The ability of monocytes to travel through the bloodstream, traverse tissue barriers, and aggregate at disease sites endows these cells with the attractive potential to carry therapeutic genes into the nervous system. However, gene editing in primary human monocytes has long been a challenge. Here, we applied the CRISPR/Cas9 system to deliver the large functional Hutat2:Fc DNA fragment into the genome of primary monocytes to neutralize HIV-1 transactivator of transcription (Tat), an essential neurotoxic factor that causes HIV-associated neurocognitive disorder (HAND) in the nervous system. Following homology-directed repair (HDR), ~10% of the primary human monocytes exhibited knockin of the Hutat2:Fc gene in the AAVS1 locus, the “safe harbor” locus of the human genome, without selection. Importantly, the release of Hutat2:Fc by these modified monocytes protected neurons from Tat-induced neurotoxicity, reduced HIV replication, and restored T cell homeostasis. Moreover, compared with lentiviral transfection, CRISPR-mediated knockin had the advantage of maintaining the migrating function of monocytes. These results establish CRISPR/Cas9-mediated Hutat2:Fc knockin monocytes and provide a potential method to cross the blood-brain barrier for HAND therapy.

INTRODUCTION

In recent years, the lifespan of HIV-infected patients has been prolonged because of the advancement of combination antiretroviral therapy (cART). However, approximately 50% of patients suffer from varying levels of HIV-associated neurocognitive disorder (HAND) ranging from undetectable neurocognitive impairments to severe dementia, which greatly affects their survival rate and quality of life. The progression of HAND is related to several factors, and HIV transactivator of transcription (Tat) is one of the most important. On the one hand, HIV-Tat contributes to the release of several inflammatory factors that induce oxidative stress and excitotoxicity, ultimately resulting in neurodegeneration. On the other hand, HIV-Tat increases the levels of viral transcription in HIV-infected astrocytes and microglial cells, forming a positive feedback loop. Given the role of HIV-Tat in the progression of HAND, efforts to block its effects on the CNS might be exploited as an adjuvant strategy to reduce HIV-induced neuropathogenesis and improve patients’ quality of life.
types without known adverse effects. However, it has not yet been verified if this system is applicable in primary human monocytes.

In the present study, we generated Hutat2:Fc knockin (KI) primary human monocytes using CRISPR/Cas9 and investigated the biological activity of secreted Hutat2:Fc to neutralize HIV-Tat in vitro. Based on our data, Hutat2:Fc was successfully inserted into the AAVS1 locus and effectively expressed in primary monocytes. Secreted Hutat2:Fc protected mouse primary cortical neurons, limited viral replication and spread, and restored T cell homeostasis. Moreover, compared with traditional lentiviral transduction methods, CRISPR-mediated KI did not affect the normal functionality of monocytes, particularly their migrating ability. Thus, CRISPR-mediated Hutat2:Fc KI monocytes (KI-Ms) may represent a promising strategy for HAND therapy in the future.

RESULTS
CRISPR/Cas9-Mediated KI of the Hutat2:Fc Gene Fragments into the AAVS1 Locus in Various Cell Lines and Primary Human Monocytes

We designed six single guide RNAs (sgRNAs) (Table S1) targeting the AAVS1 locus to generate specific DSBs (Figure S1A). T7E1 and universal CRISPR activity assays were both employed to select the most active guide RNA, which was identified as sgRNA2 (Figures S1B and S1C). Notably, the activity of sgRNA2 was dose dependent, and 2 μg of sgRNA2 achieved the highest knockout efficiencies of up to ~20% in HeLa cells (Figure S1B) and ~12% in primary monocytes (Figure S3B).

A donor plasmid containing the CAG promoter, Hutat2:Fc fragment, GFP, the selective marker Puro, and flanking sequences of ~1.5 kb on each side homologous to the sgRNA2 targeting region was designed to achieve high HDR efficiency (Figure 1A). Although the expression of Hutat2:Fc could be driven by the PPP1R12C promoter itself, the CAG promoter was included to enhance its expression. The genotyping of HeLa cells after co-transfection and selection with Puro revealed that donor templates were successfully knocked into the genome (Figures 1B–1D).

Furthermore, we performed PCR using a pair of primers, GT-F and GT-R, which were located at a region of the chromosome outside of the homology arms, to determine the optimal ratio of sgRNA2 to donor plasmids. Without screening, ~35% in HeLa cells (Figure 1E, upper panel) and ~45% in 293T cells (Figures 1F and S2A, upper panel) after selection with Puro. Additionally, without screening, ~20% of U937...
cells (Figures 1G and S2B, upper panel) and ~10% of monocytes (Figures 1H and S3C, upper panel) exhibited Hutat2:Fc insertion following electroporation.

The proportion of GFP+ cells was analyzed by FACS to confirm the insertion of the target fragments. Consistent with the semiquantitative PCR results, up to 32.40% of GFP+ cells were detected in HeLa cells (Figure 1E, lower panel). Although the donor plasmids themselves might produce GFP in cells without insertion, less than 1% of cells treated with donor plasmids alone expressed GFP (Figure 1E, lower left panel), and no transgenic chromatid bands were detected in that sample by PCR (Figure 1E, upper left panel). It is likely that long-term culture might eliminate such interference.20 Similarly, with optimal sgRNA2 to donor plasmid ratios, the percentages of GFP+ cells were 40.20% in 293T cells (Figures 1F and S2A, lower panel), 21.00% in U937 cells (Figures 1G and S2B, lower panel), and 9.12% in monocytes (Figures 1H and S3C, lower panel). Furthermore, Sanger sequencing of both boundaries confirmed that the full-length DNA template was completely inserted into the AAVS1 locus targeted by sgRNA2 (Figure S4). Based on these data, CRISPR/Cas9 mediated successful insertion of the Hutat2:Fc gene into the AAVS1 locus of the HeLa, 293T, and U937 cell lines and monocytes. Moreover, specific KI was achieved in primary human monocytes without selection, albeit at relatively low rates.

Finally, the off-target (OT) effects on these transduced monocytes were evaluated. Recent studies suggested that CRISPR/Cas9 frequently induces unwanted OT mutations.21 These mismatches might exert negative effects on the normal functions of the targeted cells. Here, we examined the top four potential OT sites for sgRNA2 (Table S1). Indeed, only one OT mutation, OT-3, was detected in the T7E1 assay (Figure S5). Due to the specific design of the homology arms, no misinsertion of the DNA template was observed (Figure S5).

**Evaluation of the Stable Expression and Secretion of Hutat2:Fc**

Although successful insertion was confirmed, we needed to ensure that the functional Hutat2:Fc fragment was effectively expressed and secreted from the transduced cells. To this end, we conducted a preliminary panel of experiments to detect intracellular Hutat2:Fc expression. First, in HeLa cells, GFP was localized to both the nucleus and cytoplasm, whereas Hutat2:Fc was mainly distributed in the cytoplasm (Figure 2A). In monocytes, the expression of GFP was shown in Figure 6A. The levels of Hutat2:Fc and GFP transcription among the transformed cell lines and monocytes were compared by RT-PCR. Consistent with the KI efficiency, 293T cells exhibited the highest levels of expression, whereas monocytes exhibited the lowest expression levels (Figure 2B). This finding was confirmed by western blot analyses using cell lysates (Figure 2E).

Second, the release of Hutat2:Fc from the HeLa, 293T, and U937 cell lines and monocytes was quantified by ELISA. The analysis of HeLa and 293T cells revealed that the concentration of secreted Hutat2:Fc increased exponentially over time and reached a plateau on day 4 (704.23 ± 44.09 ng/mL for HeLa cells and 923.27 ± 43.54 ng/mL for 293T cells). For U937 cells, the concentration peaked on day 5 (194.30 ± 17.76 ng/mL) (Figure 2D), and similarly to the findings for U937 cells, a delay in the peak concentration was observed in monocytes on day 5 (113.30 ± 8.82 ng/mL) (Figure 2C). Thus, the cell culture supernatants were collected on day 5 and subjected to western blot analysis to confirm the release of Hutat2:Fc (Figure 2F).

Hutat2:Fc secretion was detected significantly earlier than GFP, which was apparent on day 9 and maximal on day 12, indicating that co-expression through the internal ribosome entry site (IRES) element was weaker than the expression of promoter-proximal genes.22

**Effects of Hutat2:Fc on Tat-Induced Neurotoxicity, HIV-1 Challenge, and T Cell Homeostasis**

Even though Hutat2:Fc was released extracellularly and accumulated to a detectable concentration in the culture medium, we wanted to ascertain whether it had biological activity for HAND therapy. Therefore, we subsequently determined the effects of Hutat2:Fc on Tat-induced neurotoxicity, HIV-1 replication, and T cell homeostasis in the presence of HIV-1 infection.

First, based on dot-immunobinding assay (DIBA) data, Hutat2:Fc secreted in the conditioned media specifically and sufficiently bound to Tat and acted as a functional antibody (Figure 5A). Cell Counting Kit-8 (CCK8) and apoptosis assays were subsequently performed to measure the viability and apoptotic status of HTB-11 cells after exposure to Tat in the presence or absence of conditioned media for 3 days. Despite the low levels of secreted Hutat2:Fc, the media collected from KI-Ms significantly increased the relative cell viability (p < 0.05) and reduced the percentages of 7-aminoactinomycin D (7-AAD)+ and Annexin V+ cells (p < 0.05) (Figures S6B–S6D). Moreover, primary mouse cortical neurons were isolated and treated using the same approach as HTB-11 cells to more directly assess the protective effects of Hutat2:Fc. As shown in Figure 3A, Tat-treated mouse neurons exhibited an increase of cellular atrophy and apoptosis, as well as a shorter length of dendrite. However, treatment with the media of transduced monocytes partly reversed the conditions and resulted in a higher proportion of survival neurons than treated with Tat alone (p < 0.01) (Figure 3B).

Second, we examined the ability of secreted Hutat2:Fc to restrict HIV-1 spread and replication. Both KI-Ms and their culture media (KI-M-Hutat2) were added to peripheral blood mononuclear cells (PBMCs) isolated from healthy donors after exposure to full-length infectious HIV-1pHSL. The HIV-1 p24 levels were quantified for 8 days to determine the viral titer in the culture media. On day 8, p24 levels were dramatically decreased in KI-M and KI-M-Hutat2 groups (by 9- to 20-fold) compared with those in the negative control group (Figure 4A). At the same time, total HIV DNA copies per 10^6 cells were determined to reflect the size of HIV reservoirs. Consistent with the results of p24 levels, a reduction in the total HIV DNA levels was also observed in KI-M and KI-M-Hutat2 groups (p < 0.01) (Figure 4B). Notably, the levels of both p24 and total HIV-DNA in
the KI-M group increased faster than that in the KI-M-Hutat2 group within the first 4 days. This result might be attributed to the relatively low levels of Hutat2:Fc secreted in the early period of time posttransfection (Figure 2C).

Third, to investigate the regulatory effects of secreted Hutat2:Fc on T cell stability in the presence of HIV-1Ba-L, the distributions of T cell subsets in six healthy donors were assessed. Overall, compared with the HIV-1Ba-L-infected group, the distributions of both CD4+ T cells and CD8+ T cells subsets in the KI-M-Hutat2 group were much closer to the normal control. For CD4+ T cells, treatment with KI-M-Hutat2 led to a lower percentage of effector memory (EM) CD4+ T cells and a higher percentage of naive CD4+ T cells than the merely HIV-1Ba-L-infected group (p < 0.05) (Figure 5A). But for CD8+ T cells, an increase in the proportion of naive CD8+ T cells and a reciprocal reduction of the proportion of terminally differentiated (TD) CD8+ T cells were observed (p < 0.05) (Figure 5B).

Because HIV is characterized as inducing global T cell dysfunction and death, we further evaluated the effects of secreted Hutat2:Fc on T cell exhaustion and apoptosis after exposure to HIV-1Ba-L. The exhaustion status was determined by FACS to examine the frequency of the activation markers CD38 and human leukocyte antigen-antigen D related (HLA-DR), as well as the inhibitory markers, programmed cell death protein 1 (PD-1), programmed cell death-ligand 1 (PD-L1), and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4), expressed on the surface of CD4+ and CD8+ T cells. As shown in Figures 5C and 5D, after exposure to HIVBa-L, PBMCs treated with KI-M-Hutat2 group exerted a notable reduction in the percentage of CD4+ T cells expressing PD-1 and PD-L1, as well
First, we initially compared the transduction efficiency among CRISPR/Cas9-mediated Hutat2:Fc KI-Ms, lentivirus-mediated transduced monocytes (LT-Ms), or MDMs (LT-MDMs). GFP+ monocytes were sorted by flow cytometry 9 days after transduction and the percentages of GFP+ cells were calculated to equal 86.49% ± 2.75% for LT-Ms, 95.36% ± 2.14% for LT-Ms, and 92.81% ± 2.31% for LT-MDMs. However, the percentage of Annexin V+ cells, as compared with HIV-1Ba-L exposure alone group (p < 0.05). Moreover, the apoptotic status of PBMCs was also detected by FACS. Despite a different degree of loss in cell viability on 3 days after sustained exposure to HIV-1Ba-L, KI-M-Hutat2 supplementation slightly decreased the percentage of Annexin V+ cells, as compared with HIV-1Ba-L-treated alone (p < 0.05) (Figures S7A and S7B). Because apoptosis was partly suppressed, KI-M-Hutat2 slowed the reduction in the number of CD4+ T cells and facilitated the restoration of the balance between CD4+ T and CD8+ T cells (Figures S7C–S7E).

Based on the data shown above, secreted Hutat2:Fc partially protected neurons from Tat-induced neurotoxicity, limited HIV replication, and maintained T cell homeostasis, although not as effectively as the commercial anti-Tat antibody. These results revealed that Hutat2:Fc exhibits excellent biological activity and has the potential to exert therapeutic effects on HAND.

Comparison of the Transduction Efficiency and Potential Adverse Effects of Different Gene-Editing Strategies on Primary Monocyte Cells

Both the transduction efficiency and monocyte functionality, including migration, differentiation, and cytokine secretion, should be considered to ensure that Hutat2:Fc functions in the CNS. Therefore, we compared the efficiencies and potential adverse effects between two transfection methods, CRISPR/Cas9 and traditional lentiviral transduction, the latter of which has been used extensively to transfect hard-to-transduce monocytes and monocyte-derived macrophages (MDMs).

First, we initially compared the transduction efficiency among CRISPR/Cas9-mediated Hutat2:Fc KI-Ms, lentivirus-mediated transduced monocytes (LT-Ms), or MDMs (LT-MDMs). GFP+ monocytes (not LT-MDMs) were sorted by flow cytometry 9 days after transduction to eliminate interference from non-transfected cells. After sorting, the percentages of GFP+ cells were calculated to equal 96.42% ± 3.07% for KI-Ms, 95.36% ± 2.81% for LT-Ms, and 51.28% ± 1.42% for LT-MDMs (Figure 6A). A prominent decrease in the number of cells was observed in the KI-M group, and this effect can be attributed to the transduction method of electroporation, in which approximately 40% of cells are killed by the high-intensity discharge. The levels of Hutat2:Fc produced by these three transduced cell types were further measured by western blot analysis (Figures 6B and 6C). Although slightly higher than those of LT-MDM, the levels of Hutat2:Fc secreted by KI-Ms were approximately half of the levels produced by LT-M.

Second, the potential adverse effects of gene editing on monocytes were assessed. Fifteen monocyte-related genes, including molecular signal genes, apoptosis-induced genes, inflammatory genes, and chemokine receptor genes, were quantified by real-time PCR to evaluate the function of monocytes. Overall, the LT-MDM group was quite different from the other groups. Notably, the expression levels of P53 (2.65% ± 0.68%) and STAT-1 (3.19% ± 0.82%) were significantly increased in LT-M compared with those in normal monocytes, whereas no differences were observed between KI-Ms and non-transduced monocytes (Figure 6D). Next, the levels of IL-10, TNF-α, and IL-1β in culture media were subsequently measured by ELISAs to confirm the changes in cytokine secretion among the four groups. Consistent with the real-time PCR results, the release of both IL-10 and IL-1β was greatly increased while the release of TNF-α was declined in the LT-MDM group compared with other groups. No variations were observed among KI-Ms, LT-Ms, and non-transduced monocytes (Figures 6E–6G). A transendothelial migration (TEM) assay was subsequently employed to evaluate the migrating ability of monocytes. The percentage of migrated cells in the KI-M group (82.30% ± 2.31%) was similar to that in normal monocytes (86.49% ± 2.75%) (Figure 6H). However, the percentage...
of transmigrated cells was decreased to different extents in LT-M (71.46% ± 1.67%) and LT-MDM (43.64% ± 1.88%) groups (Figure 6H). Moreover, we noticed that, compared with MDM, the percentage of transmigrated cells in LT-MDM was much less (Figure 6H).

Thus, despite the inferior transduction efficiency, KI-Ms were superior to LT-Ms and LT-MDMs in terms of their conservation of normal monocyte functionality, which is critical for modified monocytes to function in the CNS.

**DISCUSSION**

CRISPR/Cas9 technology has been widely employed to edit various eukaryotic cells because of its convenient manipulation and relatively high efficiency. Here, we constructed Hutat2:Fc KI-M via HDR induced by CRISPR/Cas9, and ~10% of monocytes exhibited successful insertion into the AAVS1 locus without selection. Furthermore, secreted Hutat2:Fc neutralized Tat-induced neurotoxicity, suppressed HIV replication, and restored T cell homeostasis without inhibiting the migrating ability of monocytes. These data collectively provide support for the wide application of monocytes for the delivery of therapeutic genes to treat HAND.

Although gene editing has been effective in various cell lines, induced pluripotent stem cells (iPSCs), and primary T cells, little attention has been paid to modifying the genomes of monocytes because of their poor ability to proliferate and express genes in vitro.12–15 In the present study, we introduced a successful multi-kilobase functional gene KI method in primary human monocytes using CRISPR/Cas9. We identified the best of six sgRNAs and assayed multiple concentrations to determine the experimental conditions with optimal editing efficiency. In addition, due to the use of electroporation with a maximum amount of donor plasmids, including extended homology arms, Hutat2:Fc was successfully inserted into monocytes, despite at a low proportion. The efficiency of this strategy was consistent with that observed in the previous studies.24,25 Recently, there is a wonderful double-cut donor gene-editing strategy based on CRISPR/Cas9, which is most likely to yield a higher KI efficiency in hard-to-transduce cells, such as astrocytes, neurons, as well as mouse and monkey embryos both in vitro and in vivo.26,27 More attempts will be performed to increase the efficiency of the new strategy in the future.

Additionally, the non-specific mutation, OT-3, was identified using T7E I assay (Figure S5). OT-3 is located on the Chromosome 13: +106612910. Neither long non-coding RNA (LncRNA) or protein had been found to be associated with this locus until now. Additionally, based on the comparison of the expression of 15 monocyte-related functional genes, secretion of 3 cytokines, as well as the migrating ability (Figures 6D–6H) between KI-Ms and normal monocytes, we considered that OT-3 did not appear to cause any adverse effects on primary monocytes.

As a class of lymphocytes, monocytes migrate through the bloodstream to tissues and then differentiate into resident macrophages. These characteristics endow these cells with the ability to deliver therapeutic genes to locations that are out of reach to drugs, such as the CNS, and to function persistently at disease sites.12 Based on the results of the present study, Hutat2:Fc KI-M exhibited excellent biological activity in vitro, although merely ~10% cells contained an insertion. Notably, because Hutat2:Fc expression was enhanced by the exogenous CAG promoter, the selection of modified monocytes was avoided, which greatly increased the suitability of utilizing these cells for therapeutic applications, such as autotransplantation.

Although the expression was clearly enhanced, Hutat2:Fc was still secreted at a relatively low level in the monocytes medium, with levels nearly 8- or 11-fold lower than those obtained with engineered HeLa or 293T cell lines (Figures S6B and S6C). However, this concentration was sufficient to protect neurons, restrict the virus, and balance the immune system, despite not being as effective as cell lines or commercial anti-Tat antibodies. Nevertheless, the low levels of Hutat2:Fc released from monocytes were considered beneficial as a therapeutic strategy to treat HAND in vivo. On the one hand, given the effective viral suppression achieved by cART, the residual Tat in the CNS is so little that these modified monocytes might be sufficient to neutralize...
them. On the other hand, the low levels of secreted Hutat2:Fc might minimize the disturbance to the CNS microenvironment, improving the safety of this strategy.

Traditional editing strategies, such as lentiviral-vector-mediated gene transfer, have also been widely utilized in monocytes and MDMs. Compared with CRISPR/Cas9, transduction mediated by lentiviral vectors induced higher levels of Hutat2:Fc expression but inhibited the normal function of monocytes to varying extents. Several factors might contribute to this phenomenon. First, the genomic integration sites of HIV-based lentiviral vectors are random but are more likely to occur in actively transcribed chromatin regions. Interactions between exogenous DNA fragments and self-genomic sequences might affect normal gene transcription and expression in monocytes. Second, lentivirus-mediated integration of multiple gene copies at multiple loci might influence the stability of the whole genome. Thus, the single-copy KI induced by CRISPR/Cas9 at the AAVS1 locus presents an advantage over lentiviral strategies for maintenance of the normal function of monocytes.

The ability to edit specific DNA sequences in the AAVS1 locus of monocytes using the CRISPR/Cas9 system, as demonstrated in the present study, provides the possibility of delivering target genes to the CNS. However, owing to the lack of animal models of HAND, as well as the specificity of genomic sequences among each species, we did not examine whether the edited human monocytes were able to reach the injured sites of the brain tissue to alleviate HIV-Tat-induced neurotoxicity in an animal model. Next step, we are going to generate Hutat2:Fc KI mouse monocytes in Rosa26 locus and detect in an HIV-Tat-induced brain inflammation mouse model. Looking forward, the present study on Hutat2:Fc editing in primary monocytes using CRISPR/Cas9 represents a

Figure 5. Effects of Secreted Hutat2:Fc on the Distributions of T Cell Subsets and Exhaustion Status after Exposure to HIV-1Ba-L
(A) Representative FACS plots of the distributions of CD4⁺ and CD8⁺ T cell subsets after HIV-1Ba-L infection, with or without KI-M-Hutat2. Commercial anti-Tat antibodies were used as a positive control. (B) Comparison of CD4⁺ and CD8⁺ T distributions following treatment with or without each type of conditioned media (n = 6). (C) Expression of CD38 and HLA-DR in CD4⁺ and CD8⁺ T cells treated with or without each type of conditioned media (n = 6). (D) Expression of PD-1, PD-L1, and CTLA-4 in CD4⁺ and CD8⁺ T cells treated with or without each type of conditioned media (n = 6). Wilcoxon matched-pairs signed-ranks tests were used to statistically compare the data shown in (B)–(D); *p < 0.05. The results are presented as the medians from independent experiments.
potentially powerful strategy as an adjuvant to cART for the treatment of HAND.

MATERIALS AND METHODS

Plasmid Preparation
N_{20}NGG motifs in the AAVS1 locus were scanned, and candidate sgRNAs that fit the rules for U6 Pol III transcription and the PAM recognition domain of Streptococcus pyogenes Cas9 were identified.34 The top six sgRNA candidates were selected from the MIT CRISPR Design Tool (http://crispr.mit.edu). Using the same procedure, potential OT sites were also predicted. The sequences are listed in Table S1. Oligonucleotides were annealed and cloned into Bbs I-digested pX330-U6-Chimeric_BB-CBh-hSpCas9 (Addgene plasmid #42230). The resulting plasmids containing sgRNAs were further confirmed by Sanger sequencing.

The donor plasmid was generated by cloning the Hutat2:Fc and GFP genes from the pHR-Hutat2:Fc-IRES-GFP plasmid, which was kindly provided by Dr. Yuanan Lu,29 into the PTA2 vector. Additionally, ~1.5-kb homology arms were ligated on both sides of the target fragments (Hutat2:Fc and GFP) using T4 ligase (Thermo Scientific).

Generation of Lentiviral Vectors
The pHR-Hutat2:Fc-IRES-GFP plasmid, which co-expresses the Hutat2:Fc fusion protein and GFP from the CMV promoter, was co-transfected with pCMV-ΔR8.2 and pCMV-VSV-G plasmids into 293T cells using Lipofectamine 2000 (Invitrogen). The vector concentrations were determined as previously described.35 Finally, high-titer HR-Hutat2 lentiviral vectors (~5 × 10^8 IU/mL) were prepared for transduction.

Cell Culture
All cell lines were kindly provided by the Stem Cell Bank, Chinese Academy of Science. HeLa, 293T, and HTB-11 cell lines were maintained in DMEM (Corning Life Science) supplemented with 100 IU/mL penicillin (Sigma-Aldrich), 0.1 mg/mL streptomycin (Sigma-Aldrich), and 10% fetal bovine serum (FBS) (HyClone). U937 cells were maintained in RPMI 1640 (Corning Life Science) supplemented with 100 IU/mL penicillin, 0.1 mg/mL streptomycin,
and 10% FBS. Human umbilical vein endothelial cells (HUVECs) were cultured in endothelial cell medium (ECM) (ScienCell). The culture media were replaced every 2–3 days, and the cells were passaged using EDTA solution containing 0.25% trypsin (Corning).

**Primary Human Monocytes**

Total PBMCs were obtained from healthy donors (serum-negative for HIV-1, hepatitis B, and hepatitis C). CD14+ cells were purified by negative selection using EasySep magnetic beads (STEMCELL Technologies) (Figure S3A). The isolated monocytes were maintained in RPMI 1640 supplemented with 100 IU/mL penicillin, 0.1 mg/mL streptomycin, and 10% FBS. Half of the culture media was replaced every 3–5 days.

**Human MDMs**

PBMCs were cultured in RPMI 1640 supplemented with 100 IU/mL penicillin, 0.1 mg/mL streptomycin, 10% FBS, and 20 ng/mL M-CSF (PeproTech). The culture medium was replaced every 3 days, and non-adherent cells were removed.36

**Mouse Primary Neurons**

Primary neurons were isolated from the cortices of early post-natal (P0) C57BL/6j mice using a previously described method.37 The cells were cultured in Neurobasal medium (GIBCO) supplemented with B27 (Invitrogen), L-glutamine (Sigma-Aldrich), 100 IU/mL penicillin, and 0.1 mg/mL streptomycin. Half of the culture medium was replaced every three days.

**Cell Transduction**

HeLa and 293T cells (1 × 10^5 cells) were transfected with different amounts of plasmids using Lipofectamine 2000 (Invitrogen), according to the manufacturer’s protocol. The efficiency of CRISPR-mediated knockdown was analyzed 3 days after transfection with sgRNA1-6 plasmids. For CRISPR-mediated KI, the cells were co-transfected with sgRNA2 and donor plasmids for 3 days, and selected with 400 ng/mL puromycin for 9 days prior to the final analyses.

U937 cells and monocytes (5 × 10^5 cells) were transfected with plasmids by electroporation (Bio-Rad). In brief, the cells were suspended in 200 μL of electroporation buffer (Bio-Rad) and then electroporated (1,000 V, 50 ms, one pulse for U937 cells; 1200 V, 20 ms, three pulses for monocytes). The efficiency of CRISPR-mediated knockdown was analyzed 9 days after electroporation. For CRISPR-mediated KI, the cells were co-electroporated with sgRNA2 and donor plasmids and cultured for 9 days, and GFP+ monocytes were then sorted by flow cytometry using a FACS Aria II cell sorter (BD Biosciences).

MDMs (1 × 10^5 cells) were infected with HR-Hutat2 vectors at an MOI of 10 in the presence of 8 μg/mL polybrene for 4 hr. The transduction efficiency was evaluated 9 days after transfection.

In addition, 5 × 10^5 monocytes were infected with HR-Hutat2 vectors at an MOI of 10 in the presence of 8 μg/mL polybrene with rotation at 1,500 × g for 4 hr. GFP+ monocytes were then sorted by flow cytometry 9 days after transfection.

**Genomic DNA Extraction, PCR, Purification, and Sequence Analysis**

Genomic DNA was extracted from different groups of cells using a QIAamp DNA Blood Mini Kit (QIAGEN) according to the manufacturer’s recommended protocol. Genomic DNA was subjected to PCR (Takara) using primers listed in Table S1. The general reaction conditions were 95°C for 10 min followed by 30 cycles of 95°C for 30 s, annealing at 61°C for 30 s, and extension at 72°C for 30 s. After each reaction, 200 ng of the PCR products was purified using a QIAquick PCR Purification Kit (QIAGEN), subjected to T7E1 assays, and then analyzed by agarose gel electrophoresis. The interest bands were extracted and cloned to pUC118 vector by Mighty cloning Reagent (Takara) and confirmed by Sanger Sequencing.

**T7 Endonuclease I Assay**

As described before,39 200 ng of the purified PCR products was denatured and re-annealed in 1 × NEB Buffer 2 with or without T7E1 (New England Biolabs). The reaction mixtures were then separated by 2% agarose gel electrophoresis. The knockdown efficiency (KO%) was determined using the following formula: \( \text{KO}% = 100 \times \left(1 - \frac{b}{a+b}\right)^{1/2} \), where a is the integrated intensity of the undigested PCR product and b is the combined integrated intensity of the cleavage products.

**Universal CRISPR Activity Assay**

Universal CRISPR activity assays were performed according to the manufacturer’s protocol. In brief, PCR amplicons of the genomic DNA templates encompassing the target sites of sgRNA1-6 were cloned into pUC vectors (Luc) (Bioxyten). After 24 hr of co-transfection with sgRNA and its pUCA (Luc) into 293T cells, the luciferase activity was detected to reflect the ability of sgRNA to cut the target site.

**Western Blot**

For the western blot assays, 5 × 10^5 cells with or without transduction and their supernatants were harvested and resuspended in 1 × SDS buffer. The samples were separated by 10% SDS-PAGE, transferred to polyvinylidene difluoride membranes, and incubated with immunoglobulin G (IgG) (H+L) (Rockland) overnight at 4°C. After washing, the membranes were treated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Abcam) for 2 hr at room temperature (RT). Finally, the membranes were exposed to 3,3′-diaminobenzidine (DAB) substrate (Pierce) for detection. As a control, β-actin was detected with rabbit anti-β-actin antibodies (Rockland).

**ELISA**

Plates were coated with goat anti-human IgG Fc capture antibodies (Rockland) overnight at 4°C. After washing, diluted supernatant samples were added for 1 hr, and the plates were then incubated with goat anti-human IgG Fc biotin-conjugated detection antibodies (Abcam) for 1 hr. Finally, streptavidin-HRP and tetramethylbenzidine (R&D Systems) were added, and the plates were then incubated for
The optical density at 450 nm was read with a microplate reader (Bio-Rad).

The levels of IL-10, TNF-α, and IL-1β in the supernatants of monocytes, KI-Ms, LT-Ms, and LT-MDMs were all determined using ELISA Kits (R&D Systems).

Dot-Immunobinding Assay
For each DIBA, 200 ng of recombinant HIV-Tat protein (Abcam) was spotted onto a nitrocellulose membrane and incubated with conditioned media collected from KI-HeLa, KI-293T, KI-U937, or KI-M. The membrane was incubated with rabbit anti-human IgG (H+L) (Rockland) and goat anti-rabbit IgG HRP (Abcam) before exposure to DAB substrate. Anti-Tat antibodies (Abcam) served as a positive control.

Real-Time PCR
Total mRNA was extracted from KI cells using TriPure (Roche) according to the manufacturer’s instructions. cDNA was synthesized with a Transcriptor First-Strand cDNA Synthesis Kit (Roche) and then used for PCR using the following primers: Hutat2, 5’-ACATC TGTGGTTCTTCTCTCCT-3’; EGFP, 5’-GGTAGCAAGGGCGAGGAG-3’; ACTB, 5’-GGTGTCGAGATGAACT-3’; and ACTB, 5’-AGGTGACACTATAAATAGGACTC-3’. The PCR products were analyzed by 2.5% agarose gel electrophoresis. The primers that were used to profile the expression of 15 genes in monocytes have been described as previously except that ACTB was listed above. Three reference genes, ACTB, GK and Ezrin, were used for normalization.

Real-time PCR was performed on a LightCycler 480 instrument (Roche) using FastStart Universal SYBR Green Master Mix (Roche) according to the manufacturers’ instructions. Fold changes were determined using the arithmetic comparative method (2^−ΔΔCt).

Fluorescent Immunocytochemistry
Cells were fixed with 4% paraformaldehyde and blocked with 1% BSA in PBS containing 0.1% Triton X-100 (Sigma-Aldrich). IgG Fc was stained by primary rabbit anti-human IgG Fc (Rockland) and secondary goat anti-rabbit IgG Cy3-conjugated antibodies (Abcam). For mouse neuronal staining, the cells were incubated with rabbit anti-MAP2 antibodies (Abcam) followed by the same secondary antibody. To detect neuronal apoptosis, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) assays were performed using an In Situ Cell Death Detection Fluorescein Kit (Roche) according to the manufacturer’s instructions. Finally, all cells were incubated with DAPI to stain the nuclei. Images were captured using an Olympus DP70 immunofluorescence microscope and analyzed using ImageJ.

Primary Neuron Protection Assay
As previously described, mouse primary neurons were treated with 500 ng/mL HIV-Tat and conditioned media from KI-HeLa, KI-293T, KI-U937, or KI-M for 3 days. Neurons treated with normal medium were used as a normal control, cells treated with Tat alone were used as a negative control, and cells treated with Tat plus commercial anti-Tat antibodies (Abcam) were used as a positive control. The cells were subjected to fluorescence immunocytochemistry. The surviving neurons, which displayed intact cell bodies (red) and nuclei (blue), but not TUNEL labeling (green), were counted to determine the survival rate. The rate observed in the normal control was defined as 100%. The relative neuronal survival rate was evaluated for each treatment.

HIV-1 Challenge
HIV-1/Helix was obtained from the NIH AIDS Reagent Program. PBMCs from six healthy donors were isolated, and after HIV-1/Helix (final concentration of p24 was 8 ng/mL) was added, the cells were incubated at 37°C for 4 hr. Subsequently, the cells were washed three times, and KI-M-Hutat2, KI-M, or anti-Tat antibodies were added. Half of the volume of each culture’s supernatant was replaced every 3 days. Viral replication was evaluated by monitoring the p24 levels in the culture supernatants using an HIV-1 p24 Antigen ELISA 2.0 (Petrotec), according to the manufacturer’s instructions. The viral reservoirs were evaluated by quantifying the total HIV-DNA levels in the genomic DNA of each cell sample using an HIV-DNA Detection Kit (Supbio) as previously described.

Cell Viability Assay
HTB-11 cells were treated with HIV-Tat (500 ng/mL) and conditioned media from KI-HeLa, KI-293T, KI-U937, or KI-Ms. PBMCs isolated from healthy donors and HIV-1/Helix (final concentration of p24 was 8 ng/mL) were added, and the mixtures were incubated at 37°C for 4 hr. Subsequently, the cells were washed three times, and KI-M-Hutat2, KI-Ms, or anti-Tat antibodies were added. Normal medium was used as a negative control, whereas anti-Tat antibody was used as a positive control.

HTB-11 and PBMCs were harvested 3 days after treatment. At the time of harvest, the cells were stained using a PE Annexin V Apoptosis Detection Kit I (BD Biosciences) and then analyzed immediately on a FACSCalibur flow cytometer (BD Biosciences). HTB-11 cells were further analyzed using a CCK8 (MCE), according to the manufacturer’s instructions.

Flow Cytometry
The percentages of GFP+ HeLa cells, 293T cells, U937 cells, and monocytes were evaluated by suspending the cells in PBS and fixing them with 2% paraformaldehyde for 10 min prior to flow cytometry analysis using a FACSAria II cell sorter (BD Biosciences). Intact cells were gated using forward scatterplots versus side scatterplots, and further gating for cell was performed based on the presence of control samples expressing GFP using fluorescein isothiocyanate (FITC) versus side scatterplots. The number of GFP+ cells in control samples ranged from 0% to 1%.

At the time of harvest, PBMCs were stained with antibodies against the following proteins: CD45-PerCp (2D1), CD4-phycoerythrin (PE)/Cy7
onto the gels at a density of 2 × 10^6 cells/mL. HUVECs were co-incubated for 4 hr to allow polymerization. HUVECs were seeded at a density of 2 × 10^5 cells/mL and cultured for 48 hr until a confluent HUVEC monolayer was formed. Normal monocytes, KI-Ms, LT-Ms, and LT-MDMs were plated on the HUVECs at a density of 2 × 10^5 cells/mL, and the mixtures were incubated for 1.5 hr at 37°C and then washed three times to remove unattached cells. After 48 hr, the collagen gels were digested with collagenase D (Roche). The proportion of GFP+ cells was analyzed by flow cytometry. The proportion of migrated monocytes in the MDM population was calculated using the following formula: transmigrated cells (%) = GFP+ cells (%)/transduction efficiency. The transduction efficiency was determined based on PD-1, PD-L1, and CTLA-4. The gates for activation markers and inhibitory markers were determined using isotype control antibodies.

**TEMASSay**

TEM assays were performed as described previously. In brief, three-dimensional collagen matrices containing 1.25 mg/mL Cultrex type I bovine collagen (R&D Systems), 35.7 mM NaOH, and 10% ECM were co-incubated for 4 hr to allow polymerization. HUVECs were seeded onto the gels at a density of 2 × 10^6 cells/mL and cultured for 48 hr until a confluent HUVEC monolayer was formed. Normal monocytes, KI-Ms, LT-Ms, and LT-MDMs were plated on the HUVECs at a density of 2 × 10^5 cells/mL, and the mixtures were incubated for 1.5 hr at 37°C and then washed three times to remove unattached cells. After 48 hr, the collagen gels were digested with collagenase D (Roche). The proportion of GFP+ cells was analyzed by flow cytometry. The proportion of migrated monocytes in the MDM population was calculated using the following formula: transmigrated cells (%) = GFP+ cells (%)/transduction efficiency. The transduction efficiency was determined based on CD38, HLA-DR expression, whereas the inhibitory status was determined based on PD-1, PD-L1, and CTLA-4. The gates for activation markers and inhibitory markers were determined using isotype control antibodies.

**Statistics**

Statistical analyses were performed using SPSS Version 16.0. The results from T cell exhaustion studies were treated as nonparametric data and assessed using the Wilcoxon signed-rank test. For independent data, Student’s t test was performed to determine the statistical significance. For multiple comparisons, one-way ANOVA was performed and confirmed using the Kruskal-Wallis test. Comparisons of each group with the control were performed using Dunnett’s t test. Two-tailed p values less than 0.05 were considered statistically significant.

**SUPPLEMENTAL INFORMATION**

Supplemental Information includes seven figures and one table and can be found with this article online at https://doi.org/10.1016/j.omtn.2018.01.012.

**AUTHOR CONTRIBUTIONS**

B.W., Wen Kang, and Y.S. designed the research; B.W. and J.Z. performed the research; B.W., Q.W., J.L., and C.W. analyzed the data; Wenzhen Kang, Z.L., Y.Z., Y.L., and B.D. contributed with the reagents/materials/analysis tools; B.W., Wen Kang, Y.L., and Y.S. wrote the paper. All authors read and approved the final manuscript.

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