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RESEARCH ARTICLE

# Differential Expression of CREM/ICER Isoforms Is Associated with the Spontaneous Control of HIV Infection

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**ABSTRACT** A rare subset of HIV-infected individuals, termed elite controllers (ECs), can maintain long-term control over HIV replication in the absence of antiretroviral therapy (ART). To elucidate the biological mechanism of resistance to HIV replication at the molecular and cellular levels, we performed RNA sequencing and identified alternative splicing variants from ECs, HIV-infected individuals undergoing ART, ART-naive HIV-infected individuals, and healthy controls. We identified differential gene expression patterns that are specific to ECs and may influence HIV resistance, including alternative RNA splicing and exon usage variants of the CREM/ICER gene (cyclic AMP [cAMP]-responsive element modulator/inducible cAMP early repressors). The knockout and knockdown of specific ICER exons that were found to be upregulated in ECs resulted in significantly increased HIV infection in a $CD4^+$ T cell line and primary $CD4^+$ T cells. Overexpression of ICER isoforms decreased HIV infection in primary $CD4^+$ T cells. Furthermore, ICER regulated HIV long terminal repeat (LTR) promoter activity in a Tat-dependent manner. Together, these results suggest that ICER is an HIV host factor that may contribute to the HIV resistance of ECs. These findings will help elucidate the mechanisms of HIV control by ECs and may yield a new approach for treatment of HIV.

**IMPORTANCE** A small group of HIV-infected individuals, termed elite controllers (ECs), display control of HIV replication in the absence of antiretroviral therapy (ART). However, the mechanism of ECs' resistance to HIV replication is not clear. In our work, we found an increased expression of specific, small isoforms of ICER in ECs. Further experiments proved that ICER is a robust host factor to regulate viral replication. Furthermore, we found that ICER regulates HIV LTR promoter activity in a Tat-dependent manner. These findings suggest that ICER is related to spontaneous control of HIV infection in ECs. This study may help elucidate a novel target for treatment of HIV.

**KEYWORDS** CREM/ICER gene, HIV, elite controllers, host factor

A small group of HIV-infected individuals, termed elite controllers (ECs), display control of HIV replication in the absence of antiretroviral therapy (ART) (1). This is in contrast to what occurs in most humans, in whom HIV infection progresses to AIDS unless treated with ART. ECs can spontaneously control HIV replication without ART and maintain HIV RNA levels in the blood below the level of detection (1, 2). Some aspects of the natural course of infection in ECs that contribute to this resistance,

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including protective HLA alleles and effective CD8$^+$ T cell cytotoxicity, both of which inhibit viral replication, have been described (3–6). It has also been suggested that infection with a virus that has low replicative fitness may explain viral control in some ECs, but viruses isolated from a subset of ECs have been found to be fully competent and replicate vigorously *in vitro* (7).

Together, these data suggest that the restricted replication capacity of HIV in ECs is likely driven by host-specific differences that prevent viral replication and/or disease progression. Several host restriction factors have been identified to inhibit viral replication. For example, TRIM5$\alpha$ (8), SAMHD1 (9), and APOBEC3G (10) were found to protect human and nonhuman primates against retroviral infection. Host-specific differences in these proteins have been linked to protection against infection and are thought to provide a significant barrier against cross-species transmission, but these host factors have not been reported to account for HIV resistance in ECs. Host factor-driven mechanisms of resistance to HIV in ECs remain largely unexplored.

Previous studies found that both monocyte-derived macrophages and CD4$^+$ T cells from HIV controllers had low susceptibility to HIV infection *in vitro* (11, 12). However, the restriction of viral replication in CD4$^+$ T cells from HIV controllers can be overcome by high-dose viral challenges (11, 13). Additionally, some reports have found that CD4$^+$ T cells from ECs can be infected by autologous or laboratory strains of HIV (13, 14). A previous study identified potential host factors regulating viral replication in the transcriptomes of CD4$^+$ T cells in comparisons between ECs and ART-naive HIV-infected individuals (15). While the transcriptional profile broadly correlated with the viral set point of the patients, it was unclear if this profile was driven by different levels of infected T cells being sampled or by systemic differences within the individuals. Compared to CD4$^+$ T cells, myeloid-derived cells, such as monocytes, macrophages, and dendritic cells, are generally more resistant to HIV infection, in part due to the increased expression of innate immune factors (16).

On the basis of the results of these previous studies, we hypothesized that specific genetic programs in monocytes from ECs might provide information on cell resistance to infection. As monocytes are not the main target of infection, sequencing of the monocyte population would further avoid confounders driven by infection-dependent changes. To test this hypothesis, we performed transcriptomics and analyzed gene expression profiles of monocytes derived from ECs, HIV-infected individuals undergoing ART, ART-naive HIV-infected individuals, and healthy controls. We focused on the genes that exhibited increased or decreased expression specifically in ECs relative to those in the other three control groups (non-ECs). Here, we show increased expression of specific exons of the ICER (inducible cyclic AMP [cAMP] early repressor) locus specifically in ECs. Knockout of ICER in a CD4$^+$ T cell line increased HIV infection; overexpression or knockdown of these isoforms in primary CD4$^+$ T cells decreased or increased HIV infection, respectively. These findings suggest that specific genetic programs in ECs, including altered splicing of ICER, may regulate cellular resistance to HIV infection.

## RESULTS

**Screening of the genes controlling HIV infection in ECs.** To identify host factors specific to ECs that might be involved in controlling HIV infection, we performed RNA sequencing (RNA-Seq) to compare the transcriptomes of monocytes from ECs and non-ECs. Peripheral blood mononuclear cells (PBMCs) were obtained from 44 individuals (see Table S1 in the supplemental material), comprised of 10 ECs, 12 aviremic HIV-infected ART-treated individuals, 11 HIV-infected ART-naive individuals, and 11 healthy controls without HIV infection. Monocytes were enriched from each sample, and RNA was extracted for RNA-Seq. To explore critical molecular factors and pathways, we compared the distinct gene expression pattern in ECs with those in the other three control groups (non-ECs) (Fig. 1A). Overall, 2,836 genes exhibited significantly increased or decreased levels of expression in ECs ($\log_2$ fold change $>$ 1.4, $q$ value $<$ 0.05) compared to those in

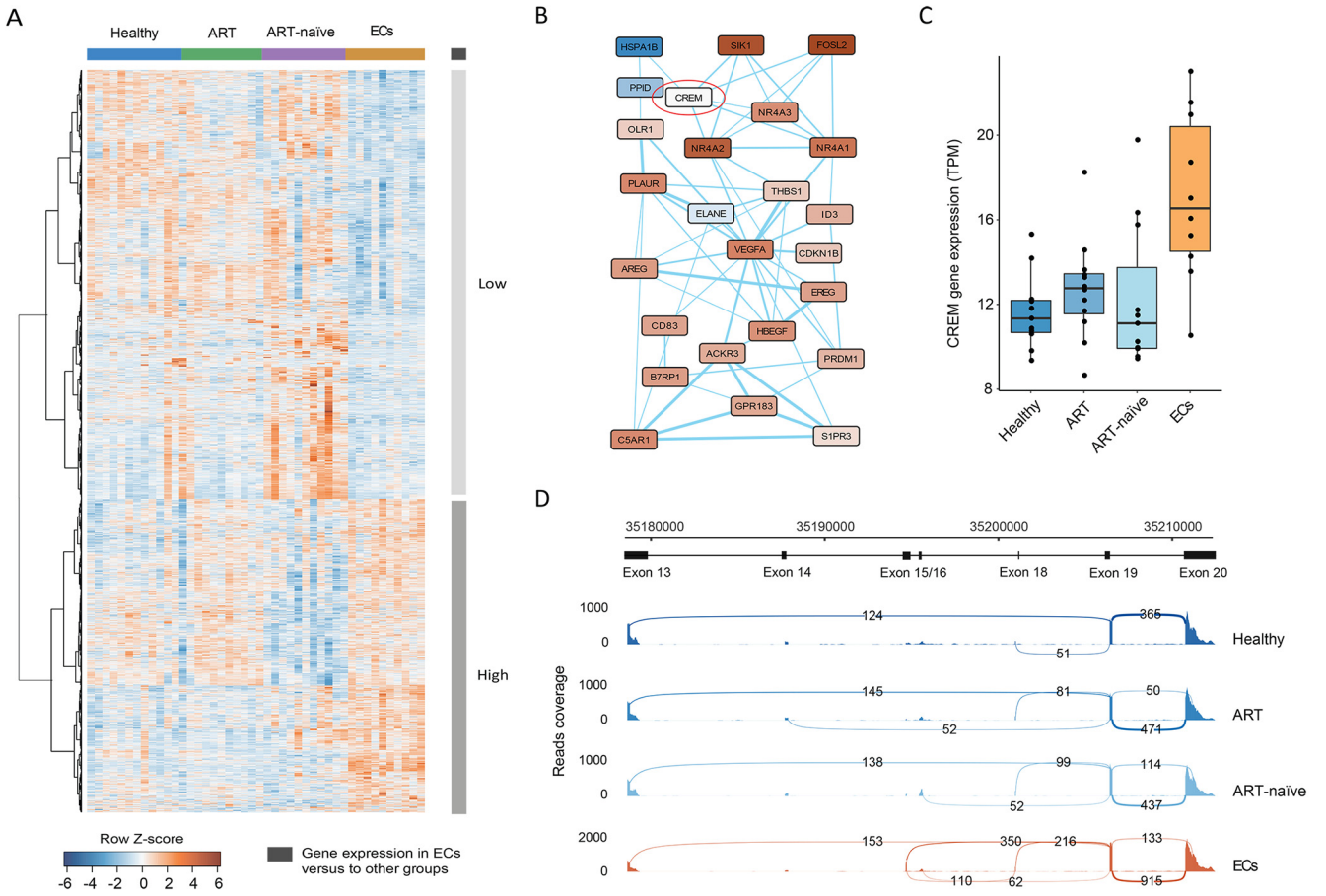


**FIG 1** Unique gene expression in ECs. Gene expression was analyzed by RNA-seq in purified monocytes from ECs and from ART-treated and virus-suppressed (ART) and ART-naive individuals, as well as healthy controls (Healthy). (A) Heatmap demonstrating the hierarchical clustering of the distinctly expressed genes in numbers of transcripts per million (TPM) in the four study groups. In total, 2,836 genes met the gene selection criteria ($\log_2$ fold change $>$ 1.4, FDR-adjusted $P < 0.05$), and the genes were significantly different from those in at least one of the EC/ART, EC/ART-naive, and EC/Healthy comparisons. (B) Functional gene network showing the genes increased (red) or decreased (blue) in ECs compared with those in the other three groups simultaneously. The edge width reflects the interaction score between two genes. The minimum required interaction score was >0.3. (C) Overall RNA expression levels (TPM) of CREM in ECs and in ART, ART-naive, and healthy control subjects. (D) Sashimi plot of CREM exons 13 to 20, displaying raw mapped RNA sequencing reads. Each group contained 10 pooled samples.

ART-treated individuals, ART-naive individuals, or healthy control individuals. Using gene ontology (GO) to functionally characterize these genes, we found that "regulation of metabolic process," "regulation of transcription by RNA polymerase ii," and "regulation of apoptotic process" represented the top functional entities of genes that were differentially expressed between ECs and healthy control or ART-treated individuals. In comparisons of ECs with ART-naive individuals, the top functional entities were "response to stress," "defense response," and "regulation of metabolic process" (Table S2). Among these, we found 53 candidate genes in ECs that were differentially expressed from those in each non-EC group (Table S3) and that might contribute to the different outcomes of HIV infection in ECs independently of HIV infection or ART status.

Next, the identified genes were subjected to gene function and network linkage analyses. We found that "cell cycle," "cellular process and activation," and "apoptotic process" represented the top functional entities that distinguished ECs from non-ECs (Fig. S1A). Using STRING to analyze interaction networks, we identified a notable cluster that includes several of the most markedly variable genes, such as the most highly upregulated genes, *SKI1B* and *NR4A2*, and the most highly downregulated gene, *HSPA1B* (Fig. 1B). To determine if the differentially expressed genes in ECs confer HIV resistance phenotypes to other cells, we overexpressed the 12 most significantly altered genes (Table S4A). Each of the genes was cloned into a lentiviral expression

vector and used to make high-titer lentivirus. Interferon alpha 1 (IFNA1) was used as an HIV-resistant positive control (17, 18); CD8 cells or an empty vector was used as a negative control. Lentiviruses containing target genes were used to transduce primary $CD4^+$ T cells isolated from 3 different non-HIV-infected donors. Transduced cells were subsequently infected with HIV NL4-3. Percentages of infected cells were quantified by intracellular HIV p24 staining 72 h after HIV infection. Except with IFNA1, which showed significant resistance to HIV challenge, we failed to observe differences in HIV infection after overexpression of the differentially expressed genes (Fig. S1B).

In the gene interaction network cluster, the cAMP-responsive element modulator (CREM) gene interacted with both the most highly upregulated and the most highly downregulated genes. While not among the most highly differentially expressed genes, the overall RNA expression level of CREM was significantly increased in ECs compared to that in non-ECs (Fig. 1C). CREM encodes the cAMP-responsive element modulator, a bZIP transcription factor that plays a critical role in cAMP-regulated signal transduction. CREM has a large number of alternatively spliced transcript variants that are regulated posttranscriptionally (>40 in humans). Some of these isoforms serve as transcriptional activators, and the others work as repressors (19). Globally, to identify compositional dissimilarity among the alternative splicing events, unsupervised principal-component analysis (PCA) was applied to calculate sample distances. ECs had distinct alternative splicing events compared with those of the other non-EC groups (Fig. S1C). Indeed, visualizing the RNA sequencing data from ECs in Integrative Genomics Viewer (IGV) showed several unique splicing junction events, particularly on chr10:35,195,000 to chr35,213,000, which spanned the CREM gene from exon 15 to exon 20 (Fig. 1D). Based on the central nature of CREM in the regulatory network and its known function as a transcriptional activator/repressor, we hypothesized that CREM might be the key upstream molecule that regulates differentially expressed genes in ECs.

**CREM/ICER isoform-enriched variants and alternative splicing in ECs.** The human CREM gene contains 20 exons, which use different promoters and transcription factors to generate complex alternative mRNA splicing events (Fig. S1E). The alternatively spliced variants generate different CREM isoforms that may exert opposing effects on target gene expression depending on the absence or presence of the transactivating domains (20). Therefore, identification of the exon usage and alternative splicing isoforms is essential to interpreting changes in RNA expression from this locus. To determine which transcripts may be differentially regulated, we compared unique reads to each isoform and calculated the expression of each transcript per million reads (TPM). Four specific CREM isoforms were found to be enriched in ECs over non-ECs (Fig. S1D). Notably, all of the isoforms enriched in ECs were inducible cAMP early repressors or ICERs. These isoforms displayed preferential usage of exons 15 and 16 (Fig. 2A). This same enrichment was observed when results were calculated as an isoform fraction (IF) (Fig. 2B). On the other hand, two CREM isoforms were enriched in non-ECs compared to ECs, one of them encoding a long noncoding RNA (lncRNA) and the other spanning exon 2 to exon 20 (Fig. 2A). We also quantified the alternative splicing events within CREM by analyzing the percent spliced (in Psi or $\Psi$) (Fig. 2C). Consistently with the IF analysis, the Psi results showed that ECs had an alternative first exon (AFE) preference for usage of exon 16 (median Psi = 90.2%), short exon 16 (median Psi = 89.0%), and exon 15 (median Psi = 4.6%). In ECs, when the first exon of CREM was exon 15, exon 18 had a highly skipped exon (SE) fraction (median Psi = 25.2%).

**Knockout of ICER exons using CRISPR-Cas9 increases HIV infection in a $CD4^+$ T cell line.** Given the many CREM/ICER isoforms, we used a CRISPR-Cas9 system to knock out the CREM/ICER gene in HuT78 cells to explore its role in HIV replication. We designed several synthetic single-guide RNAs (sgRNAs) against ICER exons 15 and 16 (Table S4B). Exons 18 and 19, which were commonly used in CREM/ICER isoforms (Fig. S1E), were also knocked out. Exon 13, which was used in most CREM isoforms but absent in ICER isoforms (Fig. S1E), was chosen as a control. CD8A was chosen as a negative control. Individual CREM/ICER exon or CD8A gene knockout clones were obtained

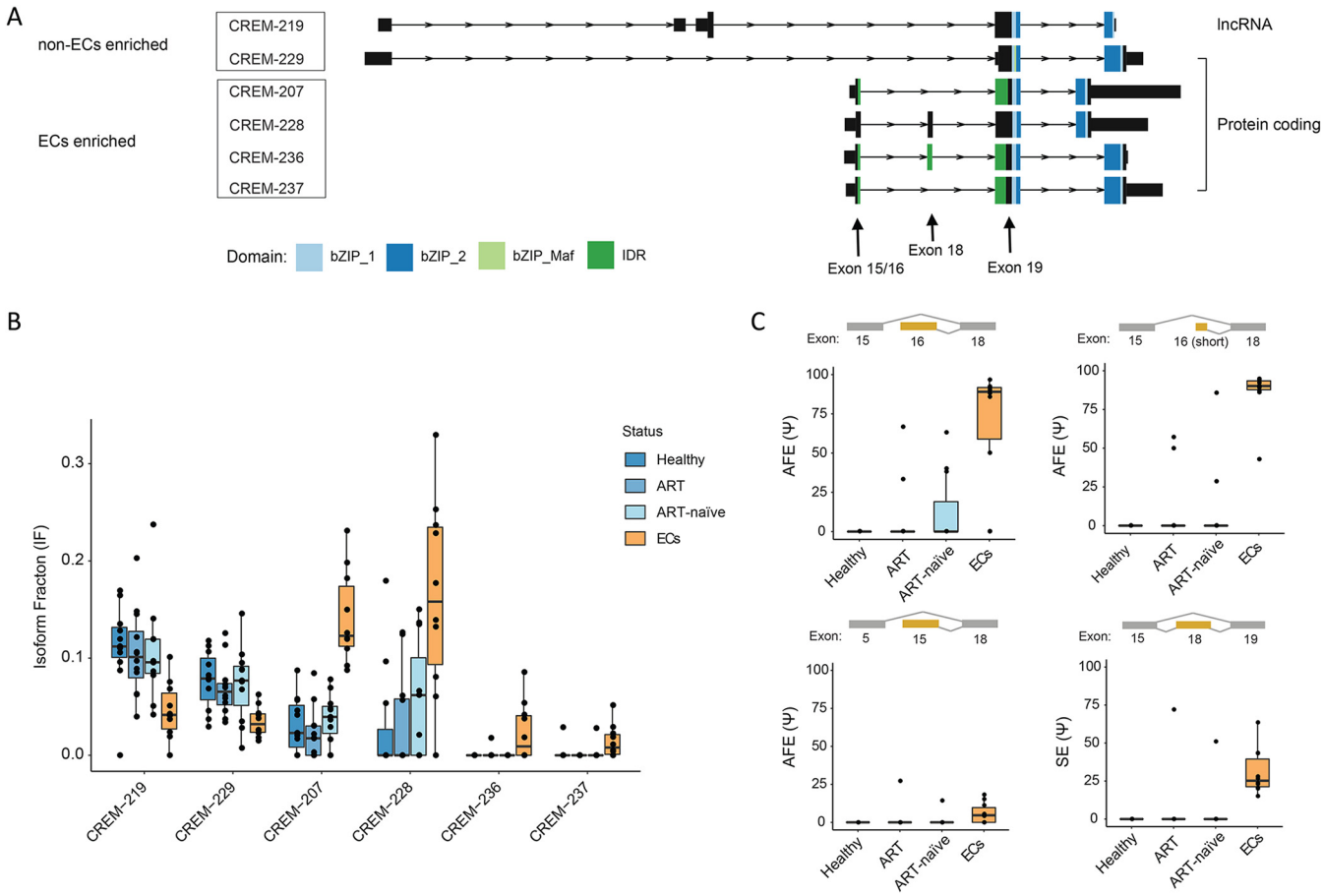


**FIG 2** EC-specific CREM/ICER isoforms and alternative splicing based on RNA sequencing. (A) Schematic illustration of CREM/ICER displaying the six relevant transcript isoforms (Ensembl ID) with their predicted functional domain structures. The expression of these six CREM/ICER isoforms was significantly increased or decreased in ECs compared to in all three control groups (non-ECs). (B) The frequencies of six CREM/ICER transcript isoform fractions (IFs) among all CREM/ICER isoforms in the four study groups. (C) Splicing patterns within the EC-relevant CREM/ICER genes quantified by the percent spliced-in (Psi or Ψ) values in the four study groups; the Psi with a significant difference is shown. The preference alternative first exon (AFE) usage and skipped exon (SE) in ECs are highlighted.

through single-cell sorting and screened by sequencing. We selected three clones for each exon or gene knockout. Next, the cell clones were challenged with HIV NL4-3, and infection was quantified by intracellular HIV p24 staining and flow cytometry at 72 h. The cell clones carrying the frameshift in ICER were more vulnerable to HIV challenge (Fig. 3A). To confirm these results, representative mutant clones that had frameshifts in each exon were challenged with increasing doses of HIV NL4-3. The productive infection was quantified. As observed above, each ICER exon's knockout significantly promoted infection on day 3 (Fig. 3B and C). Similar results were obtained using the HIV NL4-3 virus to challenge cells with each frameshift mutation after cultivation for 6 days (Fig. 3B and C). The cell clones suffered significantly, decreasing cell counts due to HIV-related cell death by the day 6 and day 9 time points (Fig. 3B and D).

**Knockdown of ICER exons using shRNA leads to increased HIV infection in primary CD4$^+$ T cells.** To determine the contribution of ICER to HIV infection in primary CD4$^+$ T cells, short hairpin RNA (shRNA)-mediated silencing was used to suppress ICER expression (Table S4C). Lentiviruses containing shRNA that target distinct exons were used to transduce primary CD4$^+$ T cells isolated from 4 different non-HIV-infected donors. The efficiency and specificity of ICER were assessed by immunoblotting (Fig. 4A). The shRNA against exons 15 and 19 was highly efficient in knocking down ICER, while shRNA against exons 16 and 18 had a low knockdown efficiency similar to that of the nontargeting control (Fig. 4A). Similar levels of cell viability were observed between CD4$^+$ T cells after ICER knockdown and control shRNA-transduced CD4$^+$ T

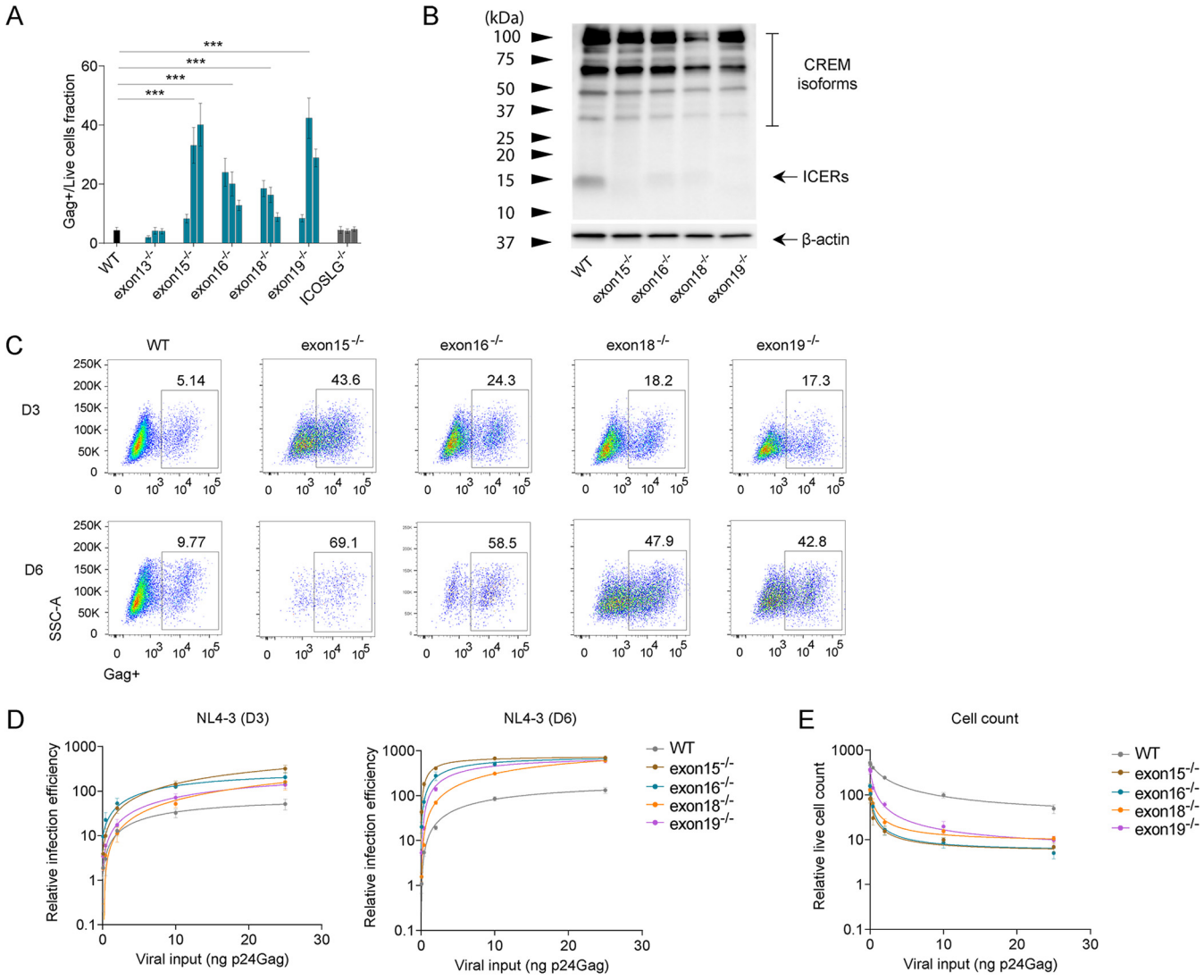


**FIG 3** Knockout of ICERs increase HIV infection. CREM/ICER exon 13, 15, 16, 18, or 19 in HuT78 cells was knocked out using the CRISPR-Cas9 system. The viral replication efficiency on each CREM/ICER exon knockout HuT78 cell clone was evaluated by HIV Gag staining using flow cytometry. (A) Fold changes of HIV infection in every three clones with CREM/ICER exon 13, 15, 16, 18, or 19 or CD8A knocked out, compared to infection levels in wild-type HuT78 cells (WT) on day 3 after HIV NL4-3 infection. (B) Expression of CREM/ICER after CRISPR-Cas9 knockout in the HuT78 cell line. (C) Dot plots representing $Gag^+$ cells on day 3 and day 6 after challenging individual exon knockouts of the HuT78 cell line with HIV NL4-3. The knockout of ICER exon 15, 16, 18, or 19 in HuT78 cells generated different resistance patterns to HIV infection. Mean levels of five replicates from three independent experiments are shown. SSC-A, side scatter area. (D, E) The representative cells with ICER exon 15, 16, 18, or 19 knocked out were infected with increasing viral inputs of HIV NL4-3. The infection efficiency was monitored on day 3 (D) and day 6 by measuring $Gag^+$ cells. (E) The live cell counts were measured on day 9. Values are means ± standard deviations (SD) (five replicates from three independent experiments). (One-way ANOVA followed by Holm-Sidak's multiple-comparison test, ***, $P < 0.001$.)

cells (Fig. S2A). Transduced cells were subsequently infected with HIV AD8 (CCR5-tropic) or HIV NL4-3 (CXCR4-tropic) virus, and infected cells were quantified by intracellular HIV p24 staining on days 3 and 5 postinfection. Upon ICER depletion, both HIV AD8 and NL4-3 infections were dramatically increased in primary T cells from all four donors compared to levels of control shRNA in transduced cells (Fig. 4B to D). The three shRNAs with the highest knockdown efficiencies, shRNA 15-1, shRNA 15-2, and shRNA 19-2, had on average 5.0-, 9.1-, and 5.8-fold increases in HIV AD8 infection, respectively, compared with levels of control shRNA on day 3 after viral challenge (Fig. 4C). Furthermore, shRNA 15-1 and shRNA 19-2 had average 4.6- and 2.8-fold increases in HIV NL4-3 infection, respectively, compared with levels of control shRNA

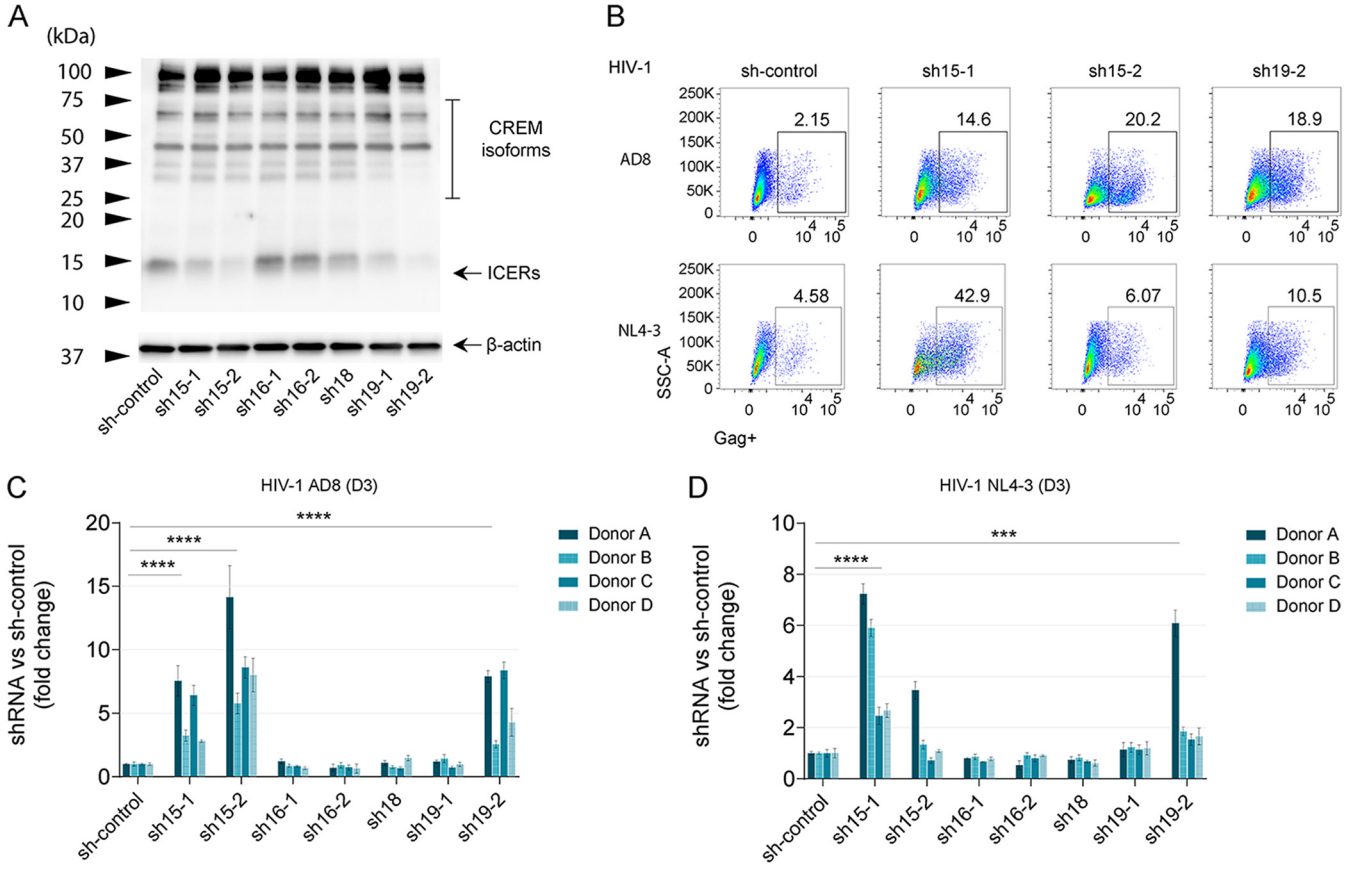


**FIG 4** Increased HIV infection in primary CD4$^+$ T cells upon ICER knockdown. Primary CD4$^+$ T cells were activated using anti-CD2/CD3/CD28 and transduced with ICER shRNAs against exon 15 (sh15-1, sh15-2), 16 (sh16-1, sh16-2), 18 (sh18), or 19 (sh19-1, sh19-2). The empty lentivirus was used as a control (sh-control). The transduced cells were selected using puromycin. (A) Expression of ICER after shRNA knockdown in primary CD4$^+$ T cells. (B) Dot plots representing percentages of Gag$^+$ cells on day 3 after HIV AD8 or HIV NL4-3 infection upon ICER exon 15 or 19 knockdown in primary CD4 T cells. (C, D) Summarized results of HIV AD8 (C) or NL4-3 (D) infection after shRNA knockdown of ICER exon 15, 16, 18, or 19 in primary CD4$^+$ T cells isolated from four different donors. Results are shown as the fold change in the percent infection of ICER shRNA-transduced cells compared to the percent infection of sh-control transduced cells on day 3 after viral challenges. Each histogram bar represents means ± SD from triplicates for each donor. (One-way ANOVA followed by Holm-Sidak's multiple-comparison test, ***, $P < 0.001$; ****, $P < 0.0001$.)

on day 3 postinfection (Fig. 4D). Similar results were obtained using the HIV AD8 and NL4-3 virus to challenge cells with each knockdown after cultivation for 5 days (Fig. S2B and S2C).

To further verify the effect of ICER exon knockdown in inhibiting HIV infection, we examined a range of different HIV strains. Human primary CD4$^+$ T cells were challenged with different strains of HIV after silencing ICER expression. The tested HIV included CCR5-tropic (JR-CSF and BaL), CXCR4/CCR5-tropic (89.6), and CXCR4-tropic (IIIB) HIV strains. Treatment with shRNAs 15-1 and 15-2 (knockdown of exon 15) and shRNA 19-2 (knockdown of exon 19) increased HIV infection of all tested strains in primary CD4$^+$ T cells from multiple independent donors on day 3 postinfection (Fig. 5A to D) and day 5 postinfection (Fig. S3A to S3D).

**Overexpression of ICER isoforms leads to resistance to HIV infection in primary CD4$^+$ T cells.** To better understand which ICER isoforms influence infection, we individually overexpressed eight different ICER isoforms in primary CD4$^+$ T cells from 4 independent donors using a lentiviral expression system. Two isoforms of CREM were used as isoform controls, and a CD8A vector was used as a negative control. The specificity of ICER isoform overexpression was assessed in HEK 293T cells (Fig. S4A), and the transduction efficiency of isoforms in primary CD4$^+$ T cells was determined by immunoblotting (Fig. 6A). Although HEK 293T cells highly expressed all tested ICER isoforms, primary CD4$^+$ T cells highly expressed only isoforms 5, 7, 8, 11, and 31. After transduction, the expression of different ICER isoforms did not affect cell viability (Fig. S4B). Next,

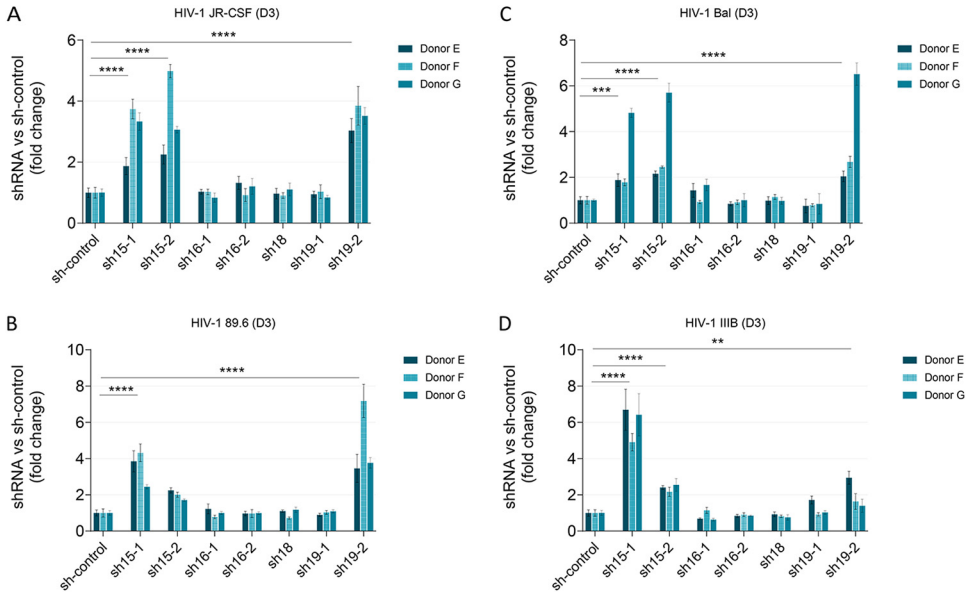


**FIG 5** ICER controls infections with different strains of HIV. Primary CD4$^+$ T cells were activated using anti-CD2/CD3/CD28, transduced with ICER shRNAs against exon 15 (sh15-1, sh15-2), 16 (sh16-1, sh16-2), 18 (sh18), or 19 (sh19-1, sh19-2), and selected with puromycin; empty lentivirus was used as a control (sh-control). The transduced cells were then infected with HIV JR-CSF (A), BaL (B), 89.6 (C), and IIIB (D). The viral replication efficiency was evaluated by p24 staining on day 3 after HIV infection. Results are displayed as the fold change in the percent infection of ICER shRNA-transduced cells from those of sh-control-transduced cells from three different donors. Each bar represents a mean ± SD from triplicates for each donor. (One-way ANOVA followed by Holm-Sidak's multiple-comparison test, **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.)

overexpression of ICER isoforms 5, 8, 11, and 31 resulted in 45.1%, 56.5%, 79.1%, and 79.5% average decreases in HIV AD8 infection on day 3, respectively, compared with the level in transduced CD8A cells (Fig. 6B; Fig. S4C). The overexpression of ICER isoforms 8 and 11 resulted in 49.2% and 39.0% decreases in HIV NL4-3 infection on day 3 (Fig. 6C; Fig. S4C). Similar results were observed on day 5; the impacts of isoforms 8, 11, and 31 on HIV NL4-3 infection were more pronounced (Fig. S4D and S4E).

In addition to assessing the overexpression of individual ICER isoforms, we assessed the effect of small-molecule agonists of ICER in the context of HIV infection. Prostaglandin E2 (PGE2) and 8-bromo-cAMP (8-Br-cAMP) have been shown to increase intracellular cAMP and induce endogenous ICER protein levels (21, 22); they were not found to alter cell viability in the current study (Fig. S4F). We found that both PGE2 and 8-Br-cAMP treatment increased cell resistance to HIV infection (Fig. 6D to F). Treatment of human primary CD4$^+$ T cells with PGE2 (100 $\mu$M) for 16 to 24 h prior to infection resulted in a 57.6% decrease in HIV AD8 infection and a 67.2% decrease in HIV NL4-3 infection compared with levels of infection in untreated cells (Fig. 6D to F). Notably, 8-Br-cAMP pretreatment resulted in a more dramatic inhibition of HIV infection in human primary CD4$^+$ T cells (Fig. 6F), consistent with its resistance to hydrolysis by phosphodiesterases. Treatment of cells with 250 $\mu$M 8-Br-cAMP resulted in 84.8% and 97.1% decreases in HIV AD8 and NL4-3 infection, respectively, compared with levels in untreated cells (Fig. 6F).

**ICER regulates HIV LTR promoter activity in a Tat-dependent manner.** CREM/ICER has been found to regulate RNA polymerase II (RNAP II) through interactions with transcription factor IIA (TFIIA) (23). HIV Tat was found to bind to the trans-activation response element (TAR) RNA element that is located in the viral 5′ long terminal repeat (LTR) sequences and recruit RNAP II elongation factor to activate transcriptional elongation (24). To evaluate the effects of CREM/ICER on RNAP II and HIV LTR promoter activity, we stably transduced TZM-B1 cells with shRNAs against CREM/ICER or overexpression of CREM/ICER isoforms; IFNA1 was used as an HIV-resistant positive control. We tested the activity of the HIV LTR promoter in the absence or presence of HIV Tat

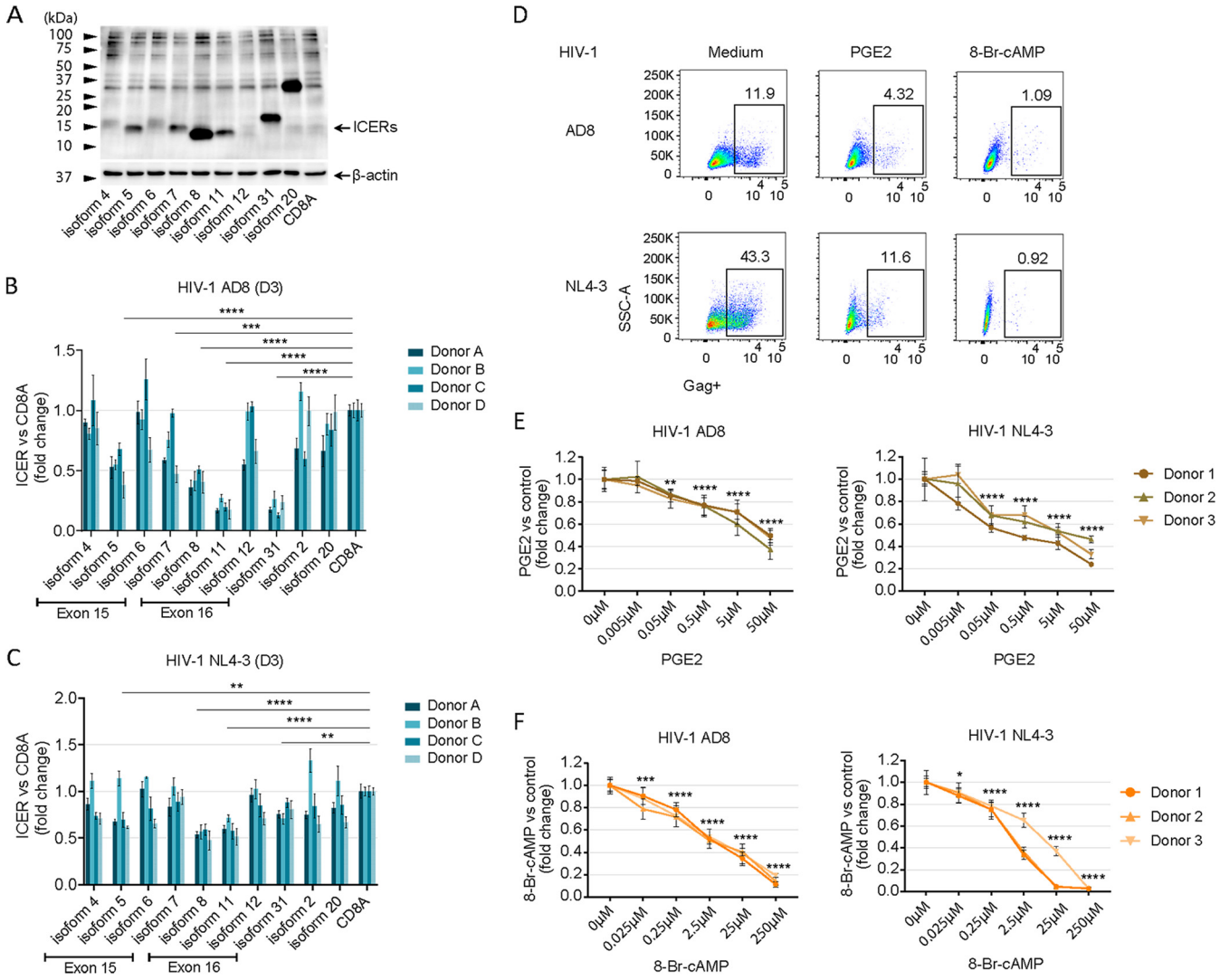


**FIG 6** Resistance to HIV infection in human primary CD4$^+$ T cells after ICER isoform overexpression. Primary CD4$^+$ T cells were activated using anti-CD2/CD3/CD28, transduced with CREM/ICER isoforms with the lentivirus system, and selected with puromycin. The expression of CREM/ICER isoforms using the lentivirus gene overexpression system is shown. (A) CREM/ICER isoform overexpression in primary CD4$^+$ T cells. (B, C) Fold changes in percent HIV AD8 (B) and NL4-3 (C) infection in human primary CD4$^+$ T cells transduced with each CREM/ICER isoform, compared to HIV infection in CD8A gene-transduced T cells. Histograms are shown in independent experiments performed with cells from four different donors. Each bar represents the mean ± SD from triplicates for each donor. (D to F) Anti-CD2/CD3/CD28-activated primary CD4$^+$ T cells were treated with PGE2 (50 $\mu$M to 0.005 $\mu$M) or 8-Br-cAMP (250 $\mu$M to 0.025 $\mu$M) for 16 to 24 h and then infected with HIV AD8 or NL4-3 for 3 days. (D) Percent infection (% Gag$^+$) in response to PGE2 (50 $\mu$M) or 8-Br-cAMP (250 $\mu$M) from one presentative donor. Dose-dependent effects of PGE2 (E) or 8-Br-cAMP (F) on HIV infection. Results are displayed as the fold change in percent HIV infection in cells treated with an ICER agonist from the percent HIV infection in untreated cells. Histograms show results in three independent experiments performed with three different donors. Each bar represents the mean ± SD from triplicates for each donor. (One-way ANOVA followed by Holm-Sidak's multiple-comparison test, *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$.)

protein. No change in HIV LTR promoter activity was found after increasing or decreasing ICER expression (Fig. 7A and B). However, in the presence of HIV Tat, the depletion of ICER exon 15 led to enhanced LTR-driven luciferase reporter activity, while coexpression of HIV Tat and the ICER isoforms 4, 5, and 8 decreased LTR-driven luciferase reporter activity (Fig. 7A to B). These results indicated that the ICER regulation of HIV infection is likely through targeting the HIV Tat-LTR axis and viral transcription; our results are also consistent with the previous work suggesting that ICER regulates RNAP II (23).

## DISCUSSION

The main characteristics of ECs are an undetectable viral load in blood and a steady CD4$^+$ T cell count in the absence of ART. Understanding the mechanism for the natural

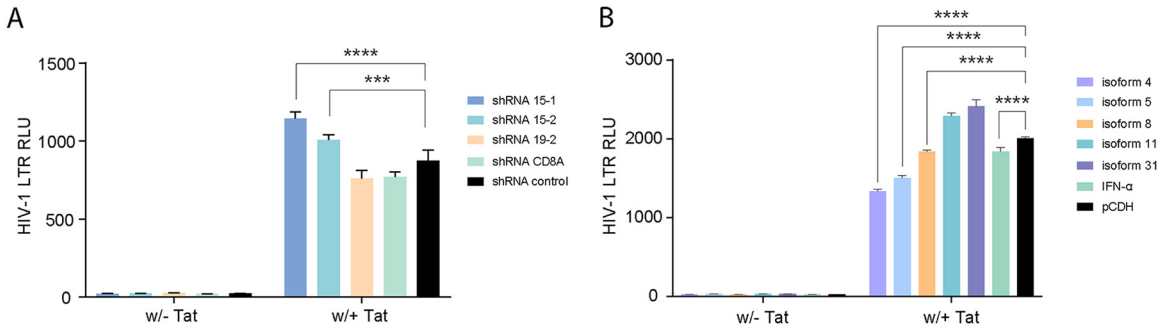


**FIG 7** ICER regulates HIV LTR promoter activity in a Tat-dependent manner. The activity of the HIV LTR promoter was evaluated by LTR-driven luciferase reporter activity. (A) TZM-bl cells were stably transduced with shRNAs against ICER exon 15 (sh15-1, sh15-2) or 19 (sh19-2) or with shRNAs against CD8A; the empty lentivirus was used as a vector control. (B) TZM-B1 cells were stably transduced with ICER isoform 4, 5, 8, 11, or 31. IFN-$\alpha$ was used as a positive control; the pCDH empty lentivirus was used as a vector control. These cells were transfected with the pQCXIP-Tat vector or the empty one. Histograms show results from three independent experiments. Each bar represents the mean $\pm$ SD. (One-way ANOVA followed by Holm-Sidak's multiple-comparison test, ***, $P < 0.001$; ****, $P < 0.0001$.)

control of HIV replication in ECs may yield new strategies for the treatment of HIV/AIDS. Here, we used a transcriptomic approach to compare levels of gene expression in monocytes isolated from ECs and non-ECs, controlling for ART and HIV infection status. We found an increased expression of specific, small isoforms of the CREM gene, all of which encoded inducible cAMP early repressors (ICERs).

To determine if the differentially expressed genes in ECs confer HIV resistance phenotypes to other cells, we overexpressed the most significantly altered genes. However, we failed to observe differences in HIV infection after overexpression of the differentially expressed genes. While individual overexpression of this gene subset did not influence infection in this model cell line, it is possible that depletion of these factors or investigation in a different model may identify unappreciated roles in replication. Alternatively, these genes may be representative of a gene expression program that renders cells resistant to infection, though these particular genes are not effectors in and of themselves. Additionally, we cannot rule out the possibility that one or more of our constructs failed to express, so we cannot definitively state that none of the genes impact HIV replication. CREM is expressed in most immune cells and encodes many distinct protein isoforms that have broad impacts on multiple signaling pathways and organ functions. CREM$\alpha$, for example, was found to perform critical functions as both an epigenetic and a transcriptional regulator for cytokine production in T lymphocytes (25). Within the CREM family, the ICERs are specific, short isoforms encoded in the latter half of the gene. ICERs inhibit T cell activation, suppress proinflammatory cytokine production in macrophages (26, 27), control systemic autoimmunity in systemic lupus erythematosus (SLE) (28), and increase apoptosis via antiapoptotic protein expression (29).

Due to the CREM gene encoding many alternatively spliced transcript variants that are regulated posttranscriptionally (19), it is essential to understand CREM alternative splicing and isoform enrichment to interpret transcriptional changes. ECs had distinct alternative splicing events compared with those of the non-EC groups. The IF and Psi calculations for the CREM gene splice sites showed that ECs widely use CREM exons 15, 16, and 18. Promoters located upstream of exons 15 and 16 drive specific ICER expression, while exon 18 encodes a short 12-amino-acid segment enriched in acidic amino acids (20, 30). The location of the promoter results in ICER proteins containing DNA-binding domains (DBDs) but lacking the upstream transactivation domain of CREM (20). Additionally, the enriched ICERs in ECs contain intrinsically disordered regions (IDRs). IDR segments include a high proportion of polar or charged amino acids and lack a unique three-dimensional structure, permitting highly specific trigger signaling events while facilitating rapid dissociation when signaling is completed (31).

To determine the function of ICER in HIV infection, we knocked out the exons of ICER using the HuT78 cell line. The knockout of ICER resulted in significantly increased

viral replication and decreased cell counts after HIV infection. However, knockout of CREM exon 13, which was widely used in CREM isoforms but absent in ICER isoforms, did not change the HIV infection. We further verified the ICER function in primary $CD4^+$ T cells. Knockdown of ICER exons 15 and 19 resulted in increases in HIV infection in primary $CD4^+$ T cells across a range of HIV strains, including both CCR5- and CXCR4-tropic viruses. Due to the short nucleotide fragment length of CREM exons 16 and 18 (20, 30), we could not identify effective shRNA against these exons. The ability of ICER to control HIV infection was also verified through overexpression of eight different ICER isoforms in primary $CD4^+$ T cells using a lentiviral expression system. Although all ICER isoforms are highly expressed in HEK 293T cells, only some ICER isoforms were well expressed in primary $CD4^+$ T cells. Consistently with the knockdown results, the EC-specific ICER isoforms inhibited HIV replication in primary $CD4^+$ T cells.

In the current study, the "response to stress," "defense response," and "response to virus" represented the different functional entities between ECs and ART-naive individuals, implying cellular responses to viral infection and related inflammation. We also observed the "regulation of metabolic process" in ECs versus ART-naive individuals, suggesting the difference in cellular metabolism. Further, we found that "regulation of metabolic process" represented the main difference between ECs and healthy controls or ART-treated individuals. Viral replication and related immune perturbations are controlled mainly after ART. Thus, our results suggested that cellular-metabolism-regulated processes remained the main difference between ART-treated individuals and ECs. The common top functional entity that distinguished ECs from each non-EC group is "regulation of metabolic process." ECs had upregulated mitochondrial function, oxidative stress, and decreased immunological activation (32). Metabolic levels were associated with the polyfunctionality of the HIV-specific $CD8^+$ T cell response (32–34). cAMP is a key mediator in the cellular metabolic process (35), and CREM encodes the cAMP-responsive element modulator. CREM is expressed in most immune cells and had many distinct protein isoforms that have broad impacts on multiple signaling pathways and organ functions. These findings were further supported by small-molecule agonists of ICER. Small-molecule agonists can rapidly induce the ICER proteins, which subsequently serve as endogenous inhibitors of gene transcription by competing for binding with cAMP response element (CRE) sequences (36). After treatment with ICER agonists PGE2 or 8-Br-cAMP, both ICER agonists strongly increased primary $CD4^+$ T cell resistance to HIV infection. These data are consistent with a model in which alternative splicing at the CREM/ICER locus in ECs provides protection against infection and decreases viral set points. PGE2-inhibited HIV replication is mediated by the activation of cAMP and protein kinase A (PKA), which suppress HIV promoter activity (37). PGE2 inhibits HIV replication in the late stages of HIV's viral cycle (38). PGE2 and 8-Br-cAMP are PKA activators, and the PKA signaling pathway plays a central role in the regulation of energy balance and immunometabolism (39), suggesting that ICER, its activated PKA pathway, and altered immunometabolism are critical for the spontaneous control of HIV infection (32, 33).

In conclusion, increased ICER isoform RNA expression was identified in ECs. The results of knockout of ICER in the HuT78 $CD4^+$ T cell line and knockdown or overexpression of ICER in primary $CD4^+$ T cells indicated that ICER is an HIV host factor that may contribute to the HIV resistance of ECs. The alternative splicing of ICER for resistance to HIV is distinct from previously reported antiviral mechanisms. Understanding the role of ICER in HIV replication may help elucidate a novel target for treatment of HIV.

## MATERIALS AND METHODS

**Subjects.** This study was conducted using cryopreserved peripheral blood mononuclear cells (PBMCs) from 10 ECs, 12 ART-treated HIV individuals, 11 ART-naive HIV individuals, and 11 healthy individuals. Samples from EC donors were received from the National Institute of Allergy and Infectious Diseases (NIAID; from Stephen Migueles and Mark Connors). ECs had been infected by HIV more than 8 years, with peripheral $CD4^+$ T cell counts above 500 cells/$\mu$l and plasma HIV RNA of $<$50 copies/ml in the absence of ART. The clinical characteristics of each individual are shown in Table S1 in the supplemental material. Twelve samples from aviremic HIV-infected individuals on ART for at least 24 weeks (a

single instance of ≤500 copies/ml was allowed) were collected from the Medical University of South Carolina (MUSC) clinical division of infectious diseases. Eleven HIV-positive ART-naive samples (plasma HIV RNA levels of 1,000 to 600,000 copies/ml) were collected from Case Western Reserve University (from Michael Lederman) and the MUSC clinical division of infectious diseases. Eleven healthy individuals were recruited from MUSC. The study received ethical approval from the MUSC review board. All recruited participants for this study provided written consent.

**RNA sequencing and analysis.** Human primary monocytes were obtained from PBMCs of 44 subjects. In brief, monocytes were enriched by negative selection using a Pan Monocyte isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany). Next, the isolated monocytes were further enriched by positive selection using CD14 microbeads (Miltenyi Biotec) according to the manufacturer's instructions. The purity of the enriched monocytes was verified by flow cytometry to be greater than 95% for the next step. Total RNA was extracted from the purified monocytes with the RNeasy Plus minikit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. After the RNA sequencing was performed, data were processed and analyzed through Trimmomatic (v0.39) (40) and STAR (v2.7) (41). Differential expression between two groups (ECs versus healthy subjects, ECs versus ART subjects, and ECs versus ART-naive subjects) was identified by the R Bioconductor package DESeq2 (v1.24.0) (42). The resulting *P* values were adjusted using Benjamini and Hochberg's approach for controlling the false-discovery rate (FDR).

**Alternative splicing analysis.** The Psi score was used for alternative splicing quantification by psichomics (1.8.2) (43). Salmon (v0.14) (44), which is based on "quasi-alignments" for mapping RNA-seq reads during isoform quantification, was performed. Calculations of IFs and prediction of functional consequences were performed with IsoformSwitchAnalyzeR (45) using standard parameters.

**Infection with HIV.** We placed $1 \times 10^5$ primary $CD4^+$ T cells in each well of a 96-U-well plate and inoculated them with an HIV NL4-3 suspension. After inoculating $CD4^+$ T cells with the AD8, JR-CSF, 89.6, BaL, or IIIB virus, the plate was spun for 1 h at 800 × *g* and 32°C and then cultured at 37°C in an incubator. After transference of the cells to a 96-U-well plate, the cells and HIV were incubated overnight at 37°C. The infected cells were washed with 200 $\mu$l of fresh culture medium. On day 3 or 5, the cells were stained with LIVE/DEAD Aqua blue (Thermo Fisher) for 20 min at 4°C to detect and exclude dead cells. Next, the cells were fixed, permeabilized, and stained for intracellular p24 using anti-p24-pycoerythrin (PE) antibodies (Beckman Coulter). Data were acquired using a FACSVers flow cytometer (BD Biosciences, San Jose, CA) and analyzed using FlowJo software 10.0.6.

**CREM/ICER Western blotting.** The primary $CD4^+$ T cells were lysed in radioimmunoprecipitation assay (RIPA) buffer with protease/phosphatase inhibitor cocktail (Cell Signaling Technology, Danvers, MA). After the protein concentration for each cell lysate was determined, an equal volume of 2× Laemmli sample buffer with $\beta$-mercaptoethanol was added to equal amounts of cell lysate. After denaturation at 95°C for 5 min, the cell lysate in the sample buffer was loaded with equal amounts of protein into the wells of an any-kilodalton mini-PROTEAN protein gel (Bio-Rad, Hercules, CA). Next, the proteins were transferred from the gel to a 0.2-$\mu$m Immobilon-PSQ polyvinyl difluoride (PVDF) membrane (Millipore). The membrane was blocked for 1 h at room temperature and incubated overnight at 4°C with CREM/ICER antibody (clone 3B5) (LifeSpan BioSciences, Seattle, WA). Mouse anti-human $\beta$-actin was used as the reference. After the membrane was washed three times using Tris-buffered saline–Tween 20 (TBST), an anti-mouse horseradish peroxidase (HRP)-conjugated secondary antibody was incubated for an additional hour. The HRP activity was determined using the SuperSignal West Femto maximum-sensitivity substrate (Thermo Fisher), and the blot was scanned using a ChemiDoc MP imaging system (Bio-Rad).

**Statistical analysis.** Conventional measurements of central location and dispersion were used to describe the data. Nonparametric Mann-Whitney U tests were applied to compare differences in continuous measurements between the two groups. A one-way analysis of variance (ANOVA) followed by Holm-Sidak's multiple-comparison test was used to compare differences among more than two categorical groups. Significance for comparisons of the expression levels of individual genes in RNA-seq data were tested using the Wald test in the DESeq2 package; *P* values denoted were adjusted using Benjamini-Hochberg correction. Comparison analysis was performed using R (version 3.3.1) or GraphPad Prism 8. A *P* of ≤0.05 was considered statistically significant.

**Data availability.** RNA-seq data in this study have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO) database under accession no. GSE157198.

## SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

**TEXT S1**, DOCX file, 0.03 MB.

**FIG S1**, PDF file, 0.4 MB.

**FIG S2**, PDF file, 0.2 MB.

**FIG S3**, PDF file, 0.2 MB.

**FIG S4**, PDF file, 1 MB.

**TABLE S1**, XLSX file, 0.01 MB.

**TABLE S2**, XLSX file, 0.03 MB.

**TABLE S3**, XLSX file, 0.02 MB.

**TABLE S4**, XLSX file, 0.01 MB.

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Z.L. and W.J. conceived the study. Z.L. wrote the manuscript. Z.L., T.L., and W.J.C. performed experiments. Z.L. and M.L. analyzed data. M.L., Z.L., Z.Y., J.F.H., J. Zhang, L.Y., L.H., and J. Zhu conceived the study and revised the manuscript. S.A.M. provided the key human specimens and revised the manuscript.

We declare no competing interests.

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