cells to suppress SIV infection of autologous CD4+ T  
689 cells<sup>67</sup> was adapted from the assay with human cells described above. CD4+ cells were stimulated for  
690 3 days with 5µg/mL Concanavalin A (ConA) in the presence of IL-2 (Miltenyi Biotec) at 100 IU/mL.  
691 CD8+ T cells were kept in culture in the absence of any mitogen or cytokine or pre-incubated with IL-  
692 15. CD4+ T cells were then superinfected with SIVmac<sub>251</sub> (MOI =  $10^{-3}$ ) in the presence or absence of  
693 CD8+ T cells (ratio CD4:CD8 of 1:1). Cells were cultured for 14 days in R10 medium containing IL-2

694 (100 IU/mL). Viral replication was monitored by p27 detection in culture supernatants (XpressBio) at  
695 day 7.

696

697 *Ethics statement*

698 Non-human primates (NHP, which includes *M. fascicularis*) are used at the CEA in accordance with  
699 French national regulations and under the supervision of national veterinary inspectors (CEA Permit  
700 Number A 92-032-02). The CEA complies with the Standards for Human Care and Use of Laboratory  
701 Animals of the Office for Laboratory Animal Welfare (OLAW, USA) under OLAW Assurance number  
702 #A5826-01. All experimental procedures were conducted according to European Directive 2010/63  
703 (recommendation number 9) on the protection of animals used for scientific purposes and national  
704 regulations.

705

706 The SIC and pVISCNTI studies were accredited under statement number 13–005, by the ethics  
707 committee “Comité d’Ethique en Expérimentation Animale du CEA” registered and authorized under  
708 numbers 01114.01 and 2453-2015102713323361v2 by the French Ministry of Education and  
709 Research. The animals were used under the supervision of the veterinarians in charge of the animal  
710 facility and were housed under controlled conditions of humidity, temperature, and light (12-hour  
711 light/12-hour dark cycles). Water was available ad libitum. Animals were monitored and fed  
712 commercial monkey chow and fruit 1-2 times daily by trained personnel. The macaques  
713 were provided with environmental enrichment, including toys, novel foodstuffs, and music, under  
714 the supervision of the CEA Animal Welfare Body.

715

716 Animals were sedated by intra-muscular injection of 10 mg/kg ketamine (Rhone-Merieux, Lyon,  
717 France) euthanized by intravenous injection of sodium pentobarbital (200 mg/Kg) and tissue  
718 samples were immediately collected during autopsy.

719

720 **Reporting Summary**

721 Further information on research design is available in the Nature Research Reporting Summary linked  
722 to this article.

723

724 **DATA AVAILABILITY**

725 All data generated or analyzed during this study are available from the corresponding author upon  
726 request. Gene expression data are included in this article and its supplementary information files..

727



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746

747 **AUTHOR CONTRIBUTIONS**

748 M.A., C.P., C.L., V.M., JCV-C, performed experiments; M.A., S.V., M-A.D., and A.S-C. analyzed the  
749 data; L.W., C.G., L.M., F.B. and O.L. contributed to inclusion of study participants, obtaining and  
750 validation of clinical information; C.P., B.V., R.L.G. and AS-C contributed to the design and  
751 development of the macaque study; G.P., MM-T, O.L. and AS-C contributed to the conception of the  
752 study; M.A., O.L. and AS-C designed the study; M.A. and AS-C drafted the article; all authors critically  
753 reviewed the manuscript.

754 **COMPETING FINANCIAL INTERESTS**

755 The authors declare no competing financial interests

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958

959 **FIGURE LEGENDS**

960 **Figure 1**

961 a) Representative examples of viral suppression assays, as previously shown<sup>8,18</sup>. Gag p24  
962 concentrations in supernatants from HIV-infected CD4+ T cells alone (green squares and lines) and  
963 CD4+/CD8+ T cell co-cultures (orange triangles and lines), using cells isolated from an HIV controller  
964 (left) and an antiretroviral-treated individual (right) on different days of culture after HIV challenge.  
965 0.1 ng/ml is considered as the lower limit of detection in our p24 ELISA. Data are means and standard  
966 deviation of three replicates. b) Gag p24 concentrations in supernatants from HIV-infected CD4+ T  
967 cells alone (green column) isolated from an HIV controller (top) or a cART-individual (bottom), in co-  
968 culture with bulk or flow-cytometry-sorted CD8+ T cell subsets on day 7 post-challenge (orange  
969 columns; N: Naïve, CM: Central Memory, TM: Transitional Memory, EM: Effector Memory). Mean  
970 and values for 3 replicates are shown. Representative results of independent experiments with cells  
971 from 3 HIV controllers and 3 cART-individuals. c) Median percentage CD38/HLADR activation marker  
972 expression by flow-sorted HIV-specific viable central memory CD8+ T cells isolated from HIV  
973 controllers (n=5 individuals) and cART (n=5 individuals). d) T-sne analyses of gene expression by flow-  
974 sorted HIV-specific central memory CD8+ T cells from HIV controllers (red circles) (n=329 cells) and  
975 cART individuals (blue circles) (n=320 cells).

976

977 **Figure 2**

978 Differentially expressed genes linked to CD8+ T cells effector functions in HIV-specific central  
979 memory CD8+ T cells from HIV controllers (red) and cART individuals (blue). Violin plots represent  
980 gene expression as Log2ex (limit of detection – threshold cycle) for each cell after data filtration. Cells  
981 negative for CD8, GAPDH and ACTB or considered as outliers (if their total expression (in log2EX) or  
982 the number of genes they express are less than two scaled median absolute deviations away from  
983 the median) were excluded from the analyses. Values for each single cell analyzed (n=329 cells for



984 HIC, and n=320 cells for cART) are represented as scatter dots. The width of the plots represents the  
985 kernel probability density of events at each given value. The proportion of cells lacking expression of  
986 a gene is represented by the wideness of the base of the violin plots. The hurdle model was applied  
987 to each gene in order to detect differentially expressed genes between cells from HIC and cART  
988 individuals. For all genes depicted a statistical significant difference ( $p < 0.05$  after adjustment  
989 according to the Benjamini and Hochberg procedure to control the false discovery rate at 5%) in  
990 either continuous, discrete or hurdle analyses was found. Red or blue color of the P values indicate  
991 higher expression in cells from HIC or cART individuals respectively.

992

993 **Figure 3**

994 Genes differentially expressed by HIV-specific central memory CD8+ T cells from HIV controllers (red)  
995 and cART individuals (blue). Violin plots represent gene expression as Log<sub>2</sub>ex (limit of detection –  
996 threshold cycle) for each cell after data filtration. Cells negative for CD8, GAPDH and ACTB or  
997 considered as outliers (if their total expression (in log<sub>2</sub>EX) or the number of genes they express are  
998 less than two scaled median absolute deviations away from the median) were excluded from the  
999 analyses. Values for each single cell analyzed (n=329 cells for HIC, and n=320 cells for cART) are  
1000 represented as scatter dots. The width of the plots represents the kernel probability density of  
1001 events at each given value. The proportion of cells lacking expression of a gene is represented by the  
1002 wideness of the base of the violin plots. The hurdle model was applied to each gene in order to  
1003 detect differentially expressed genes between cells from HIC and cART individuals. For all genes  
1004 depicted a statistical significant difference ( $p < 0.05$  after adjustment according to the Benjamini and  
1005 Hochberg procedure to control the false discovery rate at 5%) in either continuous, discrete or hurdle  
1006 analyses was found. Red or blue colors of the P values indicate higher expression in cells from HIC or  
1007 cART individuals respectively. (A) Genes upregulated in cells from cART individuals. (B) Genes  
1008 upregulated in cells from HIV controllers.

1009

1010 **Figure 4**

1011 A) Gene expression t-SNE plots. The first plot (top left) shows single cells from HIV controllers (n=329  
1012 cells) (red) and cART (n=320 cells) (blue) individuals. The remaining dot plots are colored on the basis  
1013 of gene expression values. Groups of neighbor cells identified by the t-sne analysis enriched (>90%)  
1014 in cells from HIV controllers (red) and cART individuals (blue) are indicated for visual reference. B)  
1015 Heat map of genes differentially expressed (filtered based on the coefficient of the hurdle analysis) in  
1016 cells from HIV controllers (red) and cART individuals (blue). Cells are ordered based on the first axis of  
1017 the t-sne analyses.

1018

1019 **Figure 5**

1020 (a) T-sne analysis of gene expression by cells from HIC strong responders (n=329 cells) (red), HIC  
1021 weak responders (n=348 cells) (green) and cART individuals (n=320 cells) (blue). (b-d) Genes  
1022 differentially expressed by HIV-specific central memory CD8+ T cells from HIV controllers (strong  
1023 (red) and weak (orange) responders) and cART individuals (blue). Violin plots represent gene  
1024 expression as Log<sub>2</sub>ex (limit of detection – threshold cycle) for each cell after data filtration. Cells  
1025 negative for CD8, GAPDH and ACTB or considered as outliers (if their total expression (in log<sub>2</sub>EX) or  
1026 the number of genes they express are less than two scaled median absolute deviations away from  
1027 the median) were excluded from the analyses. Values for each single cell analyzed (n=329 cells for  
1028 HIC SR, n=348 cells for HIC WR and n=320 cells for cART) are represented as scatter dots. The width  
1029 of the plots represents the kernel probability density of events at each given value. The proportion of  
1030 cells lacking expression of a gene is represented by the wideness of the base of the violin plots. The  
1031 hurdle model was applied to each gene in order to detect differentially expressed genes between  
1032 cells from HIC SR, WR and cART individuals. For all genes depicted a statistical significant difference  
1033 (p<0.05 after adjustment according to the Benjamini and Hochberg procedure to control the false  
1034 discovery rate at 5%) in either continuous, discrete or hurdle analyses was found. Genes upregulated

1035 in cells from HIC WR are in orange, genes upregulated in cells from HIC WR are in red; genes  
1036 upregulated in cells from cART individuals are in blue.

1037

1038 **Figure 6**

1039 A) Percentage of cells carrying high levels of GrZB (left) and expression levels (median fluorescence  
1040 intensity, MFI) of HIF1 $\alpha$  among HIV-specific CD8+ T CM cells from HIC (n=10 individuals) and cART  
1041 (n=7 individuals) as determined by flow cytometry. B) Flow cytometry analyses of fatty acid (BODIPY)  
1042 and glucose (NBDG) uptake by central memory CD8+ T cells from cART (blue circles, n=11 individuals)  
1043 and HIV controllers (red circles, n=10 individuals). Results are expressed as the relative MFI  
1044 compared to the MFI (set as 100) obtained with reference cells from a non-infected donor, which  
1045 were analyzed in parallel for each experiment. The ratio of fatty acid vs glucose uptake for each  
1046 individuals is also shown (right panel). (A,B) A non-parametric two-tailed Mann-Whitney test was  
1047 used to compare differences between the groups. Each symbol represents values for cells from one  
1048 individual. Medians are represented as horizontal lines.

1049

1050 **Figure 7**

1051 a) TNF $\alpha$  production and total response (sum of TNF $\alpha$ , IFN $\gamma$ , CD107a and IL2 production) by HIV-  
1052 specific CD8+ T cells from HIV controllers (n=11 individuals, red symbols) and cART (n=8 individuals,  
1053 blue symbols, [two individuals, 670115 and 350108, did not show TNF $\alpha$  responses above background  
1054 in any conditions]) against a pool of HIV-Gag peptides in control medium, in medium without glucose  
1055 and in the presence of menadione. The ratio "without/with glucose" represents the ratio between  
1056 HIV-specific CD8+ T cell TNF $\alpha$  responses in medium without and with glucose, for HIC controllers (red  
1057 symbols) and cART individuals (blue symbols), (b) Polyfunctional responses (3 or 4 functions; i.e. co-  
1058 expression of TNF $\alpha$ , IFN $\gamma$ , CD107a, or IL2) against a pool of HIV-Gag peptides by HIV-specific CD8+ T  
1059 cells from HIV controllers (n=11 individuals, red symbols) and cART individuals (n=9 individuals, blue  
1060 symbols, excluding 670115 with no Total HIV response) in control medium, in medium without

1061 glucose, and in the presence of menadione. (a,b) Each symbol represents values for cells from one  
1062 individual. Medians are represented as horizontal lines. A non-parametric two-tailed Wilcoxon  
1063 paired test was used to compare HIV-specific CD8+ T-cell responses in different conditions to the  
1064 control condition. (c) Donut plots representing the relative magnitude (represented by the size)  
1065 compared to control condition and polyfunctionality (Blue: 1 function; red: 2 functions; green: 3  
1066 functions; yellow: 4 functions) of HIV-specific CD8+ T cells from HIV controllers (n=11 individuals) and  
1067 cART individuals (n=9 individuals, excluding 670115 with no Total HIV response) in control medium,  
1068 in medium without glucose, and in the presence of menadione.

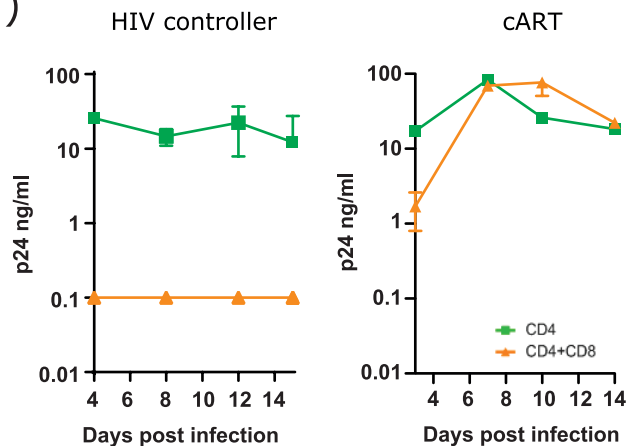
1069

1070 **Figure 8**

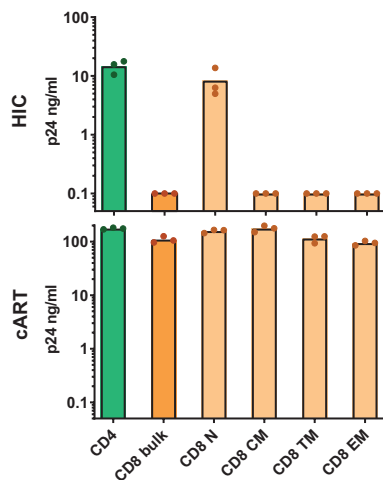
1071 a) Fatty acid (BODIPY) uptake (MFI) by central memory CD8+ T cells from cART individuals (blue  
1072 circles, n=7 individuals) after 48-72h incubation in the absence (control) or in presence of IL-15  
1073 [10ng/ml] (R&D). b) Oxygen consumption rates (two representative examples, means and standard  
1074 deviation [n=3 replicates] at each time point are shown for each analysis, left), basal glycolysis and  
1075 basal and maximal respiratory capacity (right, cells from n=8 individuals) by CD8+ T cells from cART-  
1076 individuals pre-incubated or not with IL-15. Arrows indicate the time of addition of Oligomycin, FCCP  
1077 and Rotenone/antimycin. c) TNF $\alpha$  production by HIV- and CMV-specific CD8+ T cells from cART (n=8  
1078 individuals) upon stimulation with pools of HIV-Gag (left) and CMVp665 peptides (right) in control  
1079 medium. Cells had been pre-incubated (overnight) or not with IL-15. d) Representative example (left)  
1080 and summary (cells from n=10 individuals) of viral suppression assays showing the capacity of CD8+ T  
1081 cells from cART-individual ex vivo or pretreated with IL-15 to block HIV-1 infection of autologous  
1082 CD4+ T cells. Data are means (and standard deviation for bar plots) of three replicates. (a-d) Each  
1083 symbol represents values for cells from one individual. Medians are represented as horizontal lines. A  
1084 non-parametric two-tailed Wilcoxon paired test was used to compare HIV-specific CD8+ T-cell  
1085 responses in different conditions to the control condition.

Figure 1

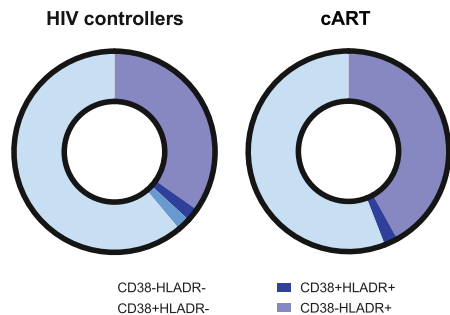
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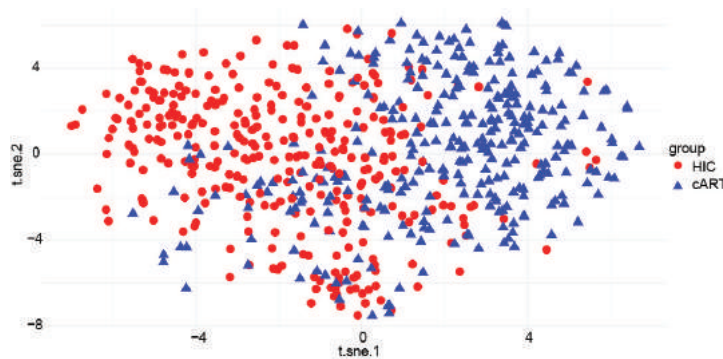
b)



c)



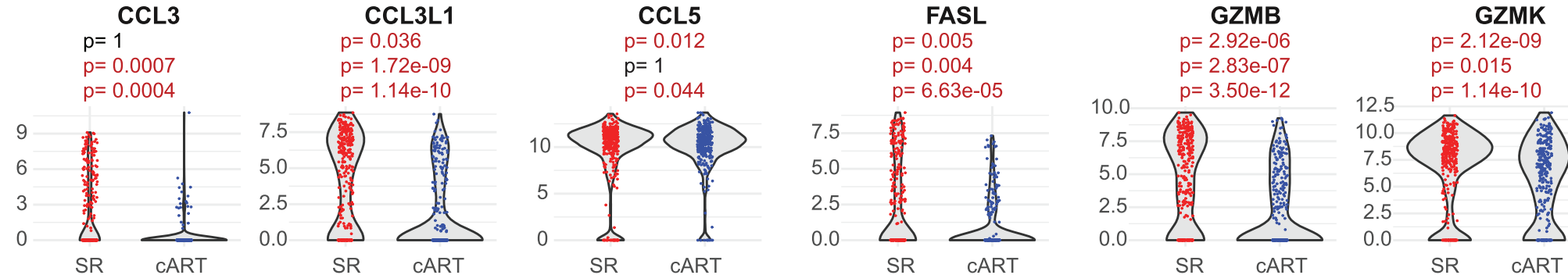
d)



# FIGURE 2

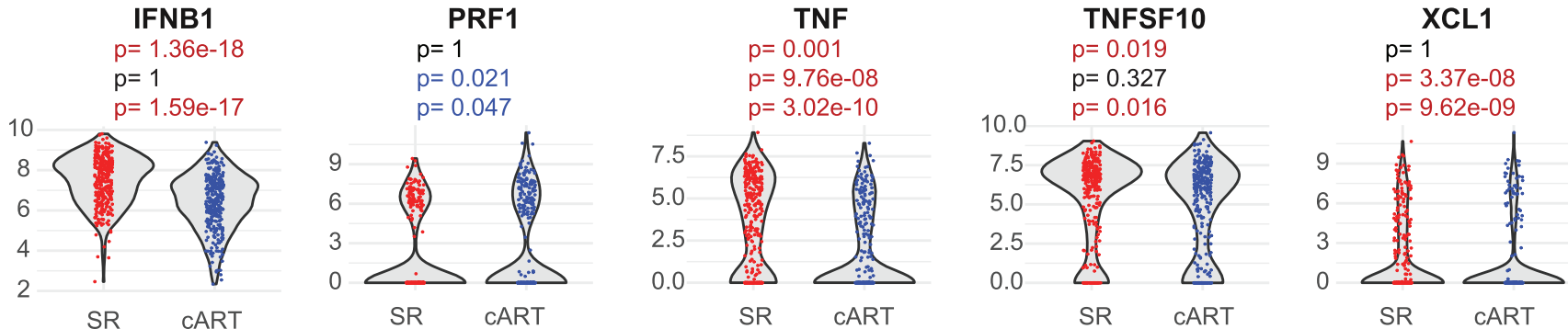
## Analysis

Continuous  
Discrete  
Hurdle



## Analysis

Continuous  
Discrete  
Hurdle



# FIGURE 3

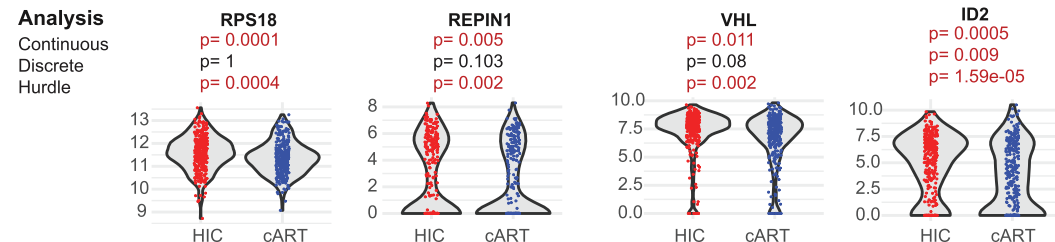
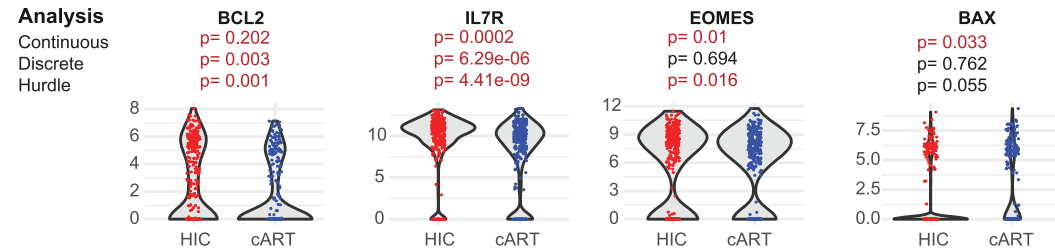
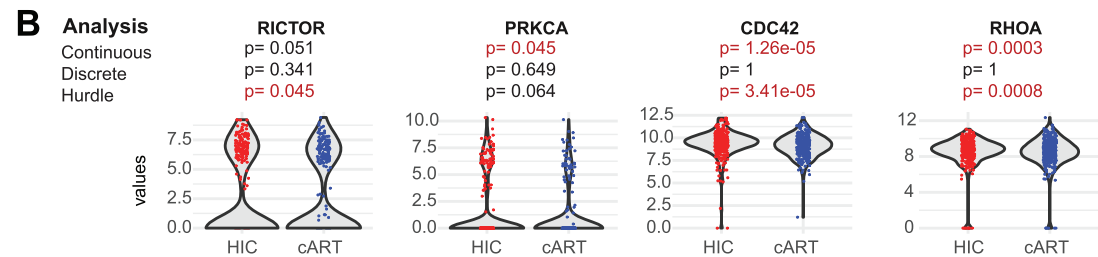
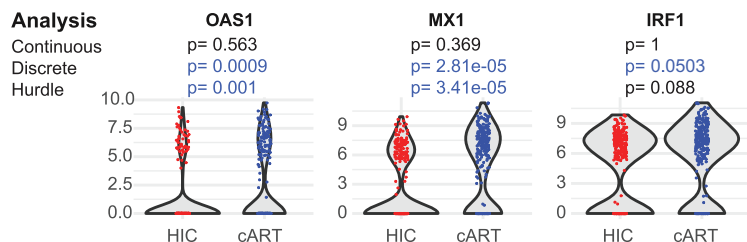
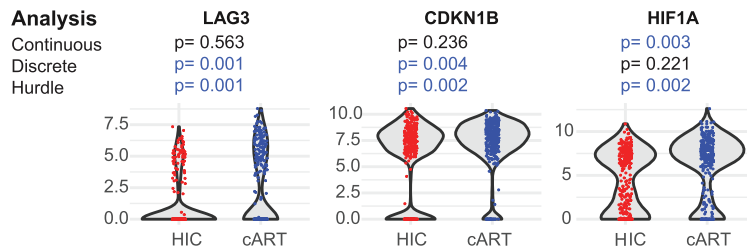
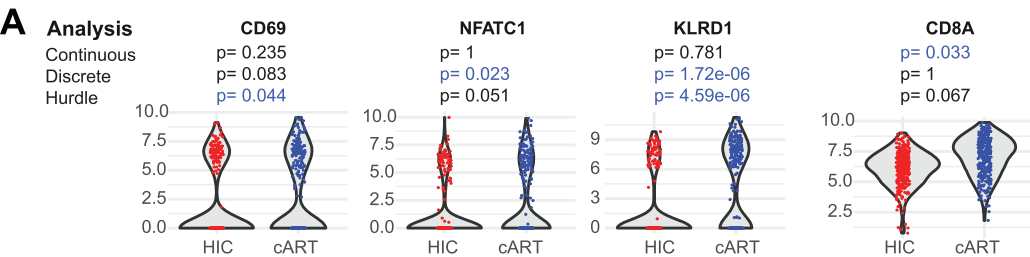
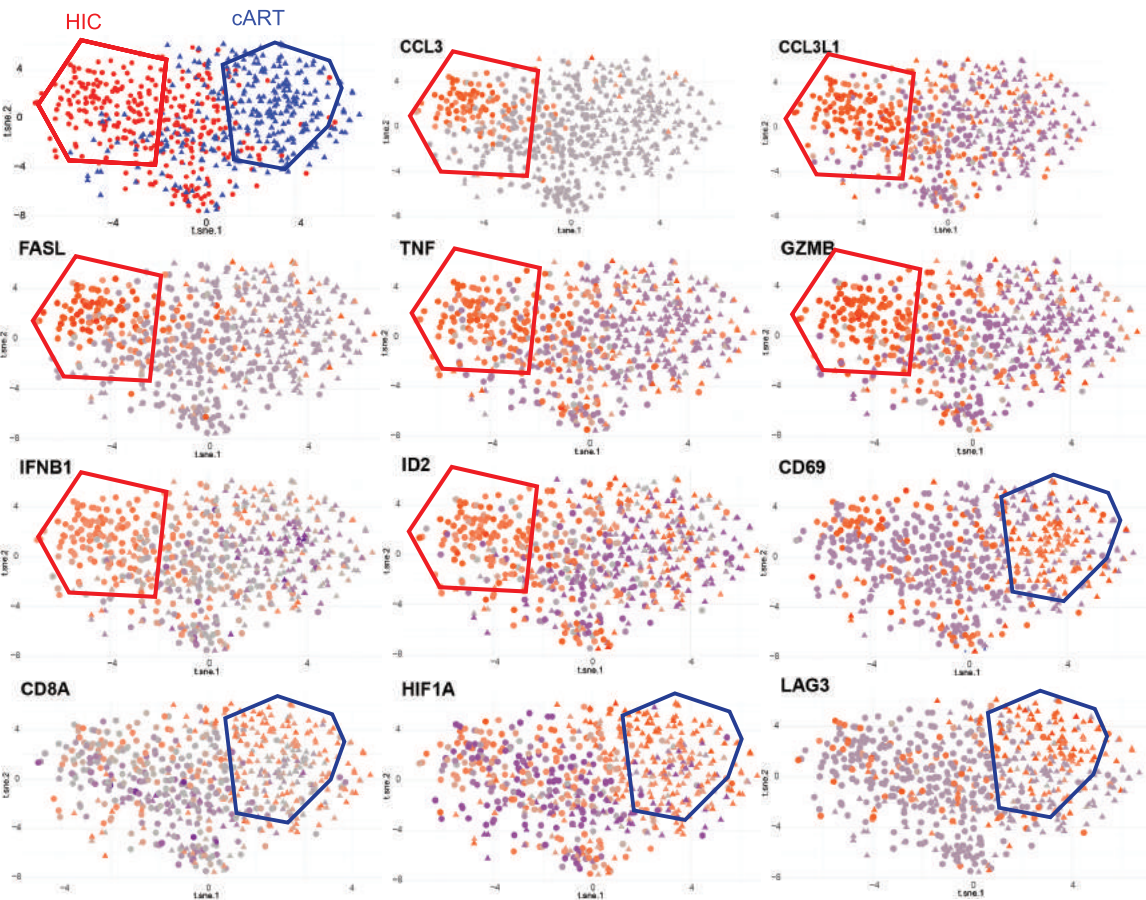
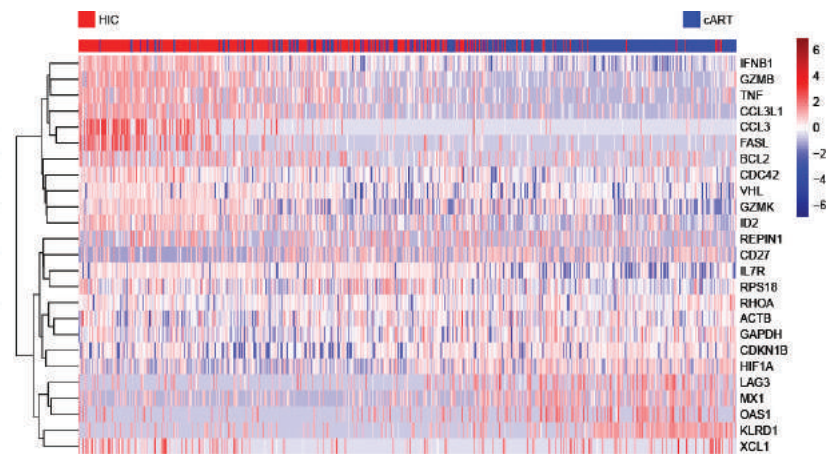


Figure 4

a)



b)





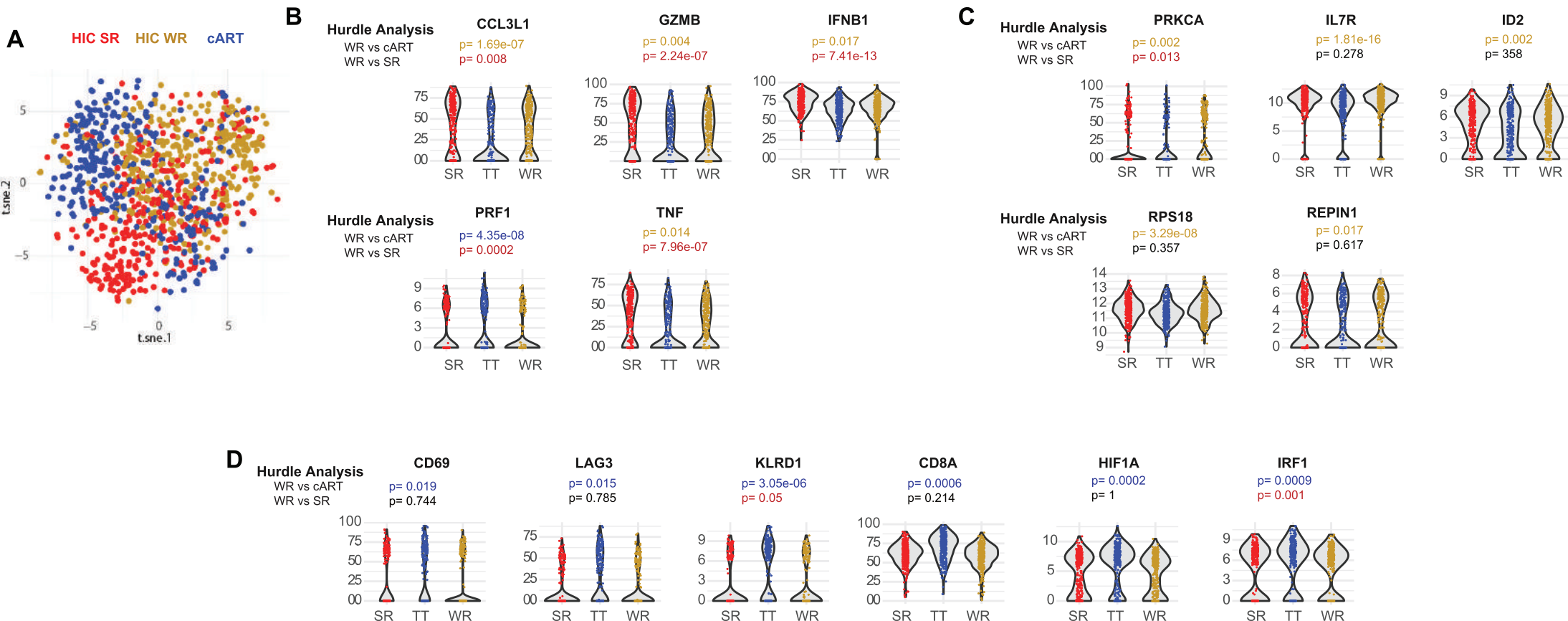
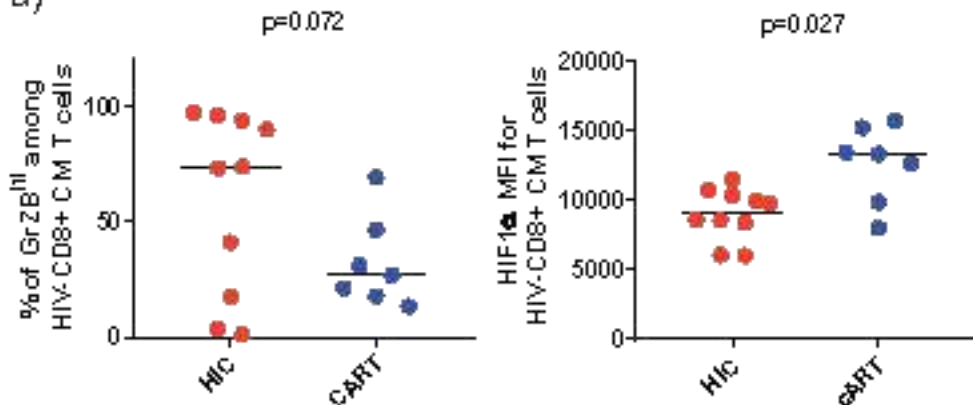
**FIGURE 5**

Figure 6

a)



b)

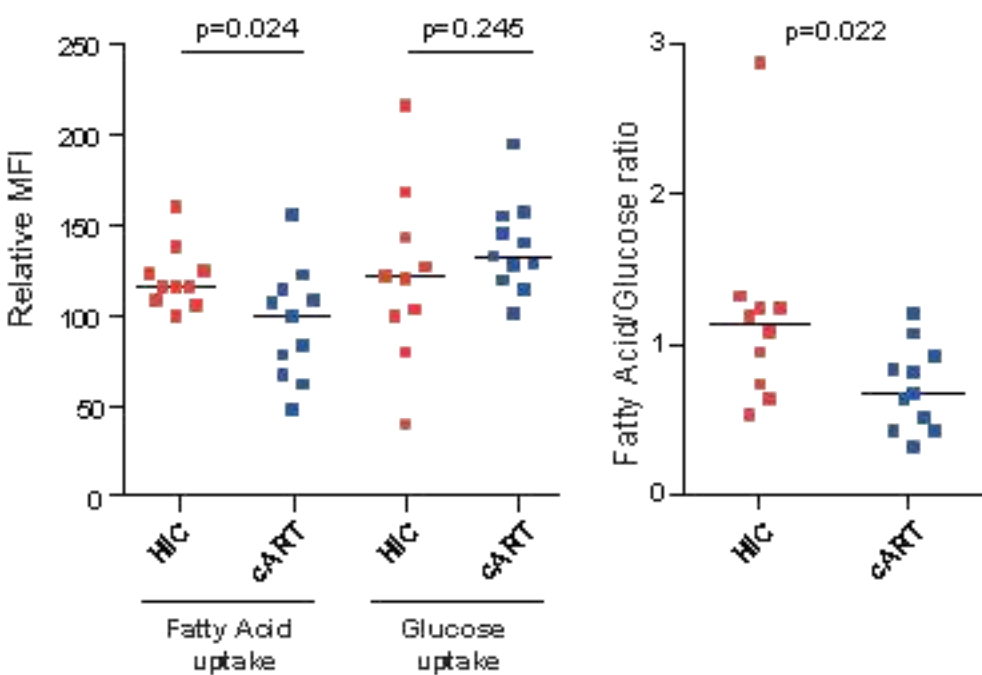


Figure 7

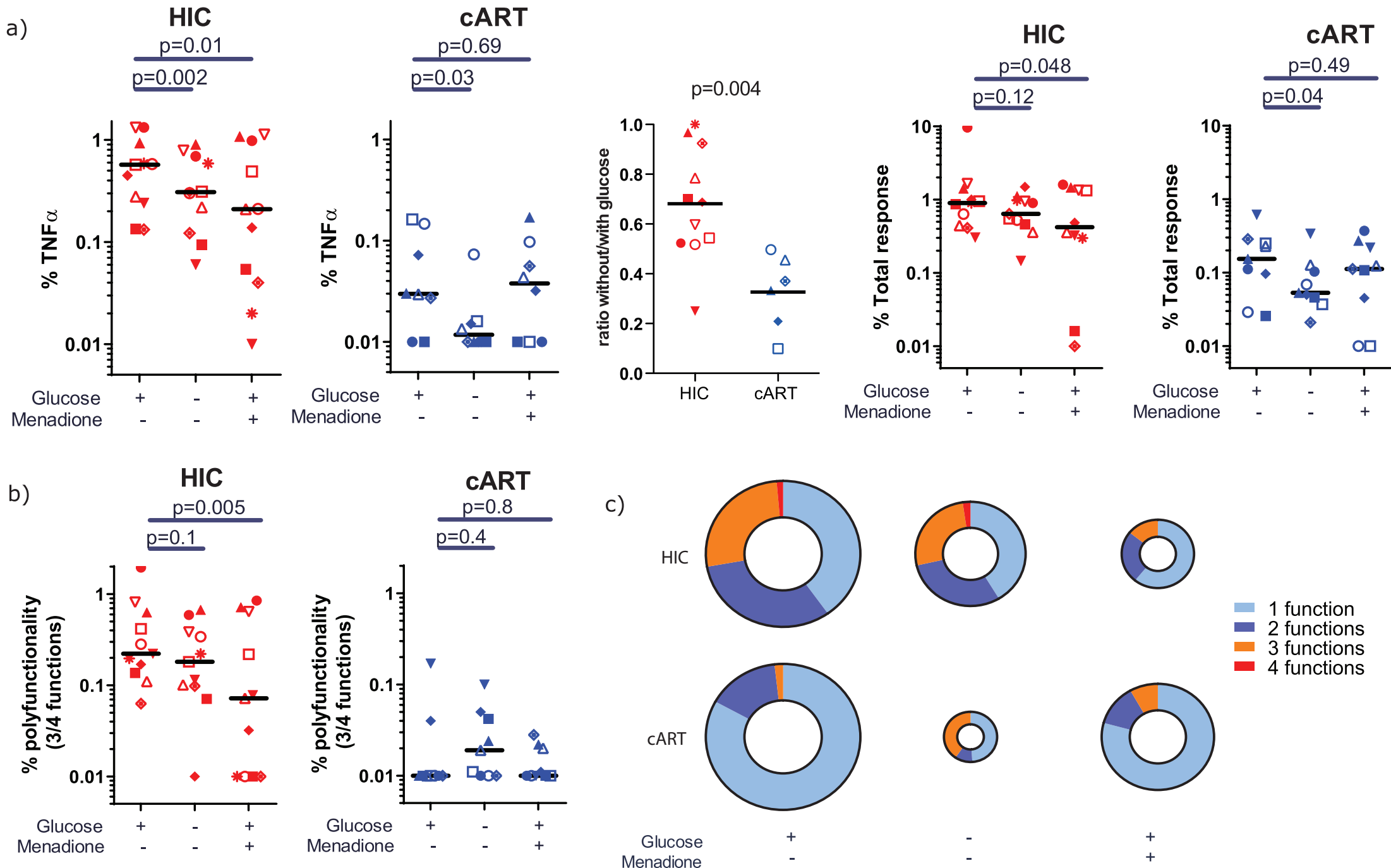


Figure 8

