

1 **Specific activation of HIV-1 from monocytic reservoir cells by bromodomain**
2 **inhibitor in humanized mice in vivo**

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1 **ABSTRACT**

2 The combination antiretroviral therapy (cART) effectively suppresses HIV-1 infection and
3 enables HIV-infected individuals to live long productive lives. However, the persistence of HIV-1
4 reservoir cells with latent or low-replicating HIV-1 in patients under cART make HIV-1 infection
5 an incurable disease. Recent studies have focused on the development of strategies such as
6 epigenetic modulators to activate and purge these reservoirs. Bromodomain inhibitors (BETi)
7 are epigenetic modulating compounds able to activate viral transcription in HIV-1 latency cell
8 lines in a positive transcription elongation factor b (P-TEFb)-dependent manner. Little is known
9 about the efficacy of activating HIV-1 reservoir cells under cART by BETi in vivo. In this study, we
10 seek to test the potential of a BETi (I-BET151) in activating HIV-1 reservoir cells under effective
11 cART in humanized mice in vivo. We discover that I-BET151 efficiently activates HIV-1
12 transcription in monocytic cells, but not in CD4⁺ T cells, during suppressive cART in vivo. We
13 further reveal that HIV-1 proviruses in monocytic cells are more sensitive to I-BET151 treatment
14 than in T cells in vitro. Finally, we demonstrate that I-BET151-activated viral transcription in
15 monocytic cells is dependent on both CDK2 and CDK9, whereas only CDK9 is involved in
16 activation of HIV-1 by I-BET151 in T cells. Our findings indicate a role of myeloid cells in HIV-1
17 persistence, and highlights the limitation of measuring or targeting T cell reservoirs alone in
18 terms of HIV-1 cure, as well as provides a potential strategy to reactivate monocytic reservoirs
19 during cART.

20

1 **IMPORTANCE**

2 It has been reported the low level of active P-TEFb critically contributes to the maintenance of
3 HIV-1 latency or low-replication in HIV-1 reservoir cells under cART. Bromodomain inhibitors
4 are used to activate HIV-1 replication in vitro but their effect on activation of the HIV-1
5 resevoirs with cART in vivo is not clear. We found that BETi (I-BET151) treatment reactivated
6 HIV-1 gene expression in humanized mice during suppressive cART. Interestingly, I-BET151
7 preferentially reactivated HIV-1 gene expression in monocytic cells, but not in CD4 T cells.
8 Furthermore, I-BET151 significantly increased HIV-1 transcription in monocytic cells, but not in
9 latently infected CD4 T cells, via CDK2-dependent mechanisms. Our findings suggest that BETi
10 can preferentially activate monocytic HIV-1 reservoir cells, and a combination of latency
11 reversal agents targeting different cell types and pathways is needed to achieve reactivation of
12 different HIV-1 reservoir cells during cART.

13 **KEYWORDS**

14 HIV-1, BET inhibitor, I-BET151, humanized mice, monocytic reservoirs

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1 **Introduction**

2 The global implementation of cART has transformed HIV infection from a fatal disease into a
3 manageable chronic illness, and successfully blunted HIV pandemic (1-3). HIV-1 replication can
4 be suppressed and maintained at a level below the detection limits by current cART regimens.
5 However, cART is still unable to cure HIV-1 infection. Drug resistance, serious non-AIDS events
6 as well as financial and societal issues associated with life-long cART highlight the urgent need
7 in finding a cure for the disease (4-7). Therefore, recent efforts have focused on interventions
8 that can yield a drug-free remission of HIV-1 replication or even eradication of replication-
9 competent HIV-1 proviruses in patients.

10 The obstacle to eradicate HIV-1 is the persistence of latent or low replicating HIV-1 reservoirs.
11 Following discontinuation of cART, HIV-1 reservoirs are able to produce infectious viral particles,
12 result in viral relapse(8-11). The molecular mechanisms associated with the maintenance and
13 reactivation of HIV latency have been extensively investigated, including transcriptional
14 interference, deleterious mutations in the viral genome, low levels of transcriptional activators,
15 inadequate Tat activity, epigenetics, transcriptional repressors engagement, nucleosome
16 positioning, mRNA splicing or nuclear export blocks as well as cellular microRNA(12, 13). It is
17 most likely that multiple mechanisms are involved in HIV-1 reservoirs persistence. And the
18 relative importance of these mechanisms in different cell types is remain to be determined.

19 The best characterized HIV-1 cellular reservoir is the long-lived resting CD4⁺ T lymphocytes
20 harboring quiescent proviral DNA that is replication-competent upon reactivation. However,
21 reservoirs in myeloid cells could be a problem for curative strategies targeting to resting CD4⁺ T

1 cells only. Macrophages are susceptible to HIV-1 infection in human and animal models(14-17),
2 and HIV-1 could be recovered from the circulating monocytes pool of patients treated with
3 cART (18, 19). In addition, HIV-1 is able to infect and replicate in brain astrocytes and microglia
4 cells in a restricted manner that could persist despite cART (20, 21). And recent reports
5 demonstrated that humanized mice with myeloid cells only allow HIV-1 persistent infection in
6 macrophages during cART in vivo(17), and integrated HIV-1 DNA can be detected in the bone
7 marrow and spleen macrophages in humanized mice with suppressive cART(22). These findings
8 highlight that, other than resting CD4⁺ T cells, monocytes or macrophages are of great clinical
9 importance in terms of HIV-1 cure.

10 It is known that bromodomain-containing protein 4 (BRD4) competes P-TEFb and disrupts the
11 interaction between Tat and P-TEFb, and forfeits the ability of Tat to trans-activate HIV-1
12 transcription(23-25). Given the important role of P-TEFb in regulating HIV gene expression,
13 different BETi have been explored and tested to activate HIV-1 gene expression in latent
14 models of primary CD4⁺ T cells, lymphocytic T cell lines and monocytic cell lines (26, 27).
15 However, little is known about the therapeutic potential of BETi in activating viral replication in
16 HIV-1 reservoirs during cART in vivo.

17 In this study, we tested how bromodomain inhibitor (I-BET151) affected viral replication in
18 HIV-1 reservoir cells in vivo during suppressive antiretroviral therapy. Our results demonstrate
19 that I-BET151 treatment leads to reactivation of HIV-1 gene expression preferentially in
20 monocytic cells during cART, which highlights the therapeutic potential of bromodomain
21 inhibitors to activate the unique monocytic HIV-1 reservoir cells in vivo.

1 RESULTS

2 HIV-1 persistence in both T and myeloid cells in NRG-hu HSC mice during cART

3 NRG-hu HSC mice (hu-mice) were infected with HIV_{JRC5F} and monitored for viral load and HIV-1
4 pathogenesis in peripheral blood until 14 weeks post-infection (wpi). Several infected animals
5 were treated with cART in mouse diet from 4-to-12 wpi. We showed that plasma viral load
6 declined rapidly after initiation of cART, concomitant with a recovery of peripheral blood CD4⁺ T
7 cells (Fig. 1A and Fig. S1A). Viral load reached undetectable levels in treated mice around 3-4
8 weeks after cART (Fig. 1A). We kept cART on board four more weeks before withdraw at 12wpi.
9 As observed in HIV-1 patients, plasma viral load rebounded rapidly after discontinuation of
10 cART (Fig. 1A), correlated with a decrease of human CD4⁺ T cells in the blood (Fig. S1A). At 12
11 wpi before cART cessation, three mice in each group were terminated for determining HIV-1
12 replication and pathogenesis in lymphoid tissues. The results demonstrated that HIV-1 gag p24
13 was significantly detected in both CD4 T cells (huCD45⁺CD3⁺CD8⁻) and monocytic cells
14 (huCD45⁺CD3⁻CD11c⁺CD14⁺) in spleens and BM in cART-naïve mice. Accordingly, cART abolished
15 p24 expression in both cell populations (Fig. 1B-E). Meanwhile, the CD4⁺ T cell level in cART-
16 treated mice was significantly recovered, whereas a marked deletion of CD4⁺ T cells was
17 observed in cART naïve-treated mice (Fig. S1A-C). The increase of CD4⁺ T cell percentage
18 correlated with a dramatic recovery of CD4⁺ T cell number in lymphoid tissues (Fig. S1D). As we
19 reported previously, cART-resistant replication-competent HIV-1 reservoirs persist (Fig. S2A),
20 though cell-associated HIV-1 RNA and DNA was remarkably decreased by cART in lymphoid
21 tissues (Fig. S2B and C) (28, 29), and viremia rebounded upon cART cessation (Fig. 1A). However,

1 the detection of p24 in monocytic cells could be due to their phagocytosis of HIV-1 infected
2 CD4⁺ T cells(30). In order to investigate whether monocytic cells could be infected by HIV-1, and
3 more importantly, play a role as HIV-1 reservoirs during cART, we purified human CD3⁻
4 CD11c⁺CD14⁺ monocytic cells and CD3⁺CD8⁻ (CD4) T cells from bone marrow cells in animals
5 with undetectable HIV-1 p24 positive cells by flow cytometry. We first measured cell-associated
6 HIV-1 DNA by real-time PCR. In addition, purified monocytic cells was stimulated with TNF- α for
7 24 hours and then co-cultured with MOLT-4 cells to detect replication-competent virus. Total
8 bone marrow cells from the same animals were used as controls. The results revealed that
9 monocytic cells harbored more HIV-1 DNA per million cells on average than CD4 T cells (Fig. 1F).
10 Notably, replication-competent viruses could be recovered from monocytic cells (Fig.1G),
11 suggesting monocytic cells as well as CD4 T cells harbored replication-competent HIV-1 during
12 cART. Therefore, HIV-1 persistent infection and cART-resistant reservoirs observed in
13 humanized mouse model resembles a situation observed in HIV-1 patients. Both CD4⁺ T cells
14 and monocytic cells can serve as HIV-1 reservoirs, contributing to HIV-1 persistence during
15 suppressive cART.

16

17 **Bromodomain inhibitor activates HIV-1 replication under suppressive cART in vivo**

18 Bromodomain inhibitors have been shown to activate HIV-1 gene expression in different cell
19 systems in vitro with latent or chronic HIV-1 infection (24-27). However, little is known about
20 how bromodomain inhibitors affect HIV-1 reservoirs in vivo during cART. In order to investigate
21 the effect of BETi on viral reservoirs in vivo, we first characterized the pharmacokinetic (PK)
22 properties of I-BET151 in NSG mice. We showed that, at dose of 18mg/kg, the concentration of

1 I-BET151 was maintained for at least 8 hours in blood and dropped below EC_{50} around 24 hours
2 after the initial administration through gavage (Fig. S3). Thus, humanized mice were treated
3 with I-BET151 once every 20 hours in the following experiments. To assess the capacity of I-
4 BET151 in activating HIV-1 reservoir cells, HIV-1 infected hu-mice were treated with I-BET151 at
5 21wpi by daily gavage after viremia was completely suppressed for 4 weeks by cART. HIV-1
6 viremia rebound was detected on day 9 (22.3wpi) after I-BET151 treatment (Fig. 2A). To catch
7 the putative HIV-1 reservoir cells responding to I-BET151 treatment, animals were terminated
8 immediately after viremia rebound was detected. Leukocytes from spleens and bone marrow as
9 well as tissues from liver and lung were harvested and subjected to the detection of cell-
10 associated HIV-1 RNA by TaqMan real-time PCR. It demonstrated that blood viral load rebound
11 was accompanied with a higher level of cell-associated HIV-1 RNA in human cells in different
12 organs or tissues (Fig. 2B). However, there was no virus spreading or secondary infection
13 occurred which is evidenced by a similar level of HIV-1 DNA in human cells in tissues upon the
14 activation of viral replication after BETi treatment (Fig. 2C). Therefore, I-BET151 treatment
15 activates HIV-1 gene expression, without spreading new infection, during cART in vivo.

16

17 **I-BET151 significantly activates HIV-1 replication in monocytic reservoir cells**

18 In order to define the HIV-1 reservoir cells that were activated by BETi, we investigated what
19 cell types were responding to I-BET151 treatment in vivo. Surprisingly, we did not observe any
20 activation of p24 production in CD4 T cells (Fig. 3A and B), but a significant percentage of
21 monocytic cells became p24-positive after I-BET151 treatment (Fig. 3C and D). And we did not
22 observe significant p24 expression in other cell types (Data not shown). To confirm this finding,

1 we performed immune-fluorescence staining on spleen tissue sections. Either human CD3/p24
2 or CD14/p24 were co-stained by specific antibodies. Consistently, we found p24 staining was
3 co-localized with CD14 positive cells in I-BET151 group but not with CD3 positive cells. In
4 comparison, no p24 positive cells were detected in cART-only mice, indicating an effective
5 suppression of HIV-1 replication by cART (Fig. 3E). Accordingly, when we normalized cell-
6 associated HIV-1 RNA to the level of human CD14 gene, a more significant increase of HIV-1
7 RNA level was observed as compare to normalization to human CD4 gene (Fig. 2B and Fig. S4).
8 We calculated CD3⁻CD11c⁺CD14⁺ monocytic cell numbers in the spleen, and found I-BET151 did
9 not alter the number of cells in treated group (Fig. 3F). However, the p24 positive monocytic
10 cell number was significantly increased by I-BET151 treatment (Fig. 3G). Similar findings were
11 also observed in the bone marrow (Fig. S5). In a separate experiment with sustained cART, the
12 elevated viral load induced by I-BET15 treatment could be inhibited again when I-BET151 was
13 stopped (Data not shown), indicating that the observed rebound of viral RNA production was
14 not due to the emergence of drug-resistant mutant viruses. In addition, neither percentage nor
15 total number of CD4⁺ T cells or huCD45⁺ cells in I-BET151-treated animals was affected in
16 comparison to non-treated mice (Fig. S6). Thus, I-BET151 treatment did not mediate significant
17 cytotoxicity in vivo in this study. Together, our results suggest that I-BET151 treatment
18 preferentially activates HIV-1 replication in CD3⁻CD11c⁺CD14⁺ monocytic cells in comparison
19 with CD4⁺ T cells under suppressive cART in vivo.

20

21 **I-BET151 activates HIV-1 reactivation more efficiently in monocytic cells than in T cells**

1 It has been reported that BETi activates HIV-1 gene transcription in latent models of both T cells
2 and monocytic cells in vitro (26, 27). To confirm our finding, we transduced either resting CD4⁺
3 T cells or monocyte derived macrophages (MDMs) with VSV-G HIV Duo-Fluo, which can
4 distinguish productive infection from latent infection. This reporter virus encodes two separate
5 fluorescent markers: an LTR-driven eGFP marker (productive infection) and an LTR-independent
6 mCherry marker driven by an EF1 α promoter (31, 32). After transduction, cells were cultured
7 for 24 hours before I-BET151 treatment. mCherry and GFP expression was determined at 72
8 hours for CD4⁺ T cells and 36 hours for MDMs after culture with I-BET151(Fig. S7), at the
9 concentration of 0.5 μ M at which I-BET151 induce minor cytotoxicity as tested on lymphocytic
10 or monocytic cell lines (Fig. S8). We chose SAHA, an HDAC inhibitor reported to activate HIV LTR
11 transcription in both cell lines and primary CD4 T cells (33-35), as positive control. The results
12 showed that I-BET151 was unable to significantly enhance HIV-1 transcription in resting CD4⁺ T
13 cells (Fig. 4A and B). In contrast, HIV-1 transcription in MDMs was significantly increased by I-
14 BET151, as evidenced by the increase in the percent of GFP positive cells observed directly
15 under fluorescence microscope, and confirmed by flow cytometry analysis (Fig. 4C-E).

16 To further compare the relative efficiency of HIV-1 transcription activation by I-BET151 in
17 T cells and monocytic cells, we performed experiments using lymphocytic clone (ACH-2) or
18 monocytic clone (U1) with latent HIV-1 provirus. The titration results demonstrate that I-
19 BET151 activated HIV-1 transcription in both ACH2 cells and U1 cells in a dose dependent
20 manner. However, I-BET151 enhanced viral gene transcription in U1 cells at about 5-to-10 fold
21 more efficiently than in ACH-2 cells using the same dose (Fig. 5A and 4B). An approximate 3-fold
22 difference in EC₅₀ was observed as calculated based on the viral transcription levels (Fig. 5C).

1 We speculated that the quiescent status of HIV-1 provirus may contribute to this
2 discrepancy observed in different cell types. Thus, we inoculated U937 cells and Jurkat cells
3 with the dual reporter virus and sorted the mCherry⁺GFP⁻ cells (latent infection). After culturing
4 for 4 days to expand the cells, we treated Jurkat-DuoFluo or U937-DuoFluo cells with I-BET151
5 (0.5 μ M) for 48 hours. The activation of HIV transcription was indicated by the percent of GFP
6 positive cells after treatment. The results demonstrated that U937-DuoFluo was more sensitive
7 to I-BET151 treatment as 64% cells became GFP positive comparing to 24% in Jurkat-DuoFluo
8 (Fig. 5D and E). These results suggested that HIV-1 transcription in latent or low replicating
9 monocytic cell reservoirs could be sufficiently activated or enhanced by I-BET151 as compared
10 to latent T cell reservoirs.

11

12 **I-BET151 activates HIV-1 gene expression in monocytic cells via CDK2/CDK9-dependent** 13 **mechanisms**

14 Inhibition of either cyclin-dependent kinase (CDK) 2 or CDK9 has been reported to suppress
15 HIV-1 transcription or replication in vitro or in vivo(36-41). Previous reports show that
16 bromodomain inhibitors induced reactivation of HIV-1 latency is dependent on CDK9 (24, 25).
17 HIV-1 replication, in CDK2-knockdown macrophages-like cells derived from pluripotent stem
18 cells, was significantly reduced(42). To further study the mechanism of I-BET151-mediated HIV-
19 1 transcription in monocytic cells and CD4⁺ T cells, we first established cell lines expressing
20 luciferase under the control of HIV-1 LTR using either U937 (U937-luc) or Jurkat (Jurkat-luc) cells.
21 We treated cells with I-BET151 in the presence of either CDK2 inhibitor (K03861)(43) or CDK9
22 inhibitor (LDC000067), and cultured for 48 hours before luciferase activity detection. In U937-

1 luc cells, either CDK2 or CDK9 inhibitor alone could down-regulate HIV-1 transcription relative
2 to mock cells. When I-BET151 was introduced, the enhancement of HIV-1 transcription was
3 abolished by either CDK2 (1 μ M) or CDK9 (25 μ M) inhibitors (Fig. 6A). Interestingly, CDK2
4 inhibitor failed to inhibit I-BET151-induced HIV-1 transcription, even increased HIV-1
5 transcription in Jurkat-luc cells. While CDK9 inhibitor was able to down-regulate HIV-1
6 transcription and inhibit the activity of I-BET151 to enhance HIV transcription in Jurkat-luc cells
7 (Fig. 6B). These results suggested that the different efficacy of I-BET151 in activating HIV-1
8 transcription in monocytic cells and CD4⁺ T cells is attributed to cell-intrinsic difference in the
9 regulation of HIV-1 transcription by CDK2.

10

11

1 DISCUSSION

2 Despite the importance of HIV-1 reservoirs in resting CD4⁺ T cells, it is impossible to achieve a
3 cure without considering reservoirs cells like macrophages in brain tissues (44, 45). The
4 existence of HIV-1 macrophages reservoirs in vivo is strongly supported by Honeycutt et al (17),
5 who demonstrated that HIV-1 replication can be sustained in macrophages in a humanized
6 mice model without T cells. Consistently, our study demonstrates that monocytes or
7 macrophages are important HIV-1 reservoir cells during suppressive cART. Importantly,
8 bromodomain inhibitors could be a novel measure adopted to activate viral replication in this
9 particular reservoir in vivo.

10 It is known that HIV-1 reservoir cells can be exist in two different statuses in vivo. One is
11 harboring latent replication-competent proviruses expressing little to no viral products. The
12 long-lived resting memory CD4⁺ T cells is the putative latent reservoir cells mostly studied to
13 date (46-48). The other kind of reservoir cells is the residual active reservoir cells which
14 consistently produce low level of viruses, because of either sanctuary sites that have low
15 accessibility to cART, or the cell types that less responsive to certain anti-retroviral drugs such
16 as monocytes or macrophages(49-51). The very recent report proved the existence of lymphoid
17 tissue sanctuary sites for harboring low level of viral replication in patients with undetectable
18 viremia (52). And the brain resident macrophages have been considered to play a pivotal role in
19 maintaining HIV-1 persistent infection since cART in brain is not as effective as in other tissues
20 (44, 45). In our study, we cannot distinguish if the increased level of HIV-1 replication in
21 monocytic cells is due to activation of HIV provirus or enhancement of residual low level of viral

1 replication. However, our findings are valuable for HIV reservoir study and development of
2 strategies targeting to different persistently infected cells during cART.

3 In this study, no reduction of cell-associated HIV-1 DNA in human cells was observed solely by
4 I-BET151 treatment on day 9 after the administration. We speculate that the poor elimination
5 of HIV-1 reservoir induced by I-BET151 in vivo is due to two reasons. First, macrophages are
6 more resistant to HIV-1 infection induced cytopathic effect comparing to CD4⁺ T cells (53, 54).
7 As I-BET151 effectively reactivates HIV-1 replication only in monocytic cells in vivo, it is likely
8 that the cells are simply not dying while producing viruses. Second, the immune system might
9 be still defective or exhausted due to chronic HIV-1 infection though viral replication being
10 suppressed by cART. Given the above possibilities, bromodomain inhibitor treatment should be
11 joint with therapy can either kill HIV-1 positive cells directly (immune-toxin) or recover immune
12 response to HIV-1 infection(29). Thus, bromodomain inhibitor therapy requires more
13 interventions to synergistically purge HIV-1 reservoir cells.

14 P-TEFb is composed of Cyclin T1 and CDK9, and converts promoter-proximally paused RNA
15 polymerase II (RNAPII) complexes into efficient elongating complexes(23). In the absence of
16 HIV-1 trans-activator Tat, RNAPII pauses after transcription of the TAR sequence. Tat recruits P-
17 TEFb via CycT1 to TAR, allowing CDK9 to phosphorylate the C-terminal domain (CTD) of RNAPII
18 and continues viral gene transcription thereafter (55). It has been reported that CDK2 can
19 phosphorylate either CDK9 or Tat, and thereby contributes to enhanced HIV-1 transcription(56,
20 57). These findings suggest both CDK9 and CDK2 are involved in the regulation of HIV-1
21 transcription. However, CDK2 expression is low or undetectable in resting CD4⁺ T

1 lymphocytes(58), which is likely to be associated with the failure of CDK2 inhibitor to exert
2 suppression on viral transcription with or without I-BET151 treatment in our study. To further
3 understand the role of CDK2 in the regulation of HIV-1 gene expression and latency in
4 monocytic cells will be beneficial for future HIV-1 cure study.

5 Although the mechanisms contributing to HIV-1 transcription quiescence have
6 investigated extensively, we still do not fully understand the relative importance of each
7 mechanism in different cell types. As we have recently reported, depletion of regulatory T cells
8 during cART in humanized mice leads to reactivation of HIV-1 replication predominantly in
9 memory CD4⁺ T cells(28). In addition, it is of particular importance to identify all possible HIV-1
10 reservoir cells in lymphoid organs in vivo. To investigate how HIV-1 maintains its low replication
11 or latency in different cell type will help guide future drug design.

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1 **MATERIALS AND METHODS**

2 **Construction of humanized mice**

3 Human fetal liver tissues were obtained from elective or medically indicated termination of
4 pregnancy through a non-profit intermediary working with outpatient clinics (Advanced
5 Bioscience Resources). Approval for animal work was obtained from University of North
6 Carolina (UNC) Institutional Animal Care and Use Committee (IACUC). We constructed hu-NRG
7 mice as previously reported (28, 59-63).

8 **HIV-1 infection of humanized mice**

9 Humanized mice were infected by intravenous (i.v.) injection with HIV-1_{JRCSF} stocks (10 ng
10 p24/mouse in 50 μ l) or with mock stocks in control mice.

11 **cART regimens in NRG-hu HSC mice**

12 Individual tablets of TRUVADA (tenofovir/emtricitabine; Gilead Sciences) or raltegravir (Merck)
13 were crushed into fine powder and manufactured as 5BXL by TestDiet based on previously
14 published (29, 64).

15 **Luciferase assay**

16 Cells were transduced with VSV-G HIV-luc, in which luciferase gene expression is under control
17 by HIV LTR, at dose of 1 MOI. After culture for 6 days, cells were treated with CDK2 or CDK9
18 inhibitor alone, or in the presence of I-BET151. At 48 hours after treatment, cells were washed
19 with PBS and lysed in 100 μ l passive lysis buffer (Promega). Cell lysis were transferred into 96
20 well plates and luciferase activity was detected by adding 50 μ l substrate into each well and
21 read by 96 microplate luminometer (GLOMAX).

1 **Cell sorting and culture**

2 For primary cells, bone marrow cells from humanized mice were harvested and submit to cell
3 sorting by flow cytometer (ARIA II, BD). Human CD45⁺CD3⁺CD8⁻ cells and CD45⁺CD3⁻
4 CD11c⁺CD14⁺ cells were sorted. For cell lines, U937 and Jurkat cells were first transduced with
5 VSV-G Duo-Fluo reporter virus at dose of 1 MOI and expand for 6 days. GFP-mCherry⁺ cells
6 were sorted by ARIA II (BD) and expand for 6 days in 10% FBS RPMI-1640 before experiment.
7 The purity of sorted cells was all over 99% (Data not shown).

8 **Quantification of HIV viral load in plasma**

9 Peripheral blood was collected with EDTA as an anti-coagulant at indicated time points after
10 HIV infection. Plasma was prepared by centrifugation and stored at -80°C until assay. Viral RNA
11 was isolated from the plasma. HIV viral road was measured by qRT-PCR as described
12 previously(28, 29, 60).

13 **Cell-associated HIV-1 DNA**

14 Total nucleic acid was extracted from cells or tissues using DNeasy mini kit (Qiagen). HIV-1 DNA
15 was quantified by real-time PCR. Genomic DNA of ACH2, which contains one copy of HIV
16 genome in each cell, was serially diluted in mouse leukocytes DNA to generate a standard
17 curve(29).

18 **Cell-associated HIV-1 RNA**

19 Total RNA was extracted from cells or tissues using RNeasy plus mini kit (Qiagen). HIV-1 RNA
20 was detected as previously described(28, 29, 60). The HIV-1 gag RNA expression were
21 normalized to human CD4 or CD14 mRNA level and relative HIV-1 gene expression levels were
22 calculated according to $2^{-\Delta\Delta CT}$ (29, 65).

1 **Cell lines and culture**

2 ACH-2, Jurkat, U-937 and U1 cells were obtained through the National Institutes of Health (NIH)
3 AIDS Research and Reference Reagent Program, Division of AIDS. Cells were grown in RPMI
4 1640 (Gibco) with 10% fetal bovine serum (FBS) (Invitrogen), 5% penicillin-streptomycin
5 (Invitrogen), and 2 mM glutamine (Invitrogen). Cells were maintained at a concentration of 10^6
6 cells/ml in T-175 flasks. Cell concentrations and cell viability were monitored throughout the
7 experiment at all time points studied.

8 **Virus outgrowth assay**

9 Splenocytes of humanized mice (1×10^6 , 2×10^5 , 4×10^4 human cells in single, duplicates or
10 triplicates) were stimulated with PHA ($2 \mu\text{g/ml}$) and IL-2 (100 units/ml) for 24 hours.
11 MOLT4/CCR5 cells were added on day 2 to enhance the survival of leukocytes and to support
12 HIV-1 replication. Culture medium containing IL-2 and T cell growth factor (29) was replaced on
13 days 5 and 9. After 7 and 14 days of culture, supernatant was harvested individually and HIV-1
14 RT-qPCR was performed to score viral outgrowth. Estimated frequencies of cells with
15 replication-competent HIV-1 were calculated using limiting dilution analysis.

16 For sorted cells, five hundred of each sorted cells were cultured in the presence of $\text{TNF-}\alpha$
17 (5 ng/ml) in 100 μl RPMI-1640 medium complemented with 10% FBS for 24 hours in U bottom
18 96-well plate and then 5×10^4 MOLT4/CCR5 cells in 100 μl same medium were added into each
19 well, and co-culture for 14 days with half medium change every 3 days. 150 μl supernatant were
20 used for measuring viral load by TaqMan real-time PCR.

21 **Primary cell isolation**

1 Human blood buffy coats were ordered from Gulf Coast Regional Blood Center and PBMCs
2 were separated with Ficoll (Ficoll-Paque™ PLUS, GE Healthcare). Resting CD4⁺ T cells were
3 purified by negative selection using a CD4 separation kit (Miltenyi Biotec) according to the
4 manufacturer's instructions. CD25⁻CD4⁺ T cells were subsequently isolated from CD4⁺ T cells by
5 negative selection using CD25 beads (Miltenyi Biotec). CD14⁺CD16⁻ monocytes were isolated
6 using the EasySep™ Human Monocyte Isolation Kit (STEMCELL). All cell purifications were
7 performed according to manufactures' instruction. Purity of CD25⁻CD4⁺ T cells was checked by
8 stain with anti-CD4-FITC and anti-CD25-PE (Biolegend) Purity of monocytes was checked by
9 stain with anti-CD3-FITC and anti-CD14-PE (Biolegend). Cell purity was analyzed by Guava
10 EasyCyte™ 8HT (EMD Millipore). The purity of each isolated population was over 90%(data not
11 shown).

12 **Primary cell transduction and culture**

13 CD4⁺ T cells were transduced with VSV-G Duo-Fluo reporter virus at 0.1 MOI and cultured in
14 RPMI-1640 complemented with 10% FBS and IL-2 (10u/ml) for 24 hours before I-BET151
15 treatment. Monocytes-derived macrophages (MDMs) were obtained by culturing monocytes
16 with M-CSF (50ng/ml) and GM-CSF (50ng/ml) in RPMI-1640 with 10% FBS for 7 days. MDMs
17 were harvested and transduced with VSV-G Duo-Fluo reporter virus at 0.1 MOI and cultured for
18 24 hours before I-BET151 treatment.

19 **Inhibitors and treatment**

20 Bromodomain inhibitor I-BET151 was obtained from GlaxoSmithKline (Research Triangle Park,
21 NC). Drug powder was dissolved in 0.5%HPMC:0.1%Tween80 to a final concentration of 1.5

1 mg/ml and pH 5. Administration of the drug was through daily oral gavage at dose of 18mg/kg.
2 The solvent without drug was used as placebo. Highly potent and specific CDK2 inhibitor
3 (K03861) and CDK9 inhibitor (LDC000067) (Selleckchem) were dissolved in DMSO and diluted to
4 the indicated concentration in medium before use.

5 **Flow cytometry**

6 For HIV-1 gag p24 staining, cells were stained with surface markers first, and then
7 permeabilized with cytofix/cytoperm buffer (BD Bioscience, cat#554714), followed by
8 intracellular staining. PE-conjugated anti-human CD11c (clone:3.9, cat#301606), PE/Cy5-
9 conjugated anti-human CD4 (clone:RP4-T4, cat#300510), PE/Cy7-conjugated antihuman CD3
10 (clone:HIT3a, cat#300316), Pacific blue-conjugated anti-human CD14 (clone:HCD14,
11 cat#325616), and APC/Cy7-conjugated anti-human CD45 (clone:H130, cat#304014) were
12 purchased from Biolegend; Pacific orange-conjugated anti-mouse CD45 (clone:HI30,
13 cat#MHCD4530), PE-TR-conjugated CD8 (clone:3B5, cat#MHCD0817), and LIVE/DEAD Fixable
14 Aqua Dead Cell Stain Kit (cat#L34957) were purchased from Invitrogen. FITC-conjugated anti-
15 HIV p24 (clone: FH190-1-1, cat#6604665) was purchased from Beckman Coulter. Flow
16 cytometry was performed using either CyAn ADP (DAKO) or BD LSRFortessa (BD Biosciences),
17 analyzed by Summit 4.3 (Beckman Coulter) or FlowJo 10 (FLOWJO, LLC), accordingly.

18 **Immunofluorescence staining of spleens**

19 Mice spleens were harvested, fixed with 10% formalin (Fisher, Fair Lawn, NJ), embedded in
20 paraffin and cut into 5µm tissue sections. Antigen retrieval was performed by incubation in Diva
21 Decloacker (Biocare Medical, Concord, CA) for 30 min at 95°C, followed by slow cooling down
22 for one hour. The tissue section was blocked with Background Sniper (Biocare Medical, Concord,

1 CA). The sections were then stained with the primary antibodies: rabbit monoclonal anti-human
2 CD3 (Life Span Bio Sciences, Seattle, WA; 1:100 dilution) or rabbit monoclonal anti-human CD14
3 (Abcam; 1:500 dilution) or mouse monoclonal anti-HIV-1 p24 (Dako, 1:5 dilution) diluted in
4 blocking buffer (PBS, 0.05% Tween 20, 5% goat serum), and then secondary antibodies: Alexa
5 Fluor 594 Donkey Anti-Mouse IgG (Life Technologies, Eugene, OR) and Alexa Fluor 488 Donkey
6 Anti-Rabbit IgG (Life Technologies, Eugene, OR). The sections were stained with DAPI and
7 mounted with an anti-fade mounting media (Abcam Cambridge, MA). Sections were analyzed
8 by confocal microscopy (Zeiss LSM 700 Confocal Laser Scanning Microscope).

9 **Statistical analysis**

10 Unpaired 2-tailed Student t tests and OneWay Anova analysis of variance with the Bonferroni
11 multiple comparison test were performed using GraphPad Prism (GraphPad Software, San
12 Diego, CA). P value of < 0.05 was considered statistically significant. All data were reported as
13 mean \pm SD (Standard deviation).

14

15

1 **FIGURE LEGEND**

2 **FIG 1. cART-resistant HIV-1 reservoirs in T cells and monocytic cells in humanized mice.**

3 Humanized mice were infected with HIV_{JRCSF}. cART was initiated at 4 weeks after infection. Mice
4 were terminated at 12 weeks after infection. Spleen and bone marrow cells were harvested for
5 flow cytometry analysis. huCD45+CD3+CD8⁻ (CD4 T cells including cells with HIV-induced CD4
6 downregulation) cells and huCD45+CD3-CD11c+CD14⁺ cells were purified by flow cytometer.
7 Sorted cells were used for either PCR detection or virus out growth assay (VOA). (A) Plasma vial
8 loads were measured weekly or every other week. (B) Histograms show percentage of HIV gag
9 p24⁺ CD4 T cells (huCD45+CD3+CD8⁻) in the spleen and bone marrow. (C) Summarized percent
10 p24⁺ cells of CD4⁺ (CD3+CD8⁻) T cells in the spleen and bone marrow. (D) Histograms show
11 percent p24⁺ of monocytic cells (huCD45+CD3-CD11c+CD14⁺) in the spleen and bone marrow.
12 (E) Summarized percent p24⁺ of monocytic cells in the spleen and bone marrow. (F) Relative
13 HIV-1 DNA (copy per cell) in either CD4 T cells or monocytic cells. (G) Total bone marrow cells or
14 purified monocytic cells were treated with TNF- α and co-cultured with MOLT-4 cells for 14 days.
15 HIV-1 RNA in supernatants on day 14 were detected. Bars in dot graphs indicate mean value.
16 Error bars indicate standard deviations (SD). * indicates p<0.05.

17

18 **FIG 2. I-BET151 treatment activates HIV-1 replication under suppressive cART in vivo.**

19 Humanized mice infected with HIV_{JR-CSF} and were treated with cART initiated at 13 wpi. Mice
20 were terminated at 22.3 wpi. (A) HIV-1 plasma viremia. (B) Relative cell-associated HIV-1 RNA
21 levels in human cells or tissues (spleen/SP, bone marrow/BM, liver/LV and lung/LU), normalized
22 to human CD4 mRNA levels. (C) Cell-associated HIV-1 DNA copy number in human cells in

1 different tissues (spleen, bone marrow, liver and lung) was measured by real-time PCR. Bars in
2 dot graphs indicate mean value. * indicate $p < 0.05$.

3

4 **FIG 3. I-BET151 treatment specifically activates HIV-1 replication in monocytic cells under**
5 **suppressive cART in vivo.** Humanized mice were treated as in Fig. 2. (A) Histograms show
6 percent HIV gag p24+ of CD4+ (CD3+CD8-) T cells in spleens. (B) Summarized percent p24+ of
7 CD4+ T cells in spleens. (C) Histograms show percent p24+ of monocytic cells (CD3-
8 CD11c+CD14+) in spleens. (D) Summarized percent p24+ of monocytic cells in spleens. (E)
9 Immunofluorescence co-staining of CD3 (green)/gag p24 (red) (upper lane) or CD14 (green)/gag
10 p24 (red) (lower lane) in spleens. (F) Monocytic cell numbers in spleens. (G) HIV gag p24+
11 monocytic cell numbers in spleens. Bars in dot graphs indicate mean value. * indicates $p < 0.05$.

12

13 **FIG 4. I-BET151 treatment activates HIV-1 transcription in MDMs but not in resting CD4+ T**
14 **cells in vitro.** CD25- resting CD4+ T cells and monocytes-derived-macrophages (MDMs) were
15 transduced with VSV-G-G pseudotyped Duo-Fluo HIV-1 reporter virus and cultured for 24 hours
16 before I-BET151 treatment. Cells were analyzed for mCherry and GFP expression at 72 hours
17 (CD4+ T cells) or 36 hours (MDMs) after I-BET151 treatment. (A) Plots show mCherry and GFP
18 expression in CD4+ T cells. (B) Summarized percentage of GFP+ cells in CD4+ T cells. (C) GFP
19 frequency in MDMs was observed under fluorescence microscope. (D) Plots show mCherry and
20 GFP expression in MDMs. (E) Summarized percentage of GFP+ cells in MDMs. Error bars
21 indicate standard deviations (SD). * indicates $p < 0.05$.

1 **FIG 5. I-BET151 activates HIV-1 transcription in monocytic cells more efficiently than in**
2 **lymphocytic T cells.** ACH-1 T cells or U1 monocytic cells were treated with various
3 concentrations of I-BET151. Cell-associated HIV gag RNA levels were detected by real-time PCR.
4 (A) Relative viral RNA levels in ACH-2 cells at 48 hours post treatment. (B) Relative viral RNA
5 levels in U1 cells at 48 hours post treatment. (C) EC50 of BET151 on ACH-2 or U1 cells calculated
6 based on Fig. 4A or Fig. 4B. Jurkat cells or U937 cells were transduced with VSV-G-G
7 pseudotyped Duo-Fluo HIV-1 reporter virus and mCherry positive only cells were purified by
8 FACS. Sorted cells were expanded and treated with 0.5uM I-BET151 for 48 hours. (D)
9 Percentages of GFP positive cells in Jurkat-DuoFuo (upper) or U937-DuoFluo (lower). (E)
10 Summarized percentage of GFP+ cells in Fig. 4D. Error bars indicate standard deviations (SD). *
11 indicates p<0.05.

12
13 **FIG 6. I-BET151-activated HIV replication in monocytic cells is dependent on both CDK2 and**
14 **CDK9.** U937 cells and Jurkat cells were transduced with VSV-G-G pseudotyped HIV-luc reporter
15 virus. After expansion for 6 days, cells were treated with CDK2 or CDK9 inhibitor with or
16 without I-BET151 treatment. At 48 hours after I-BET151 treatment, luciferase activity in cells
17 were measured. (A) Luciferase activity in U937 cells after CDK2 or CDK9 inhibitor treatment
18 with or without I-BET151. (B) Luciferase activity in Jurkat cells after CDK2 or CDK9 inhibitor
19 treatment with or without I-BET151. Error bars indicate standard deviations (SD). * indicates
20 p<0.05.

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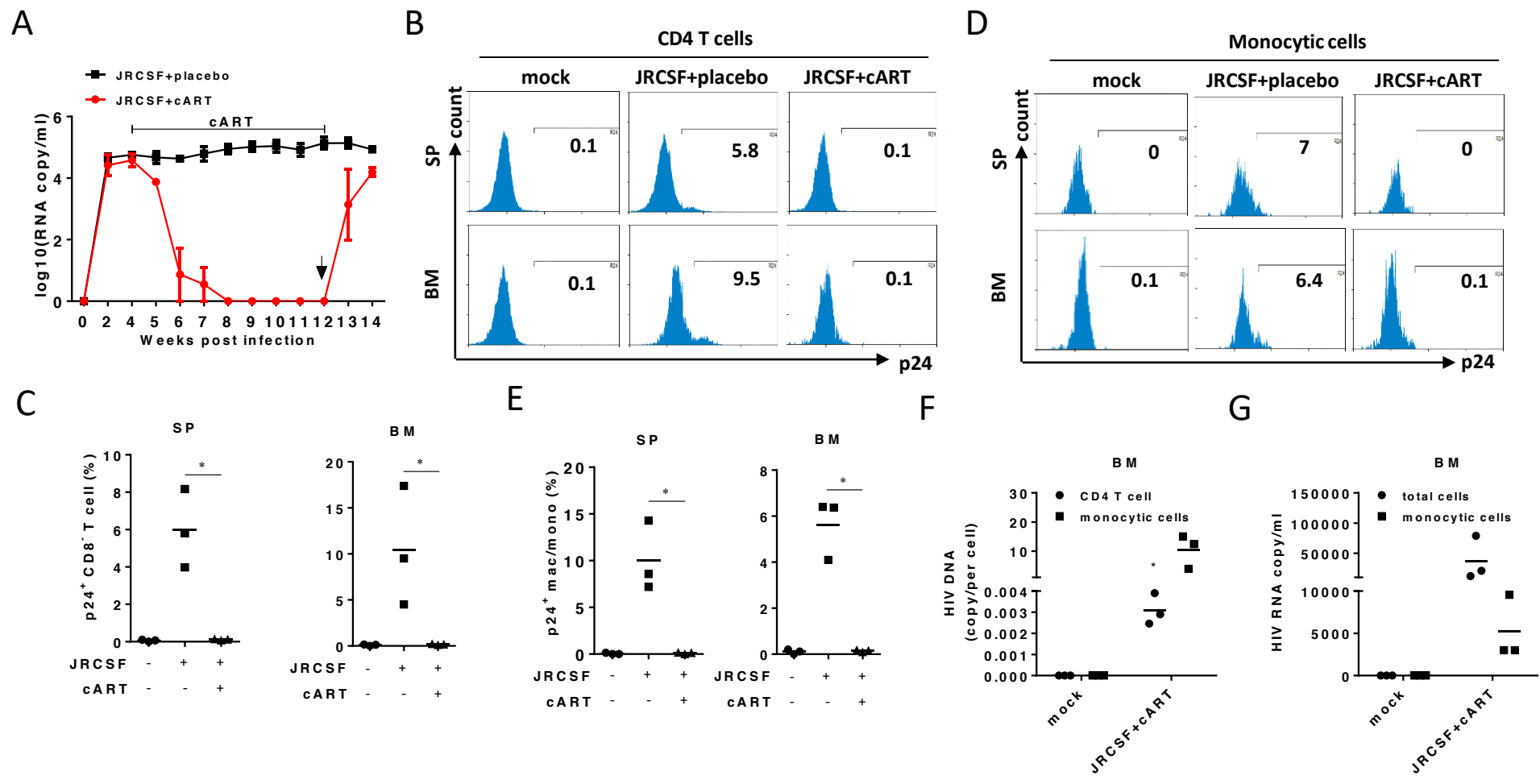
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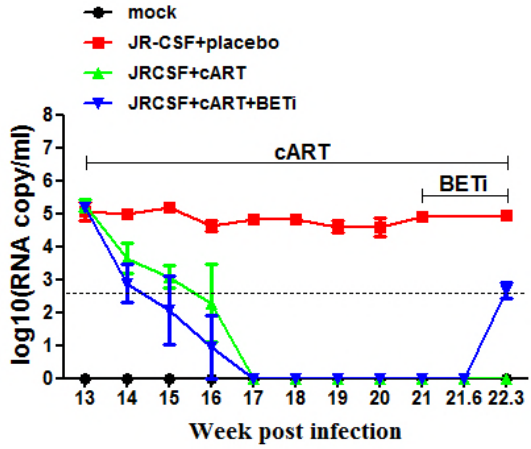
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Highlights

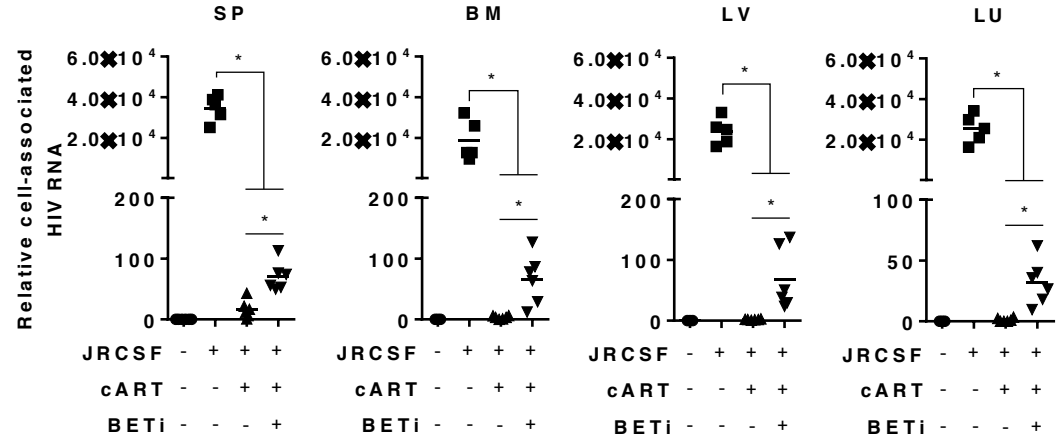
- BET-151 preferentially activate HIV-1 infection in CD3-CD11c+CD14+ monocytic cells as compare to CD4 T cells during suppressive cART in vivo .
- CD3-CD11c+CD14+ monocytic cells harbor replication-competent provirus during cART.
- BET-151 reactivate HIV-1 transcription and enhance viral replication in CD3-CD11c+CD14+ monocytic cells more than in resting CD4 T cells.
- BET151-induced activation of HIV-1 replication in monocytic cells is dependent on both CDK2 and CDK9, whereas only CDK9 is involved in the activation of HIV-1 replication mediated by BET151 in T cells.



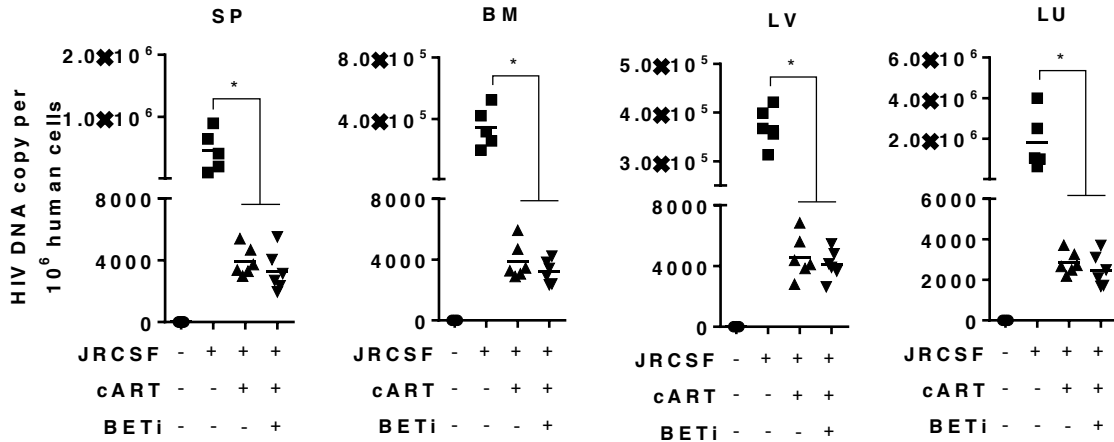
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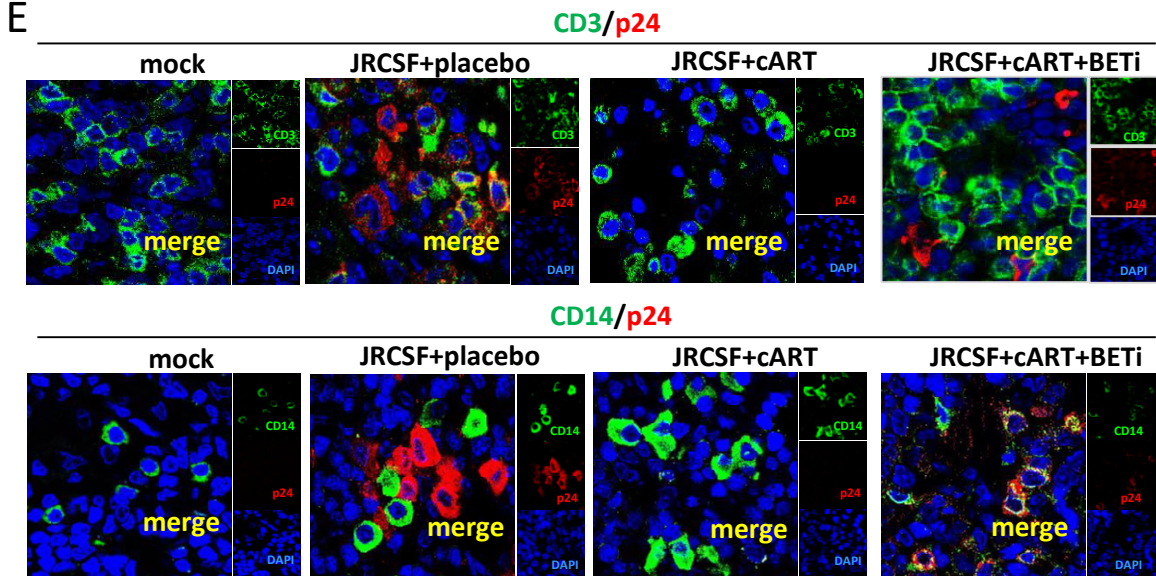
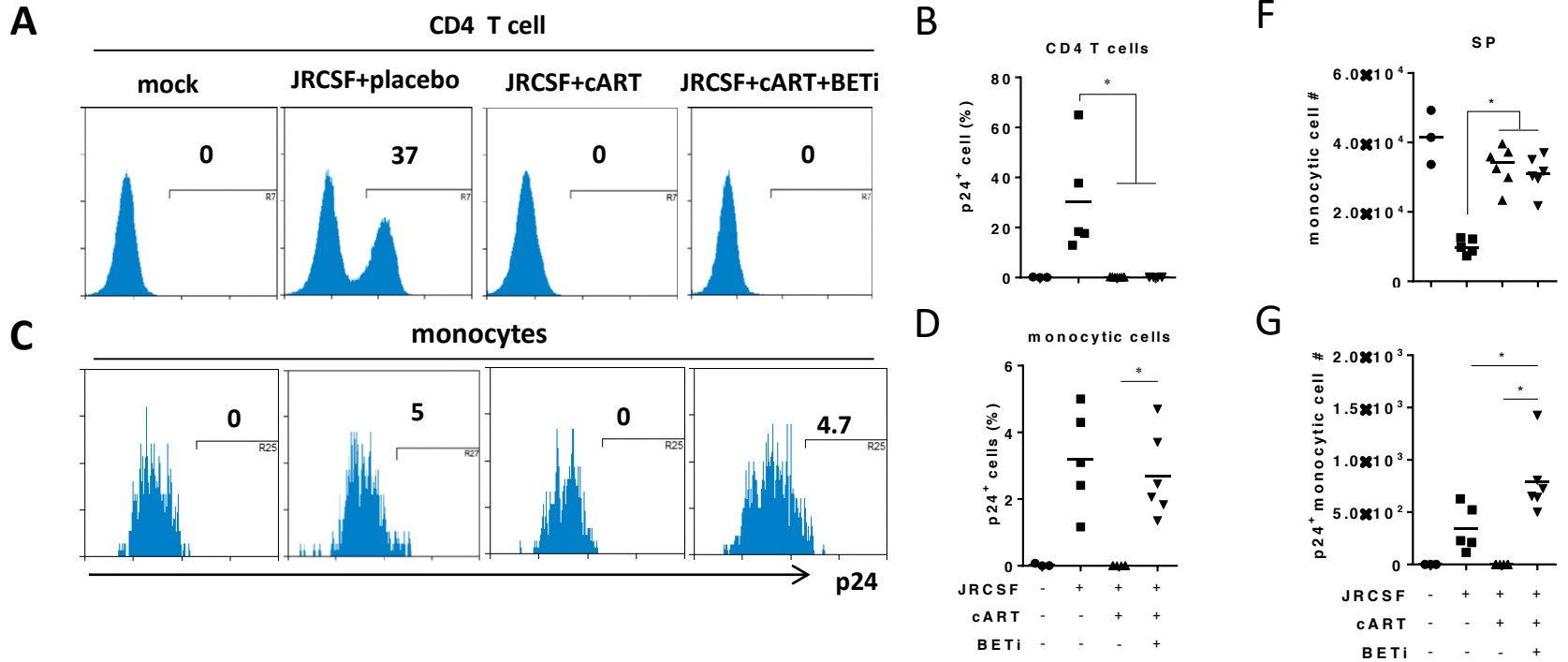


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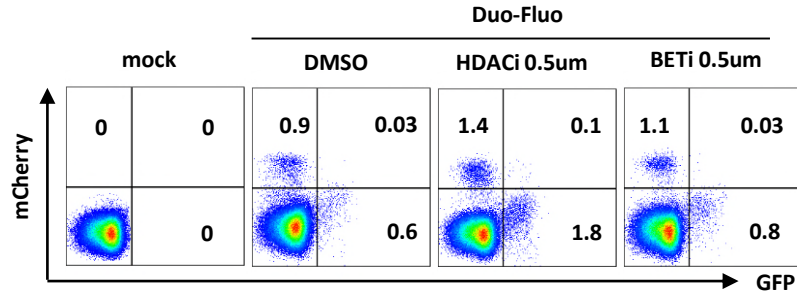


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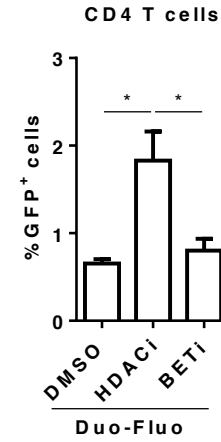




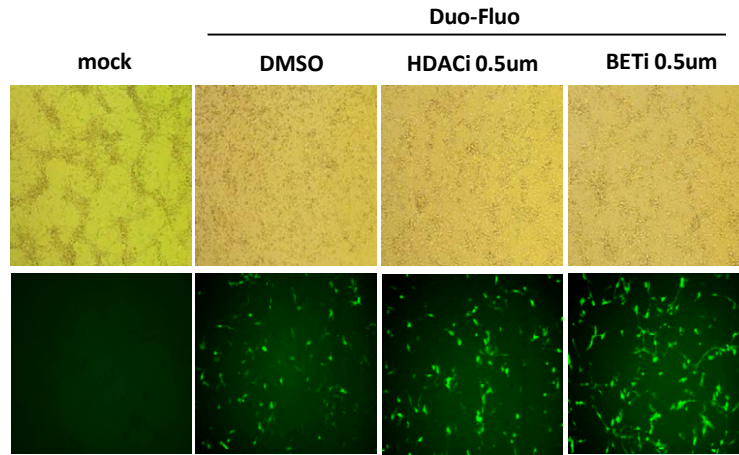
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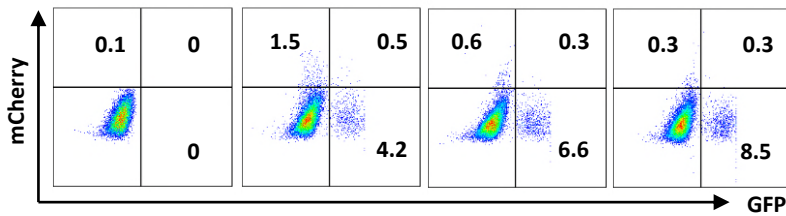
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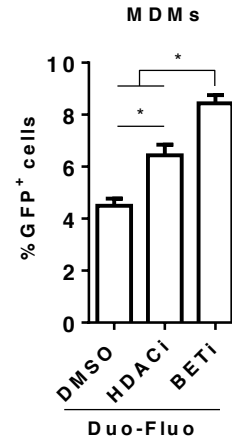
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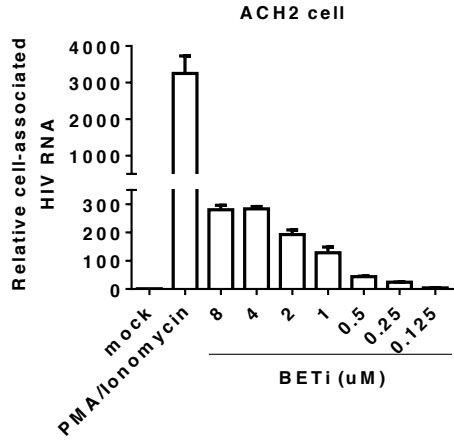
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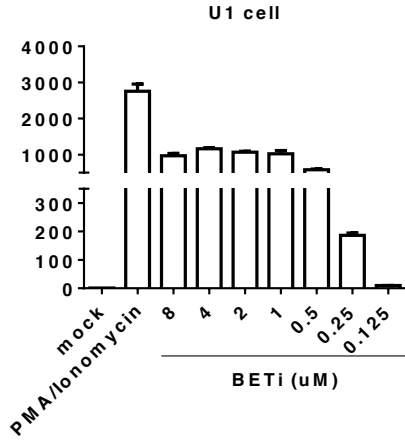
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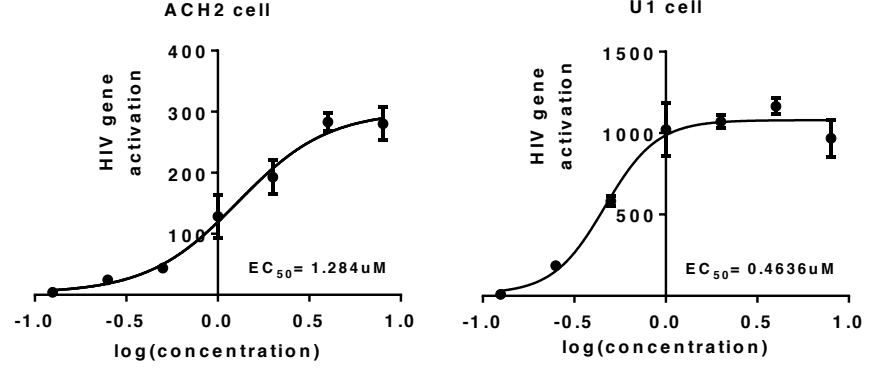
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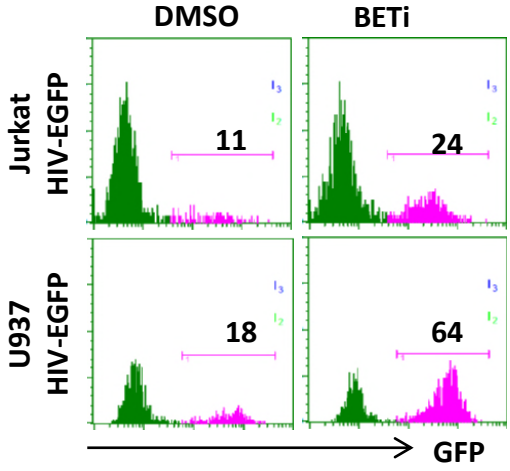
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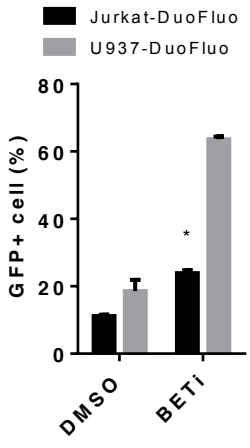
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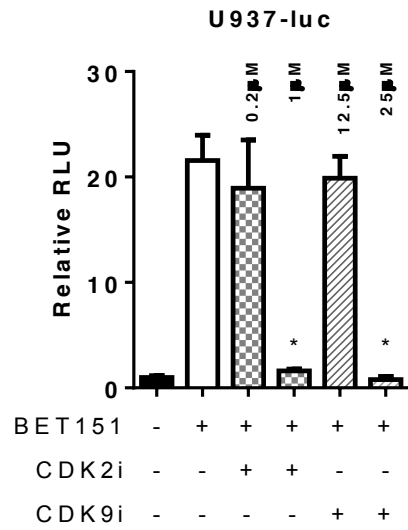
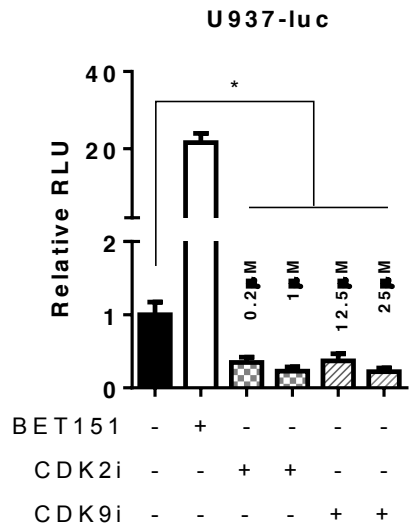
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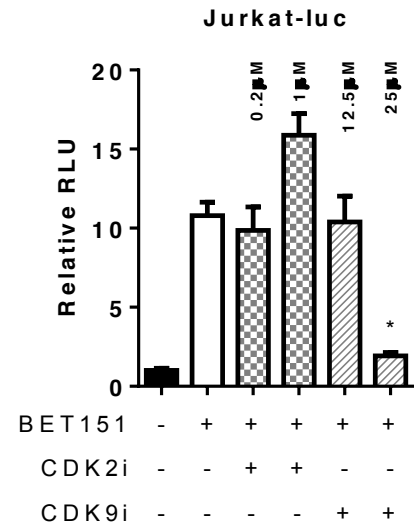
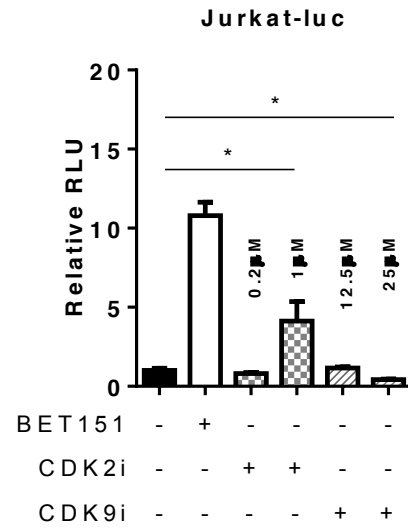
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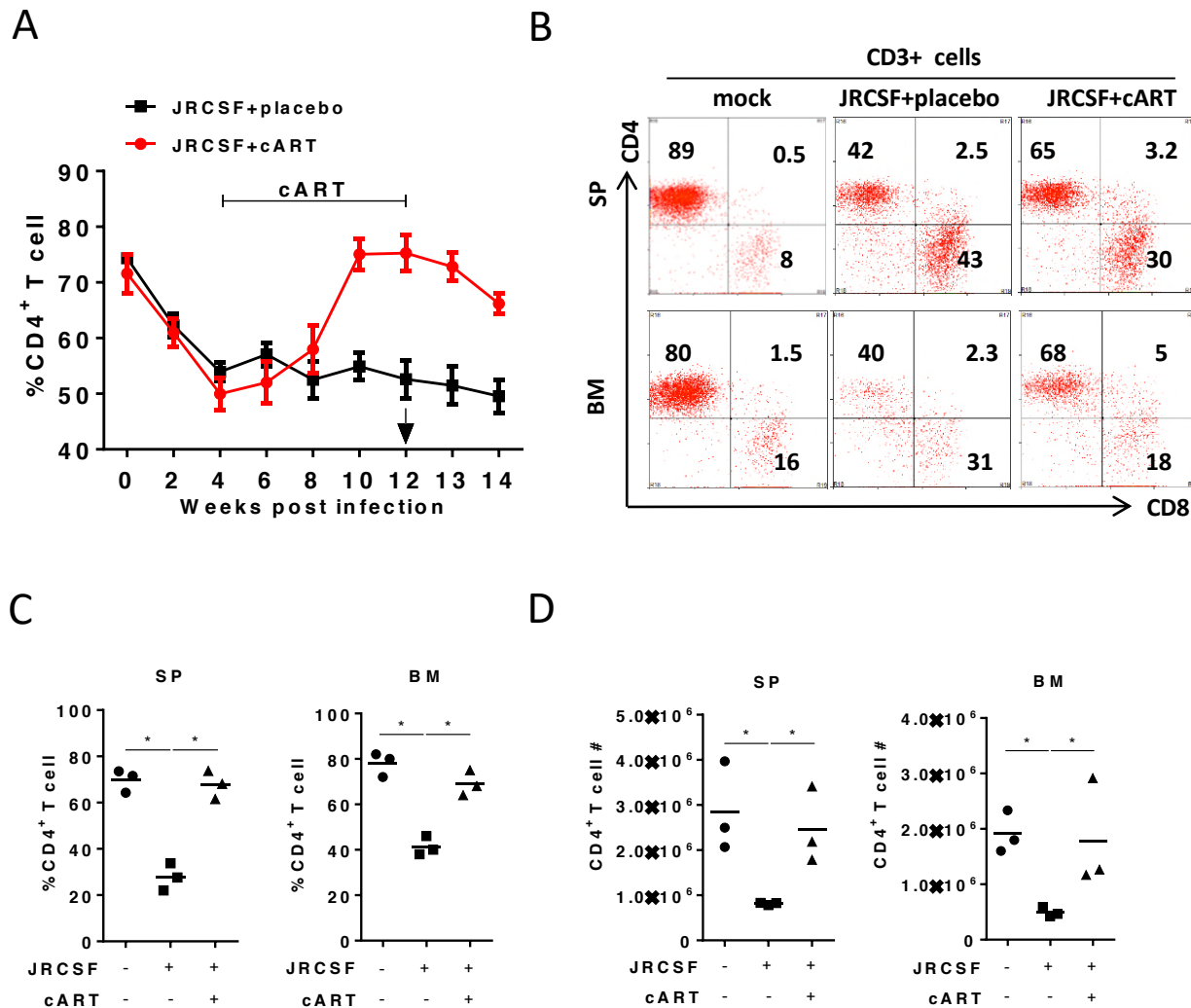


FIG S1. Humanized mice were treated and terminated as in Fig. 1A. (A) CD4+ T cell percentages in CD3+ cells over time in peripheral blood. (B) Representative plots show percentage of CD4+ T cells in CD3+ cells in spleen and bone marrow. (C) Summarized percentage of CD4+ T cells in CD3+ cells in spleen and bone marrow. (D) CD4+ T cell numbers in spleen and bone marrow. Bars in dot graphs indicate mean value. * indicate $p < 0.05$

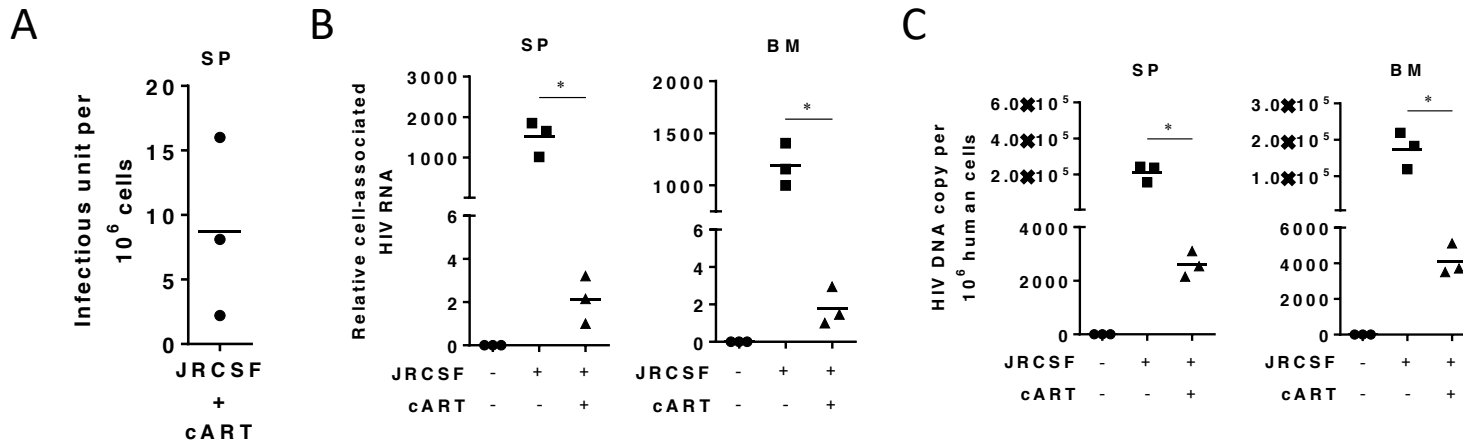


FIG S2. cART-resistant viral reservoir persist during suppressive cART in humanized mice. Humanized mice were treated and terminated as in Fig. 1A. (A) Replication-competent HIV-1 reservoir cells detected by virus outgrowth assay. (B) Relative cell-associated viral RNA in cells from spleen and bone marrow. (C) Cell-associated viral DNA in cells from spleen and bone marrow. Bars in dot graphs indicate mean value. * indicate $p < 0.05$.

PO profile of GSK1210151A in Nsg Mice

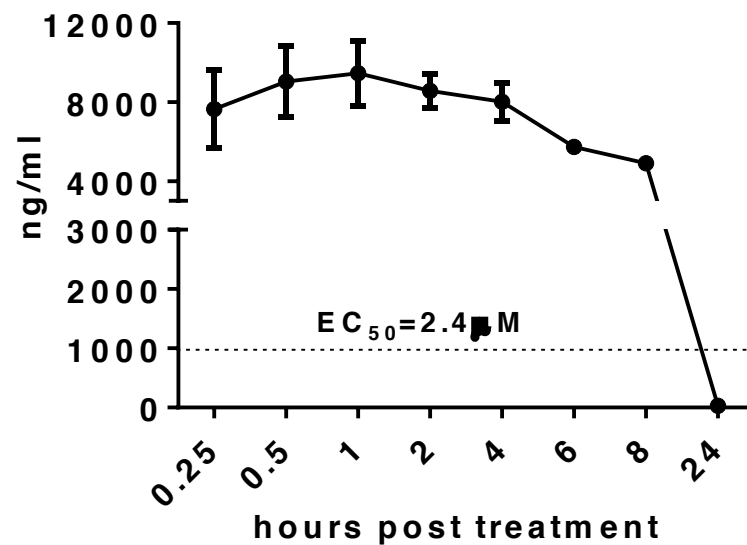


FIG S3. Pharmacokinetics of I-BET151 in NSG mice. NSG mice were treated with 18mg/kg I-BET151 through gavage and drug concentration in blood was measured by HPLC.

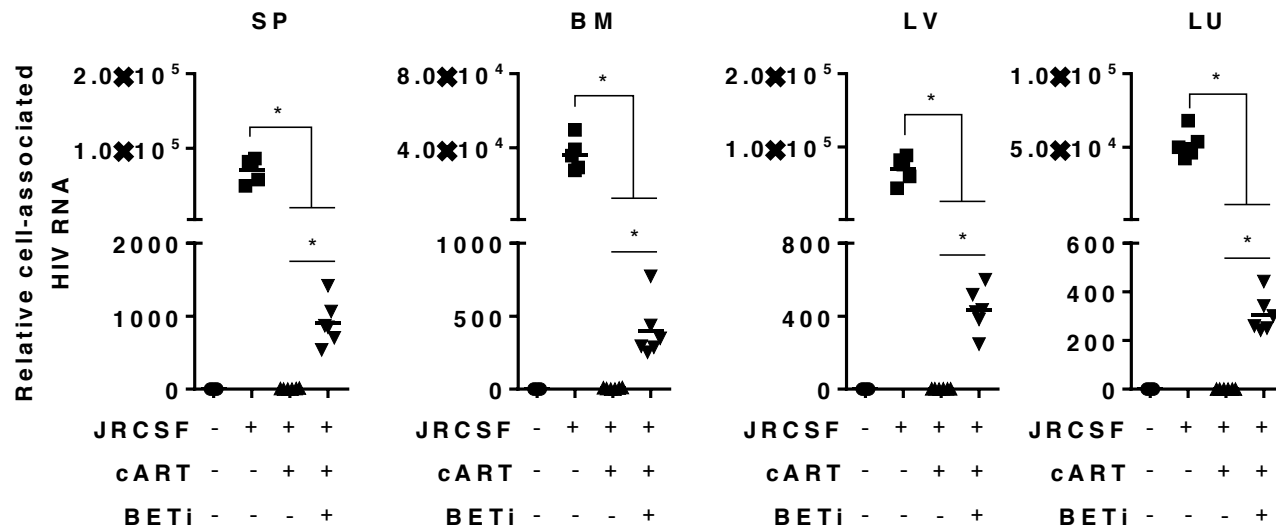


FIG S4. I-BET151 treatment activates HIV-1 replication under suppressive cART in vivo. Relative cell-associated viral RNA levels in human cells or tissues, normalized to human CD14 mRNA level. Bars in dot graphs indicate mean value. * indicate $p < 0.05$.

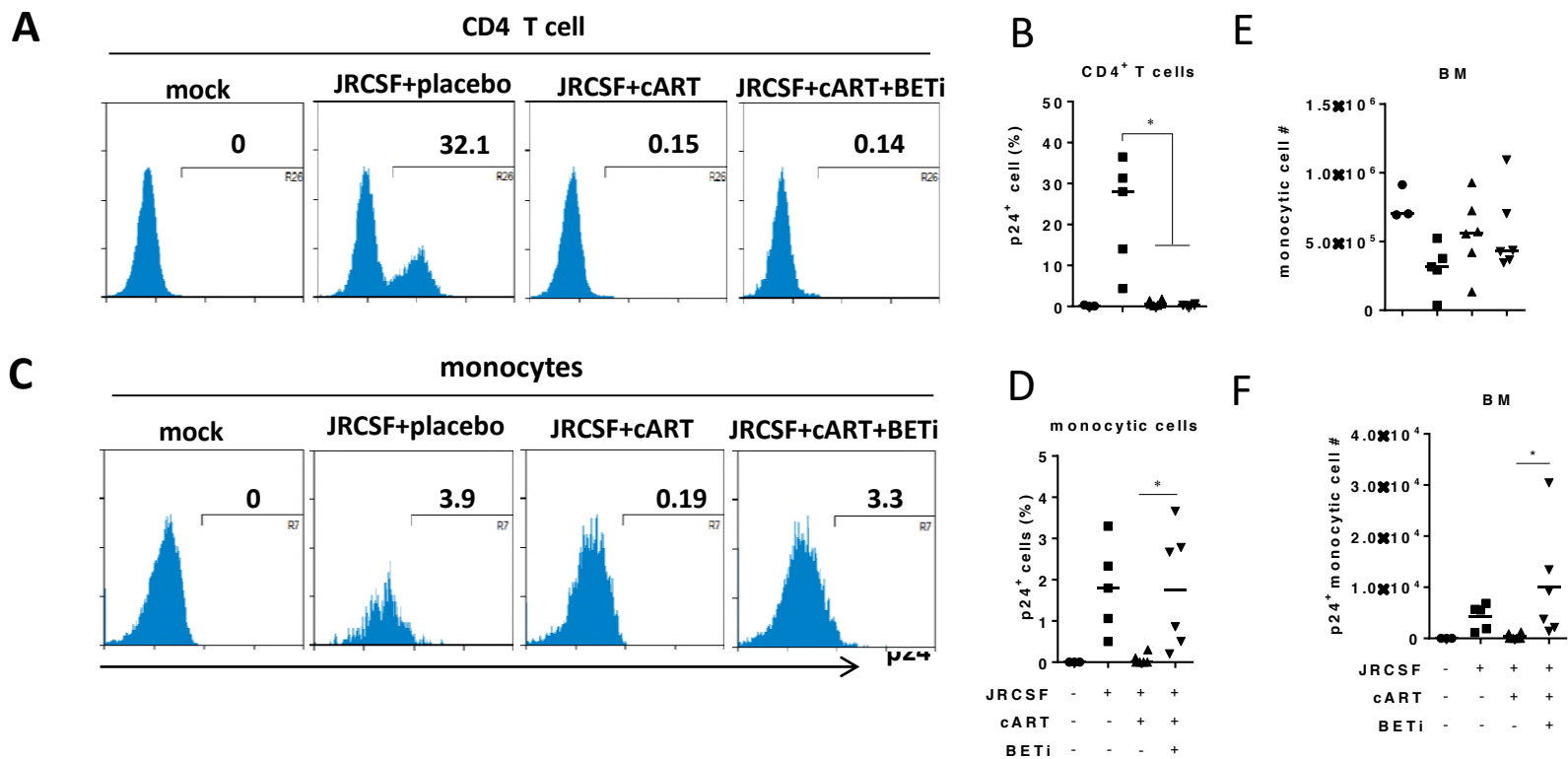


FIG S5. I-BET151 treatment preferentially activate HIV replication in monocytes under suppressive cART in bone marrow. Humanized mice were treated as in Fig.2 and bone marrow cells were investigated. (A) Histograms show percentage of HIV gag p24+CD4⁺ T cell (CD3⁺CD8⁻). (B) Summarized percentage of p24+CD4⁺ T cell. (C) Histograms show percentage of HIV gag p24+monocytic cells (CD3⁻CD11c⁺CD14⁺). (D) Summarized percentage of p24+monocytic cells. (E) Monocytic cell numbers. (F) HIV gag p24+ monocytic cell numbers. Bars in dot graphs indicate mean value. * indicate p<0.05.

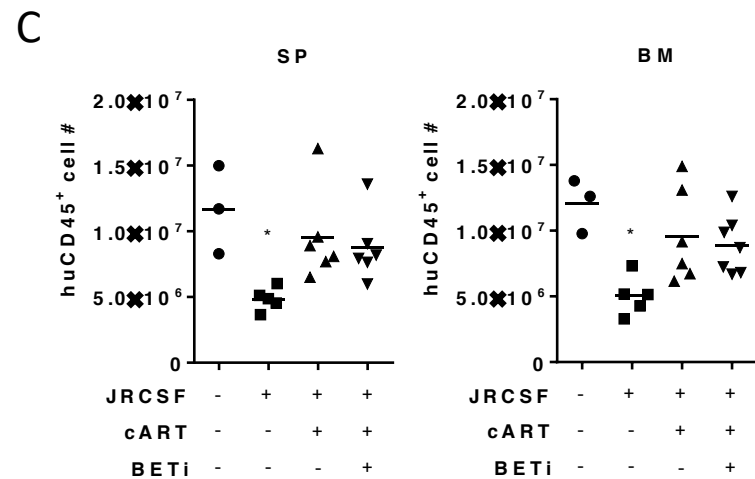
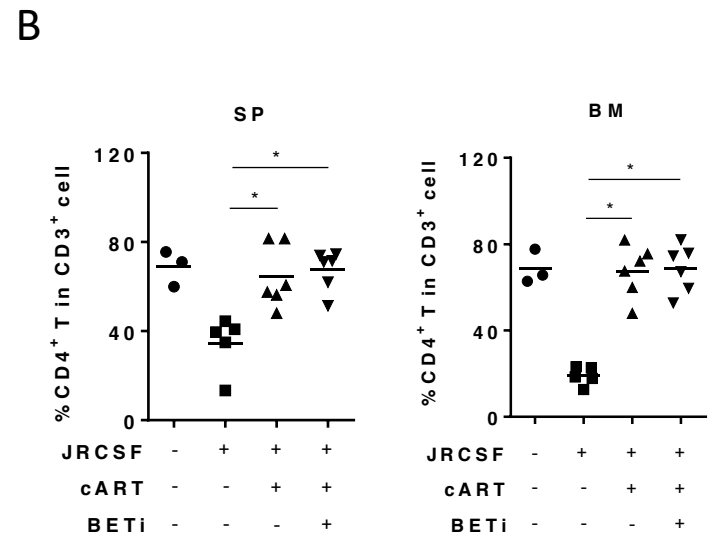
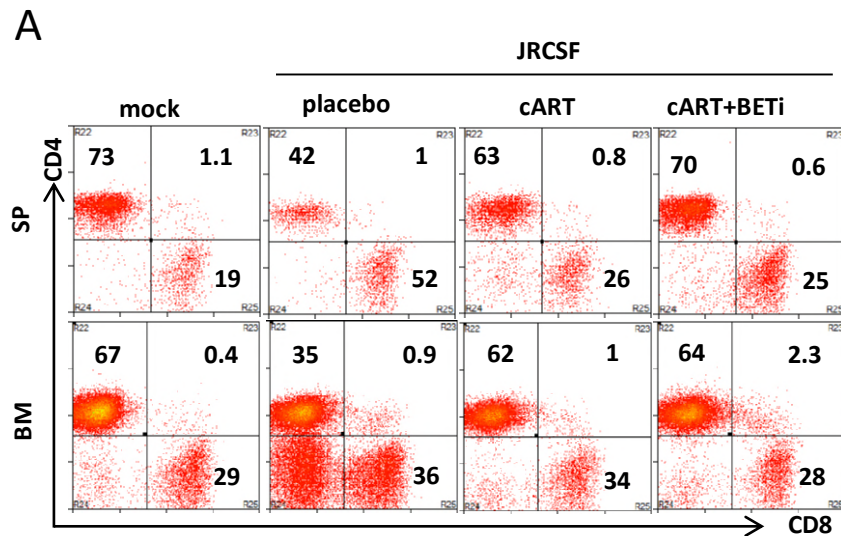
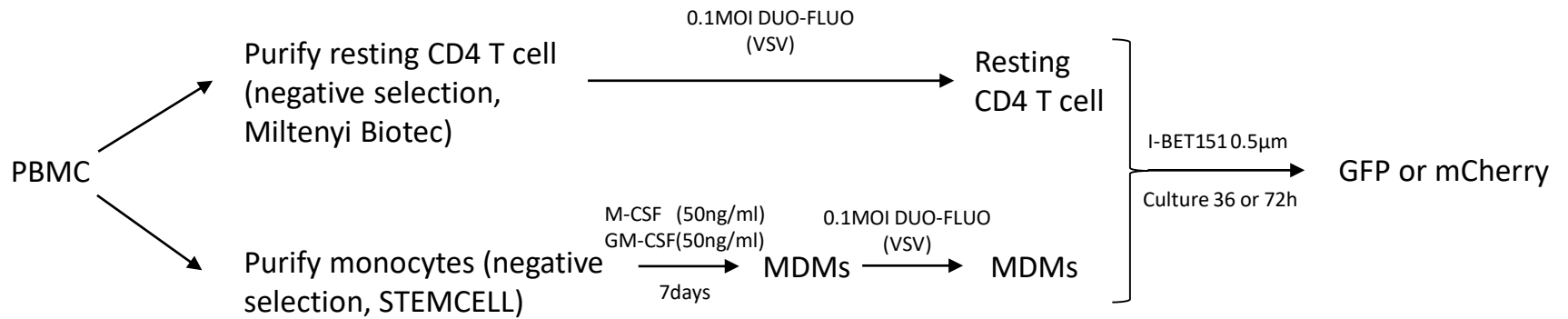
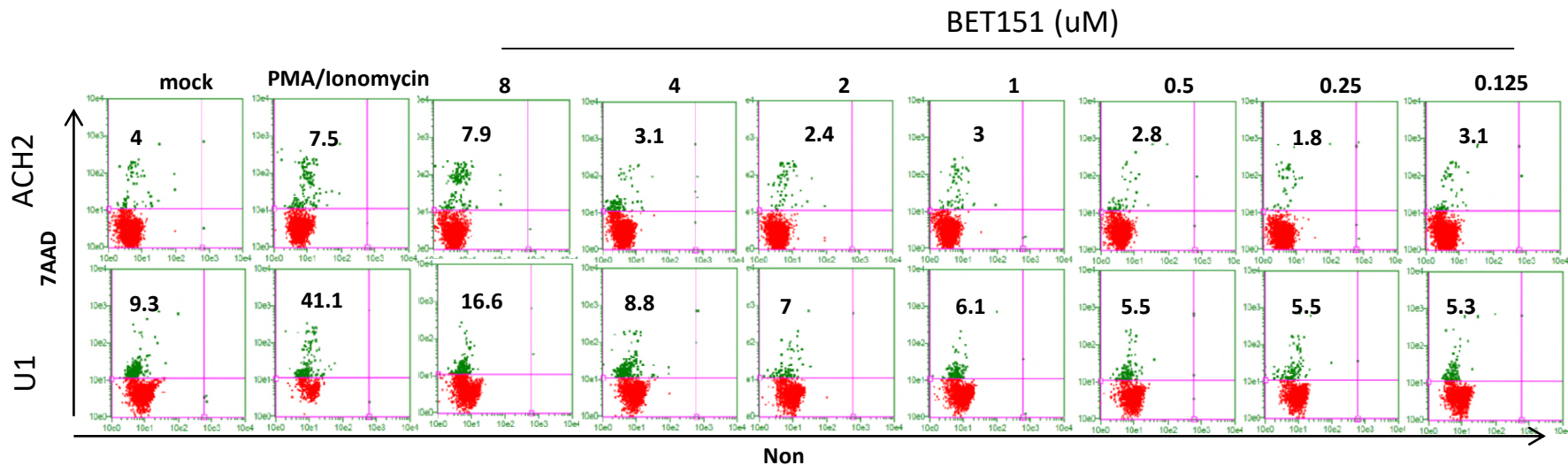


FIG S6. I-BET151 treatment does not affect T cells and total human leukocytes in vivo. On termination as in Fig.2, cells were harvested from spleen or bone marrow for flow cytometry analysis. (A) Plots show percentages of CD4⁺ T cell and CD8 T cell in CD3⁺ cells in spleen and bone marrow. (D) Summary data show percentages of CD4⁺ T cell and CD8 T cell in CD3⁺ cells in spleen and bone marrow. (C) Human CD45⁺ cells numbers in spleen and bone marrow. Bars in dot graphs indicate mean value. * indicate p<0.05.

FIG S7. Flow chart for ex vivo assay



A



B

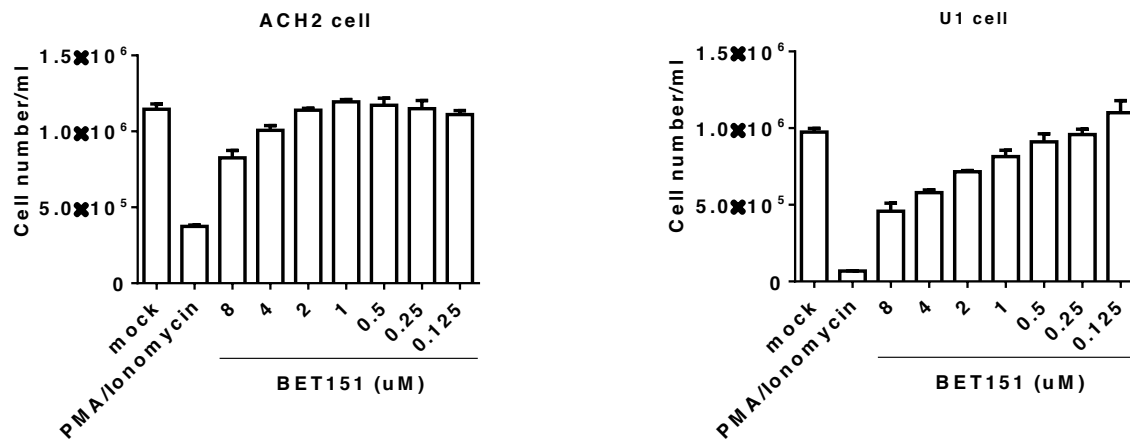


FIG S8. I-BET151 treatment induced cytotoxicity. ACH-1 or U1 cells were treated with series concentration of I-BET151 in cell culture. (A) Cell viability as evidenced by 7-AAD positive cells. (B) Live cell numbers were counted by Guava Pro system.