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1 **Title:** HIV-specific CD8 T cells from elite controllers have an epigenetic imprint that preserves
2 effector functions

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21

22 **Running title:** Epigenetic poised state allows EC HIV-specific CD8 T cell responsiveness

23

24 **Keywords:** CD8 T cells, Human Immunodeficiency Virus, elite control, DNA methylation,
25 transcriptional signature, T cell activation, T cell exhaustion, cytokine signaling

26

27 **Abstract:**

28 Several lines of evidence support a central role for CD8 T cells as key determinants in the
29 control of HIV, particularly in rare “elite controllers” who control the virus to undetectable levels
30 in the blood in the absence of antiretroviral therapy (ART). While HIV-specific CD8 T cells
31 isolated from elite controllers have enhanced antiviral cytokine production and proliferative
32 capacity in response to antigen stimulation when compared to cells isolated from viremic or
33 even aviremic ART-suppressed non-controllers, the cell-intrinsic mechanisms underlying the
34 enhanced T cell memory-like function of HIV-specific CD8 T cells in elite controllers remain
35 largely undefined. To identify the transcriptional and epigenetic pathways that regulate
36 functional capacity in HIV-specific CD8 T cells in elite controllers, we performed genome-wide
37 transcriptional and DNA methylation analysis of MHC Class I multimer+ CD8 T cells sorted from
38 aviremic elite controllers compared to aviremic non-controllers on suppressive ART. Co-omics
39 analysis revealed enrichment for gene signatures that support a multipotent differentiation state,
40 cell survival, and a long-lived effector cell fate in HIV-specific CD8 T cells from elite controllers.
41 Specifically, we observed DNA methylation programs at the transcription factor binding sites of
42 the stem-associated factors TCF-1 and LEF1 that delineate HIV-specific CD8 T cells from elite
43 controllers versus ART-treated individuals. HIV-specific CD8 T cells in elite controllers also
44 maintain T cell receptor and IL-12/STAT4 pathway signaling and have suppressed pro-apoptotic
45 TNF α pathway signaling. These findings show that HIV-specific CD8 T cells from elite
46 controllers have enhanced expression and DNA methylation programs that maintain
47 developmental potential and in turn promote long-term survival, proliferative potential, and
48 effector capacity. These data also provide new insights into the relationship between stem-
49 associated transcription factors and stable epigenetic restriction of T cell developmental
50 capacity.

51

52

53 Introduction

54 The majority of people with Human Immunodeficiency Virus (HIV) infection initially
55 generate a robust immune response but ultimately fail to control of the virus, resulting in
56 persistent infection and the subsequent development of acquired immunodeficiency syndrome
57 (AIDS). A rare group of individuals (<1% of people with HIV), known as elite controllers (ECs),
58 control the virus to undetectable levels in the blood and do not progress to AIDS. Several lines
59 of evidence, including genome-wide association studies in humans and experimental studies in
60 macaques with Simian Immunodeficiency Virus (SIV) support the notion that CD8 T cells play
61 an essential role in mediating viral control in elite controllers (1-3).

62 HIV-specific CD8 T cells isolated from ECs produce more cytokines and proliferate more
63 robustly in response to T cell receptor (TCR) stimulation than their counterparts in untreated
64 non-controllers with high viral loads (4-7). Interestingly, HIV-specific CD8 T cell functional
65 capacity in non-controllers is not completely restored by suppressive antiretroviral therapy
66 (ART) (8,9), suggesting that persistent exhaustion and impaired responsiveness to antigen
67 stimulation may be epigenetically regulated in these cells, as has been seen in other settings
68 (e.g., after virologic cure in chronic hepatitis C infection, (10-13). Based on murine models of
69 infection with lymphocytic choriomeningitis virus (LCMV), compared to functional effector and
70 memory cells generated in the context of an acute infection, exhausted virus-specific CD8 T
71 cells occupy a unique transcriptional, epigenetic, and metabolic state imprinted early during
72 chronic infection (14-16). In particular, in both mice and humans, the transcription factor (TF)
73 Tox (17), *de novo* DNA methylation by the methyltransferase DNMT3a (18) (Prinzing et al., *Sci*
74 *Transl Med*, accepted), and the preferential use of glycolytic bioenergetic pathways (16) have
75 been shown to promote CD8 T cell exhaustion in humans. In HIV infection, exhausted HIV-
76 specific CD8 T cells isolated from viremic non-controllers express higher levels of co-inhibitory
77 receptors that inhibit TCR signaling (e.g., PD-1 (19)) and lower levels of the T cell stemness and
78 memory-associated TF TCF-1 (encoded by the gene, *TCF7*) compared to the more functional

79 HIV-specific CD8 T cells in ECs (9,20). While chromatin accessibility differences persist in non-
80 controllers after HIV viral load is suppressed with ART (10), the transcriptional and underlying
81 epigenetic mechanisms that regulate persistent exhaustion in this setting have not been
82 elucidated, nor is it clear what mechanisms promote enhanced functional capacity in HIV-
83 specific CD8 T cells in ECs. Because many HIV cure strategies such as therapeutic vaccination
84 and adoptive T cell therapies seek to promote the formation of EC-like functional HIV-specific
85 CD8 T cells in ART-suppressed individuals, it is critical to understand how exhaustion and virus-
86 specific CD8 T cell functional capacity is regulated in these two clinical groups.

87 In this study, we compared the transcriptional and epigenetic profiles of MHC Class I
88 multimer+ HIV-specific CD8 T cells from aviremic ECs versus aviremic ART-suppressed non-
89 controllers to understand why HIV-specific CD8 T cells from ECs maintain long-term enhanced
90 responsiveness to TCR simulation. Using whole-genome RNA-sequencing and DNA
91 methylation profiling, we found that genes associated with distinct T cell differentiation states
92 (e.g., effector, memory/stem-like, exhausted) were differentially expressed and had regions of
93 differential DNA methylation in HIV-specific CD8 T cells from ECs compared to non-controllers
94 on suppressive ART. The retained memory/stem-like functional capacity of EC virus-specific T
95 cells was coupled to distinct DNA methylation programs at TCF-1 and LEF1 transcription factor
96 binding sites. Furthermore, functional HIV-specific CD8 T cells from ECs have transcriptomic
97 and DNA methylation signatures suggestive of a more active metabolic state and heightened
98 responsiveness to TCR, IL-12/STAT4, IL-2/STAT5 and GPCR signaling. This study provides
99 insight into transcriptional and epigenetic mechanisms that govern sustained proliferative
100 capacity, survival, and antiviral function of HIV-specific CD8 T cells in elite controllers and
101 identifies candidate target pathways for optimizing T cell-based HIV cure therapies.

102

103 **Results**

104 **HIV-specific CD8 T cells from ECs versus ART-suppressed non-controllers are**
105 **transcriptionally and epigenetically distinct**

106 During HIV infection, CD8 T cells from aviremic ECs have enhanced proliferative
107 capacity and cytokine production compared to cells from aviremic non-controllers on
108 suppressive ART (8,9). To understand the cell intrinsic mechanisms regulating exhaustion in
109 HIV-specific CD8 T cells from non-controllers on ART compared to ECs, we took a co-omics
110 approach to probe gene expression and epigenetic differences. We FACS-sorted HIV-specific
111 MHC Class I multimer+ CD8 T cells from ECs and non-controllers on suppressive ART enrolled
112 in the San Francisco-based SCOPE cohort and performed bulk RNA sequencing and whole
113 genome DNA bisulfite sequencing. To understand the relationship between the transcriptional
114 signature of HIV-specific multimer+ CD8 T cells and bulk CD8 T cell subsets, we performed a
115 principal component analysis (PCA). Bulk CD8 T cells sorted from the same EC and ART
116 donors using markers that denote classically-defined subsets (naïve [TN:
117 CD45RA+CCR7+CD27+], central memory [TCM: CD45RA+CCR7+CD27+], and effector
118 memory [TEM: CD45RA+CCR7+CD27-]) grouped according to developmental state. As
119 expected, based on their phenotype (9), HIV-specific multimer+ CD8 T cells from both the EC
120 and ART group clustered closely to TEM CD8 T cells. (Fig. 1a). There were 1002 differentially
121 expressed genes (DEGs) between the EC and ART HIV-specific CD8 T cells ($p < 0.05$; Fig. 1b).
122 Notably, genes more highly expressed in HIV-specific CD8 T cells from ECs suggest enhanced
123 responsiveness to cytokine signaling (e.g., higher expression of *IL2RA*, the gene encoding
124 CD25), faster antiviral responses upon TCR engagement (e.g., higher expression of *IFNG*
125 transcript), and enhanced homing to B cell follicles (e.g., higher expression of *CXCR5*; Fig. 1c,
126 top). In contrast to the memory-associated programming observed in the EC HIV-specific CD8 T
127 cells, genes more highly expressed in HIV-specific CD8 T cells from ART-suppressed
128 individuals suggest a cell type that is more terminally differentiated, both phenotypically (with
129 higher levels of expression of NK cell receptors that tend to mark terminal effector cells, e.g.,

130 *KLRC4*) and functionally (with expression of cytotoxic molecules, e.g., *GZLY*) (Fig. 1c, bottom).
131 From the transcriptional data we conclude that, although EC and ART HIV-specific CD8 T cells
132 both cluster with the bulk TEM subset, HIV specific CD8 T cells from ECs differentially express
133 transcripts that suggest enhanced T cell survival and homing, and a less differentiated T cell
134 state.

135 We next performed a PCA analysis on the samples in the DNA methylation dataset.
136 Once again, bulk CD8 T cell populations (TN, TCM, TEM, and stem-cell memory [TSCM:
137 CD45RA+CCR7+CD95+] CD8 T cells isolated from HIV-uninfected donors) clustered by
138 developmental state. While the HIV-specific CD8 T cells from ECs grouped near bulk CD8 TEM
139 cells as in the transcriptome dataset, the ART HIV-specific CD8 T cells clustered separately
140 (Fig. 1d). In order to identify the DNA methylation programs that delineate HIV-specific
141 multimer+ CD8 T cells from ECs compared to ART-suppressed individuals, we performed a
142 pairwise comparison of gene-associated differentially methylated regions (DMRs), defined as
143 significantly differential methylation ($p < 0.01$) at ≥ 3 CpG sites within a minimum base pair
144 length of 100, and we associated each DMR to a single gene (Fig. 1d). Applying a threshold of
145 20% methylation difference, we found 3690 DMRs between EC versus ART HIV-specific
146 multimer+ CD8 T cells with the majority of regions (64.9%) more methylated in ECs, and largely
147 located within intron or intergenic regions. Of the DMRs, we found that HIV-specific CD8 T cells
148 from ECs maintained methylation of the first intronic region within the *PDCD1* locus (encodes
149 PD-1), with a methylation pattern at this site similar to more multipotent bulk CD8 T cell subsets
150 (e.g., TN, TSCM; Fig. 1f). Taken together, these data show that HIV-specific CD8 T cells from
151 ECs versus ART-suppressed individuals can be distinguished by their DNA methylation profile.
152 Notably, we found methylation differences in the gene locus encoding the inhibitory receptor
153 PD-1, which suggests one potential mechanism for sustained enhanced TCR responsiveness in
154 HIV-specific CD8 T cells in ECs.

155

156 **HIV-specific CD8 T cells from ECs have transcriptional and epigenetic features of long-**
157 **lived memory cells**

158 To better define the gene expression and/or methylation signatures associated with HIV-
159 specific CD8 T cells isolated from either ECs or ART-suppressed previous non-controllers, we
160 performed gene set enrichment analysis on our transcriptome and methylome datasets using
161 publicly-available gene lists generated from bulk CD8 T cells in different differentiation states
162 (e.g., TN, TSCM, TCM, TEM and the recently defined long-lived effector cells [LLECs:
163 CD45RO+KLRG1+CD127^{Int}CD27^{low}] (21)), as well as functionally-distinct sub-populations of
164 antigen-specific CD8 T cells in chronic viral infection (e.g., terminally exhausted cells [Tex],
165 progenitor [Prog1, Prog2] and intermediately differentiated sub-populations (22)) (Figure 2a,
166 top). Compared to ECs, multimer+ HIV-specific CD8 T cells from ART-treated individuals had
167 both increased expression and lower DNA methylation of genes that define TEM cells
168 (compared to the less-differentiated TCM and TSCM subsets), and they also had higher
169 expression and lower DNA methylation of genes associated with more terminally exhausted
170 virus-specific CD8 T cells compared to the progenitor or intermediate sub-populations that form
171 in chronic infection in mice. Leading edge genes from these comparisons whose expression
172 was enriched in the ART CD8 T cells included targets involved in CD8 T cell dysfunction
173 (*TNFRSF1B*) (23), terminal effector function (*GZMB*, *GZMM*) (24), and terminal effector
174 differentiation (*IKZF2*, *ZEB2*) (25,26) (Fig. 2b). In contrast, leading edge genes in EC HIV
175 specific CD8 T cells included markers for lymph node trafficking (*SELL*), positive modulation of
176 T cell effector metabolism (*MYC*) (27), and follicular trafficking and CD8 T cell mediated viral
177 control (*CXCR5*) (28) (Fig 2a, top). Similarly, genes associated with DMRs that were less
178 methylated in the ART samples include the lymph node and bone marrow egress marker
179 *S1PR5* and the T cell effector differentiation marker *CX3CR1* (29) (Fig. 2c). Interestingly,
180 compared to ECs, HIV-specific CD8 T cells from ART-suppressed individuals also had higher
181 expression of genes that are more highly expressed in TCMs compared to LLECs, a recently-

182 described population of cells that are long-lived but simultaneously maintain immediate effector
183 functions (21). Taken together, our data suggest that HIV-specific CD8 T cells from ECs appear
184 to occupy a less exhausted hybrid differentiation state in which they retain some features of
185 more multipotent memory cells as well as some features of long-lived effector cells.

186

187 **Distinct regulation of T cell differentiation transcriptional and epigenetic pathways in** 188 **HIV-specific CD8 T cells from ECs**

189 Our prior work on the DNA methyltransferase, DNMT3a, has found that DNMT3a-
190 mediated *de novo* DNA methylation reinforces the terminal exhaustion state of mouse and
191 human T cells that have been chronically stimulated (18,30) . Comparing the EC versus ART
192 HIV-specific CD8 T cell DMR list with that of our established DNMT3a gene signature, we found
193 that more than 20% of the DMRs overlapped with our DNMT3a gene signature. Furthermore,
194 when we assessed the expression of transcripts that encode DNMT3a-targeted transcription
195 factors, we found that they were expressed at significantly higher levels in ART compared to EC
196 HIV-specific CD8 T cells (Fig 2XX). This pattern suggests increased DNMT3a activity may be
197 one potential mechanism by which exhaustion is reinforced in HIV-specific CD8 T cells from
198 non-controllers.

199 Because virus-specific CD8 T cell effector/memory differentiation is regulated by the
200 coordinated activity of several hallmark transcription factors (31), we were interested in the
201 broad differential regulation of transcription factor target genes between EC versus ART HIV-
202 specific CD8 T cells. ART HIV-specific CD8 T cells were enriched for the expression of STAT3
203 targets (and had lower methylation at these target genes), and they also were enriched for the
204 expression of targets of two type-2 cytokine signaling factors (STAT6 and GATA3), which have
205 been shown to impair CD8 T cell division and differentiation (32) (Fig. 2a bottom). Leading edge
206 genes from the STAT3 targets that were both more highly expressed in ART and also had an
207 associated DMR compared to the EC samples included the exhaustion-associated factors

208 *NFATC1* and *TOX* and the type-2 immunity/NK developmental TF *NFIL3* (data not shown).
209 Other leading-edge genes from the STAT3 target list that were more highly expressed in the
210 ART HIV-specific CD8 T cells suggest terminally differentiated CD8 T cells (*IKZF2*), diminished
211 proliferation and survival (*AKT2*), and DNMT3a activity (*ZBTB18*) (33). On the other hand,
212 STAT3 target genes associated with DMRs that demonstrated a trend towards higher
213 expression in HIV-specific CD8 T cells from ECs included a guanine nucleotide exchange factor
214 downstream of TCR and CD28 signaling (*VAV3*), a receptor component for the cytokine IL-12
215 (*IL12RB1*), and a stem-associated transcription factor (*BACH2*; data not shown). Lastly, from
216 the STAT3 targets we also observed differential methylation of the B cell follicle homing
217 molecule *CXCR5* ($p = 0.043$; also a differentially expressed gene, Fig. 1c). These data show
218 that, compared to ECs, CD8 T cells isolated from ART-suppressed individuals have differential
219 DNA methylation at and are enriched for the expression of genes associated with type-2
220 immune signaling, terminal differentiation, and exhaustion, suggesting additional potential
221 mechanisms that may underlie their relatively impaired ability to signal downstream of TCR and
222 cytokine stimulation.

223 To further resolve the mechanisms involved in preserving the functional capacity of HIV-
224 specific CD8 T cells from ECs, we performed Hypergeometric optimization of motif enrichment
225 (HOMER) analysis (34) to identify transcription factors (TFs) whose binding site motifs were
226 over-represented in the DMRs between EC versus ART HIV-specific CD8 T cells. As noted
227 above, the majority (64%) of our DMRs were located within intronic or intergenic regions.
228 Amongst regions that were less methylated in EC (versus ART) HIV-specific CD8 T cells, we
229 noted a striking pattern of enrichment for the binding site sequences for several stem-
230 associated TFs (e.g., HOX9, TCF-1, OCT4-SOX2-TCF-NANOG-POU, LEF1; Fig. 3a). Among
231 the genes associated with the 242 DMRs containing TCF-1 motifs, we identified targets that
232 were less methylated in ECs such as the type-2 immune TF *NFIL3* (intergenic) and the JUN and
233 FOS transcript repressor *PRDM11* (intron), and targets that were less methylated in the ART-

234 suppressed samples such as the receptor tyrosine kinase antagonist, *SPRY2* (35) (intergenic)
235 (Fig. 3b). Interestingly, this striking difference in methylation at TCF-1 motifs between EC versus
236 ART HIV-specific CD8 T cells was not accompanied by differences in the level of expression of
237 the *TCF7* transcript itself; although we have observed differences at the protein level (9).

238 In contrast to the ECs, DMRs that were less methylated in ART HIV-specific CD8 T cells
239 were enriched for the binding site sequences for several effector differentiation-associated TFs
240 (e.g., *TBX21*, *IRF4*) as well as for *FOXO3* motifs (Fig. 3c). Of note, *FOXO3* itself was
241 differentially methylated and was also among the leading-edge genes from the *STAT3* and
242 *DNMT3a* target lists (Fig. 2a). Hypomethylated DMRs in ART HIV-specific CD8 T cells included
243 markers associated with a terminal/TEM fate (*S1PR5* (36))_or CD8 T cell exhaustion (*TOX*,
244 *CD101* (37)). HOMER analysis of the DMRs found enrichment of TCF-1 motifs in DMRs that
245 were relatively hypomethylated in HIV-specific CD8 T cells from ECs and enrichment of *FOXO3*
246 motifs in DMRs that were relatively hypomethylated in ART HIV-specific CD8 T cells. In
247 addition, *FOXO3* targets, could potentially limit cell cycling and prevent homeostatic proliferation
248 required for survival of long-lasting memory T cells

249
250 **Gene expression pathways downstream of TCR and cytokine signaling are differentially**
251 **regulated in HIV-specific CD8+ T cells from ECs**

252 Given that enhanced functional capacity of HIV-specific CD8 T cells in ECs is measured
253 by increased responses to antigen stimulation (as measured by either cytokine production
254 and/or proliferation) (9), we hypothesized that HIV-specific CD8 T cells from ECs might be
255 transcriptionally and/or epigenetically poised to respond to TCR signals. Indeed, leading edge
256 genes from several of the pathways that we found are enriched in EC HIV-specific CD8 T cells
257 included several genes that encode key proteins involved in TCR signaling (e.g., *ITK*, *LCK*,
258 *SLP76/LCP2*) (Fig. 4c). Furthermore, HIV-specific CD8 T cells also had evidence of increased
259 signaling downstream of *IL-12/STAT4* (Fig. 4a,c), a cytokine that is critical for promoting the

260 acquisition of antiviral properties in T cells. Leading edge genes downstream of these pathways
261 that were enriched in the EC HIV-specific CD8 T cells (e.g., *IL12RB1*, *IL12RB2*, *TYK2*) suggest
262 that these cells may have enhanced responsiveness to IL-12. Finally, HIV-specific CD8 T cells
263 from ECs also had higher expression of gene targets of pathways that promote metabolic
264 activity and cell cycling (e.g., pathways regulated by MTOR, MYC, and E2F) (data not shown).
265 Taken together, these data show that EC CD8 T cells maintain TCR and IL-12/STAT4 signaling
266 capacity along with enrichment of metabolic pathways important for proliferation (MYC and
267 E2F), effector function (Fatty Acid Metabolism and MTORC1 signaling), and survival (unfolded
268 protein response [UPR]).

269 In contrast to ECs, ART HIV-specific CD8 T cells were enriched for TNF α Signaling via
270 NFkB pathway (Fig. 4a). Leading edge genes expressed at higher levels in ART CD8 T cells
271 included the NFkB signaling inhibitors *NFkBIA* and *TNFAIP3* (a.k.a *A20*), and the pro-apoptotic
272 molecules *KLF4* and *DRAM1*. These data suggest that HIV-specific CD8 T cells from individuals
273 on ART may have enhanced sensitivity to the negative effects of TNF α including reduced
274 signaling through NFkB and increased pro-apoptotic markers.

275 Other pathways with gene targets that were differentially methylated between EC and
276 ART HIV-specific CD8 T cells included the IL2-STAT5 signaling, IL-4 and IL-13 signaling, and
277 signaling by G protein-coupled receptor (GPCR) pathways (Fig. 4d). Many differentially
278 methylated genes within these signaling pathways suggest enhanced responsiveness of EC
279 HIV-specific CD8 T cells to TCR and cytokine signaling, including genes that encode isoforms of
280 diacylglycerol (DAG) kinases (e.g., *DGKB*, *DGKK*, *DGKH*, *DKGZ*) and protein kinase C (e.g.,
281 *PRKCA*, *PRKCD*, *PRKCH*), guanine nucleotide exchange factors (GEFs; e.g., *SWAP70*,
282 *ARHGEF3*, *VAV3*), the solute transporter *SLC2A3/GLUT3*, GTPase *RHOB*, T cell differentiation
283 TFs *IRF4* and *EOMES*, migratory molecules associated with LN egress (*S1PR5*), B cell follicle
284 homing (*CXCR5*), recruitment to inflammatory sites (*CCR2*), and terminal differentiation
285 (*CX3CR1*). These data demonstrate that HIV-specific CD8 T cells from ECs are transcriptionally

286 and epigenetically poised for rapid TCR and cytokine signaling pathway activation, and they
287 have unique epigenetic programs that regulate the expression of migratory molecules
288 associated with T cell function and developmental status.

289

290 **Discussion**

291 HIV-specific CD8 T cells in elite controllers can maintain suppressive antiviral activity for
292 decades in infected individuals (38). Although many HIV cure strategies seek to elicit T cells that
293 possess the functional capacity of HIV-specific CD8 T cells from ECs, the cell-intrinsic
294 mechanisms responsible for their ability to sustain long term effector and proliferative potential
295 are not well understood. By analyzing the transcriptome and methylome datasets from antigen-
296 specificity matched CD8 T cells isolated from ECs and aviremic ART-suppressed non-
297 controllers, our work here identified differentially regulated gene expression and epigenetic
298 pathways involved in memory differentiation and exhaustion, proliferation and survival, and TCR
299 and cytokine signaling that delineate a virus specific T cell response among EC versus aviremic
300 ART treated non-controllers.

301 Consistent with the known antiviral capacity and enhanced antigen responsiveness of
302 HIV-specific CD8 T cells in ECs (5,7,9,39,40), our analysis identified several genes significantly
303 upregulated in EC CD8 T cells involved in T cell survival (*IL2RA*), homing potential to the major
304 HIV reservoirs (*CXCR5*), and effector function (*IFNG*). These data suggest that retained
305 function of CD8 T cells in ECs allow for surveillance and control during viral latency and
306 potential reactivation (41,42). In line with the idea of sustained viral control, our key findings
307 from the epigenetic analysis included DMRs in the exhaustion-associated loci of *PDCD1* and
308 *TOX* that may act as a potential mechanisms to explain how EC HIV specific CD8 T cells
309 remain poised for TCR stimulation despite their long-term chronic infection setting.

310 From our combined expression and epigenetic pathway analysis, we observed that EC
311 CD8 T cells were enriched for the expression of genes associated with less exhausted/more

312 multipotent T cell differentiation states (e.g., TSCM, TCM, Tpex) as well as a signature for long-
313 lived effector cells (21) relative to their ART counterparts. Related to sustained long term
314 effector function, our work found expression and DNA methylation pathways that highlighted IL-
315 12 signaling in EC HIV-specific CD8 T cells. Studies have reported the potential of IL-12 to
316 enhance the function of exhausted T cell in tumor and viral infection (43,44) including better
317 tumor control and lower expression of PD-1 (44). This potentially hints at an IL-12 dependent
318 mechanism in EC HIV specific CD8 T cells that is necessary to maintain long term effector CD8
319 T cell antiviral responses while simultaneously retaining stem-like properties.

320 Previously we showed that the stem-associated TF TCF-1 contributes to viral control
321 differences between EC and ART CD8 T cells (9). Although we did not observe stem-associated
322 TF expression differences between EC and ART HIV specific CD8 T cells in this study, our
323 analysis found differential epigenetic regulation of TCF-1 and LEF1 DNA binding sites that may
324 be responsible for sustained viral control. Our work also found differential regulation of
325 transcription factors targeted by DNMT3a which we have previously found to be responsible for
326 *de novo* DNA methylation mediated exhaustion programs in LCMV chronic mouse model of
327 infection (18). The enrichment of TCF-1 binding site motifs at EC hypomethylated regions
328 suggests an EC HIV specific CD8 T cell intrinsic mechanism that is resistant to DNA methylation
329 at specific sites that enables continued binding of TCF-1. These data suggest that both DNA
330 methylation and demethylation programs may play a role in the establishment of or resistance to
331 DNA methylation-associated exhaustion programs.

332 In summary, here we have provided a co-omics approach to identify differentially
333 regulated transcriptional programs between HIV-specific CD8 T cells in ECs versus ART-
334 suppressed individuals. Our expression and methylome pathway analysis determined that EC
335 HIV-specific CD8 T cells maintained a more stem-like profile compared to those from ART. We
336 found TCR and cytokine intracellular signaling pathways that remained poised in EC HIV
337 specific CD8 T cells, specifically related to the IL-12 family signaling pathway. In addition, while

338 we didn't observe direct mRNA expression differences in the stem-associated TFs *TCF7* and
339 *LEF1*, we did find differentially epigenetic regulation of their DNA binding motifs. Future work will
340 focus on whether these findings extend to other HIV-specific epitopes and chronic infection
341 settings. It also remains to be tested as to what DMRs are necessary to phenocopy the
342 sustained antiviral response of the EC HIV specific CD8 T cells. A recent study has shown that
343 it is possible to leverage CRISPR technology to target precise regions for DNA methylation (45)
344 to test potential DMR targets from this study to attempt to copy the EC HIV specific CD8 T cell
345 phenotype. The importance in the characterization of peripheral HIV specific CD8 T cells lies in
346 their immunotherapeutic potential in light of recent findings showing that future immunotherapies
347 may be dependent on recruitable cells considering that tissue resident cells can be refractory to
348 immune checkpoint blockade (46,47).

349

350 **Methods**

351 **Human study participants and samples:** This study sampled de-identified PBMCs
352 retrospectively collected under IRB approval from participants with HIV enrolled in the
353 Zuckerberg San Francisco General Hospital clinic-based SCOPE cohort.

354

355 **Human PBMC sorting by flow cytometry:**

356 Cryopreserved PBMCs were thawed as described previously and enriched for CD8 T cells by
357 negative selection with magnetic beads (STEMCELL). HIV-specific CD8+ T cells were identified
358 via staining with peptide–MHC Class I multimers, either monomers that were provided by RPS
359 (Emory University, Atlanta, Georgia, USA) and tetramerized as described using PE, or
360 biotinylated pentamers (ProImmune) followed by staining with streptavidin PE. The multimers
361 used in this study targeted the following specificities: HLA-A*02 (SL9), HLA-B*27 (KK10), HLA-
362 B*07 (TL10). PBMCs were incubated with multimer diluted in PBS for 15 minutes at room
363 temperature (pentamer) or 20 minutes at 37°C (tetramer), followed by 20 minutes at room

364 temperature with surface antibodies (with streptavidin for stains with pentamer), along with a
365 viability dye to allow for discrimination of dead cells (Thermo Fisher Scientific). Live multimer+
366 and non-multimer bulk CD8 T cell populations were sorted using a BD FACS-Aria II.

367

368 **Genomic methylation analysis:** DNA was extracted from the sorted cells using a DNA
369 extraction kit (QIAGEN) and then bisulfite-treated using an EZ DNA methylation kit (Zymo
370 Research), which converts all unmethylated cytosines to uracils. WGBS was performed as
371 described previously. Briefly, bisulfite-modified DNA sequencing libraries were generated using
372 the EpiGenome kit (Epicentre) according to the manufacturer's instructions. Bisulfite-modified
373 DNA libraries were sequenced using Illumina HiSeq 4000 and NovaSeq 6000 systems.
374 Sequencing data were aligned to the Hg19 genome using the BSMAP v. 2.74 software. Using
375 significantly methylated CpG sites ($p < 0.01$), DMRs were defined as ≥ 3 CpG sites within a
376 minimum base pair length of 100, and each site associated with a single gene and a pairwise
377 comparison done between EC and ART CD8 T cells.

378

379 **Hypergeometric optimization of motif enrichment (HOMER)**

380 A 20% minimum difference in methylation was applied to the EC vs ART HIV-tetramer+ CD8 T
381 cell DMR list and split into DMRs that were relatively hypomethylated in ART or EC CD8 T cells.
382 These DMRs were then analyzed for transcription factor motifs using HOMER. Motifs with an
383 FDR of < 0.01 were assessed.

384

385 **DECLARATION OF INTEREST:** Drs. Youngblood and Zebley have patents related
386 to epigenetic biomarkers and methods for enhancing T cell function for cellular therapies.

387

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396

397 **Figure Legends**

398 **Figure 1. Transcriptional and DNA methylation programs delineate ART versus EC HIV-**
399 **specific CD8 T cells**

400 A. Principal component analysis (PCA) of RNAseq profiles of sorted HIV-specific multimer+ CD8
401 T cells from elite controllers (EC) versus ART-suppressed non-controllers (ART) and bulk TN,
402 TCM, and TEM CD8 T cells from the same donors.

403 B. Volcano plot of differentially expressed genes between EC (n = 4) and ART (n = 4) HIV-
404 specific CD8 T cells ($p < 0.05$)

405 C. Representative transcripts enriched in EC (top) and ART (bottom) HIV-specific CD8 T cells

406 D. PCA of DNA methylation profiles of sorted HIV-specific multimer+ CD8 T cells (EC versus
407 ART) and bulk TN, TSCM, TCM, and TEM CD8 T cells from HIV-uninfected donors.

408 E. Genes associated with differentially methylated regions (DMR)s between ART (green) and
409 EC (yellow) HIV-specific CD8 T cells (methylation difference between the two conditions versus
410 area-statistic). DMRs below the 20% threshold are in black.

411 F. DNA methylation patterns at intron 1 of the *PDCD1* locus in HIV-specific CD8 T cells from EC
412 (yellow) versus ART (green) donors (top) and percent DNA methylation in this region in bulk
413 CD8 T cell subsets compared to EC or ART HIV-specific CD8 T cells (bottom). P-value
414 calculated using average methylation from significant DMR CpG sites.

415

416 **Figure 2. Pathways that govern CD8+ T cell memory and exhaustion are differentially**
417 **regulated in HIV-specific CD8+ T cells from elite controllers versus ART-suppressed non-**
418 **controllers**

419 A. Heatmap of pathways that are differentially expressed and methylated between EC and ART
420 HIV specific CD8 T cells (Nominal p value < 0.01).

421 B. Heatmap of leading edge genes from pathways differentially expressed and methylated.

422 C. Heatmap of DMRs from pathways differentially expressed and methylated

423

424 **Figure 3. Differential methylated regions at stem-associated transcription factor binding**
425 **sites delineate ART vs EC HIV specific CD8 T cells**

426 A. HOMER analysis of relatively hypomethylated EC HIV specific CD8 T cell DMRs (FDR <
427 0.01)

428 B. Heatmap of DMR genes containing Tcf7 motifs

429 C. HOMER analysis of hypomethylated ART HIV specific CD8 T cell DMRs (FDR < 0.01)

430 D. Heatmap of DMR genes containing Foxo3 motifs

431

432 **Figure 4. Elite controller HIV-specific CD8+ T cells differentially regulate pathways**
433 **sensitivity to TCR and cytokine signaling and metabolism.**

434 A. Heatmaps of differentially expressed pathways between EC and ART HIV-tetramer+ CD8 T
435 cells (Nominal p value < 0.01).

436 B. Heatmaps of differentially methylated pathways between EC and ART HIV-tetramer+ CD8 T
437 cells.

438 C. Heatmaps of pathway leading edge genes expressed between EC and ART HIV-tetramer+
439 CD8 T cells.

440 D. Heatmaps of pathway targets differentially methylated between EC and ART HIV-tetramer+
441 CD8 T cells.

442

443

444

445

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574

Figure 1. Transcriptional and DNA Methylation programs delineate ART versus EC HIV specific CD8 T cells

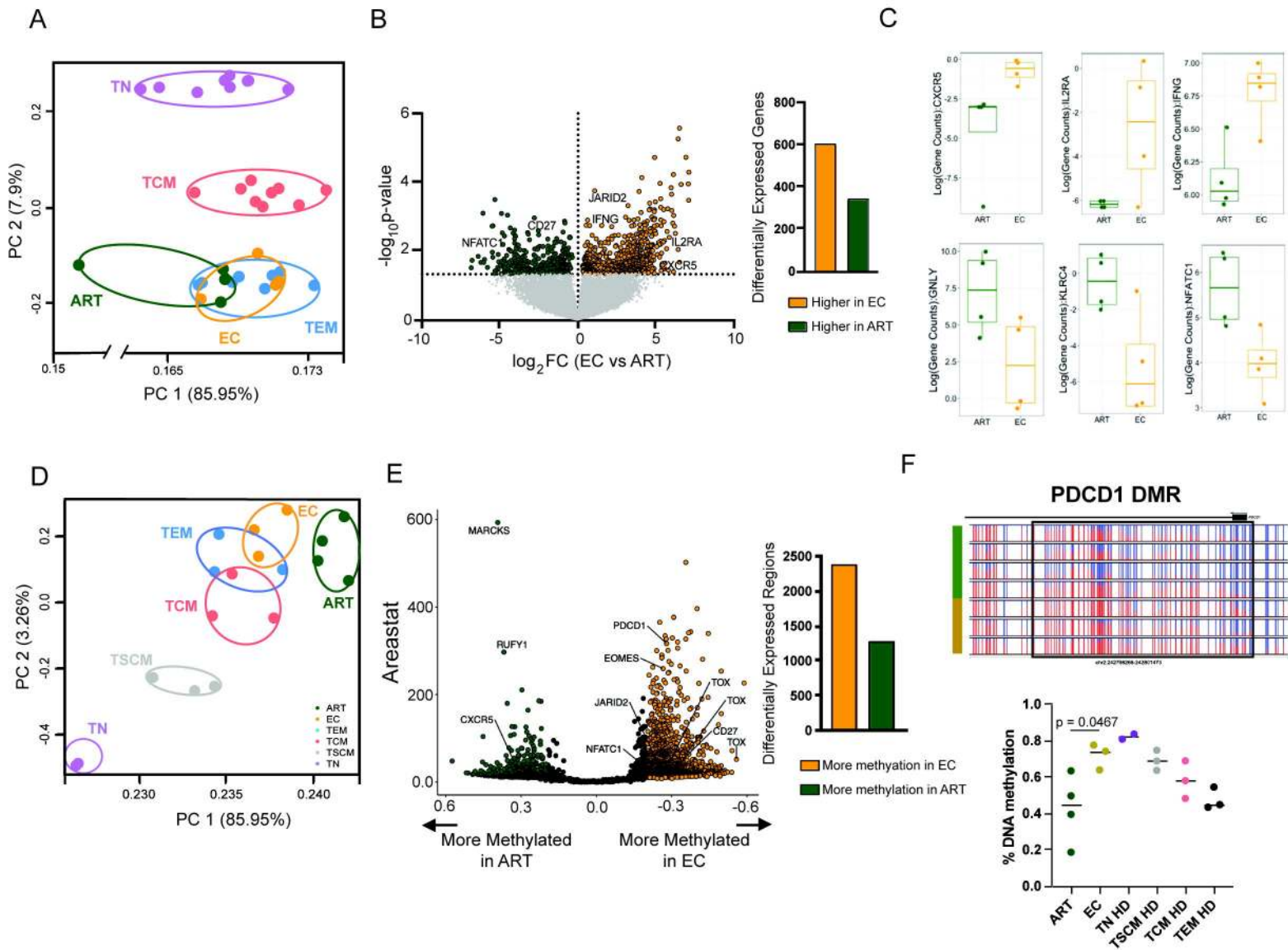


Figure 2. Pathways that govern CD8+ T cell memory and exhaustion are differentially regulated in HIV-specific CD8+ T cells from elite controllers versus ART-suppressed non-controllers.

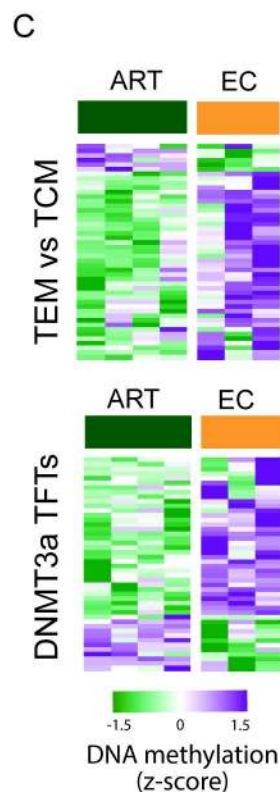
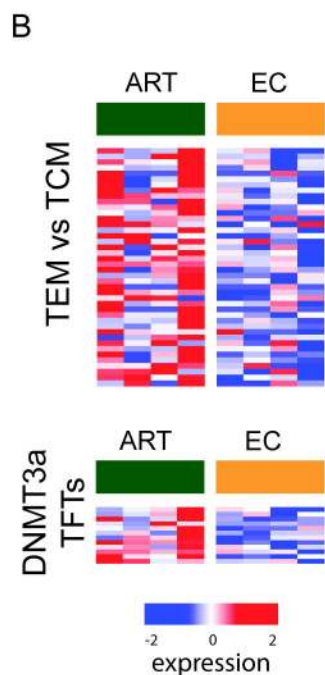
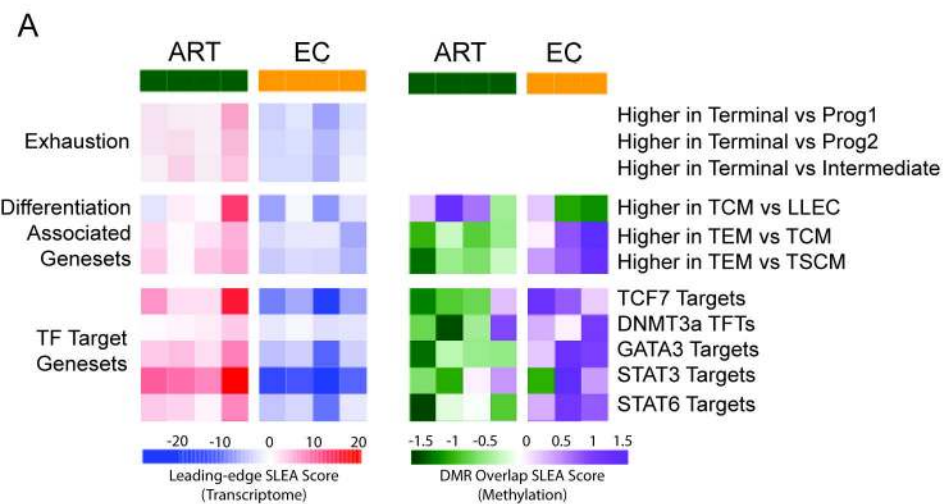


Figure 3. Differentially methylated regions at stem-associated transcription factor binding sites delineate ART vs EC HIV specific CD8 T cells

A

TF motif enrichment
EC hypomethylated DMRs

EC hypomethylated DMRs	
	Hoxc9
	Tcf7/TCF7
	OCT4-SOX4
	Brn1
	EWS:ERG
	LEF1

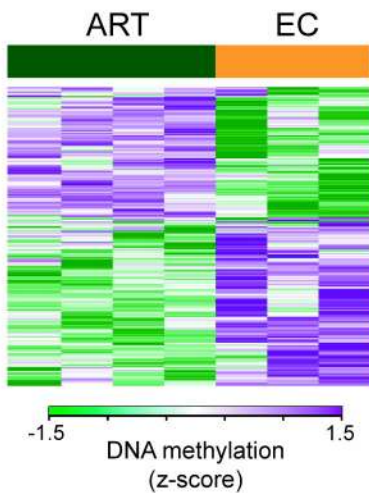
C

TF motif enrichment
ART hypomethylated DMRs

ART hypomethylated DMRs	
	Runx1
	GATA3
	Tbx21-TBX21
	IRF4
	Foxo3

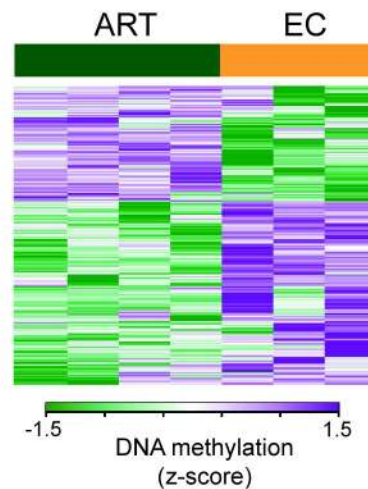
B

DMRs with Tcf7/TCF7 Motifs



D

DMRs with Foxo3 motifs



DMRs with LEF1 Motifs

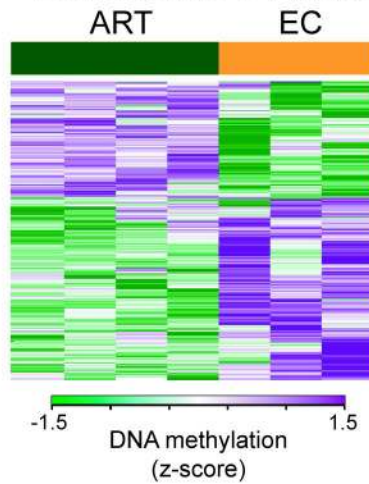
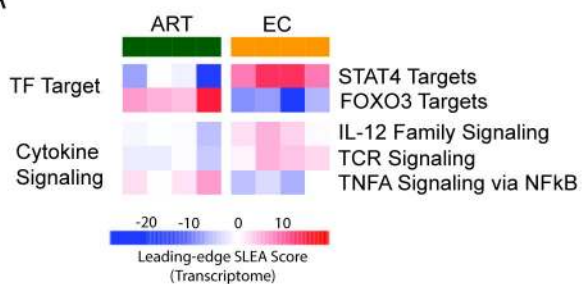
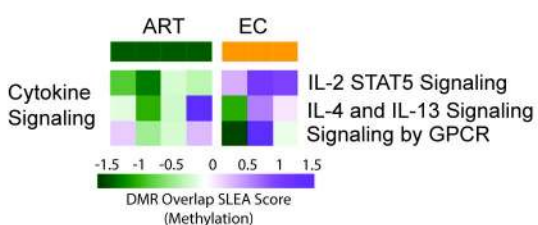


Figure 4. Elite controller HIV-specific CD8 T cells differentially regulate pathways sensitive to TCR, cytokine signaling, and metabolism

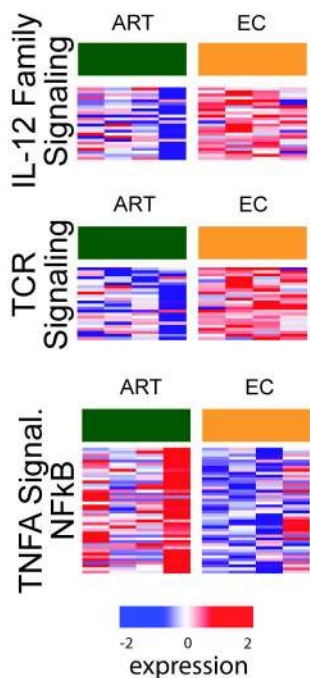
A



B



C



D

