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Identification of a Novel Small Molecule Facilitating HIV Elimination by the "Shock-And-Kill" Approach

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ABSTRACT

Antiretroviral therapy (ART) has markedly improved the prognosis of people living with HIV (PLWH); however, latent viral reservoirs remain a major barrier to a cure. The "Shock and Kill" strategy aims to reactivate latent provirus with latency-reversing agents (LRAs) and subsequently eliminate the infected cells, yet most LRAs characterized to date provide only the "shock" component. Through screening a small-molecule library, we identified 2-hydrido-2,2'-spirobi(1,3,2-benzodioxaphosphole) (2-HSB) as a novel candidate that both reactivates latent HIV provirus and selectively induces cytopathic effects in latently infected reservoir cell lines, thereby exhibiting a dual "Shock and Kill" activity within a single compound. Mechanistically, the HIV-1 *tat* protein appears to contribute to this selectivity. At the single-cell level, the two effects appeared largely independent, indicating that, under the conditions tested, reactivation was not a consequence of cell death and vice versa. Importantly, 2-HSB induced viral transcription in ex vivo $CD4^+$ T cells from ART-suppressed PLWH. Together, these findings identify 2-HSB as a dual-action candidate that reactivates latent HIV-1 and preferentially induces cytopathic effects in reservoir cell-line models, while supporting further mechanistic and ex vivo validation in primary reservoir-bearing cells.

1 | Introduction

Human immunodeficiency virus type 1 (HIV-1) primarily targets $CD4^+$ cells and causes acquired immunodeficiency syndrome (AIDS). Since the discovery of the anti-HIV activity of azidothymidine (AZT) [1], efforts have been made to control HIV infection, which has led to the development of antiretroviral therapy (ART) [2]. However, complete eradication of the virus has been achieved in very few people living with HIV (PLWH), with the "Berlin patient" being the most widely recognised case [3]. Currently, ART cannot cure HIV infection, but it decreases the viral load sufficiently to prevent the development of AIDS. Although ART has made HIV infection a controllable condition, treatment interruption typically leads to rapid viral rebound [4], making a functional cure— defined as long-term viral suppression without treatment—difficult to achieve [5]. Latent HIV reservoir cells, which maintain low-level HIV transcription, are the major obstacle preventing HIV elimination [6]. To achieve the eradication of HIV from cell

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reservoirs and truly cure the infection, multiple approaches are currently being tested, such as gene editing by the CRISPR-Cas9 system [7]. The "Shock and Kill" strategy is a different approach to eliminating HIV reservoirs [8]. As the name suggests, this strategy consists of two steps: reactivating the latent provirus ("Shock") using latency-reversing agents (LRAs) and then eliminating reactivated HIV reservoirs ("Kill") with ART and host-mediated immune responses.

While numerous LRAs have demonstrated potent HIV-1 reactivation in vitro [9], their clinical utility has been limited by the consistent failure to significantly reduce the latent reservoir size in vivo. This limited efficacy may reflect the heterogeneity of latent reservoirs, including differences in their size, anatomical distribution, and molecular state [10, 11]. However, several studies combining LRA ("Shock") and an HIV vaccine ("Kill") have shown decreased viral load/reservoir size in PLWH to some extent [12, 13], suggesting the requirement of "Kill" intervention alongside the "Shock" by LRAs in the "Shock and Kill" strategy. Thus, there is an unmet need for agents with novel modes of action that could reactivate latent HIV-1 while also promoting the elimination of reservoir cells.

Here, we report the identification of a novel LRA candidate through screening a small chemical library, as previously described [14]. This agent displays a distinctive dual profile: it reactivates the latent HIV-1 provirus and, in cell-line reservoir models, induces cytopathic effects that preferentially target provirus-bearing cells. This molecule may therefore be regarded as a candidate LRA that couples provirus reactivation with preferential cytopathic effects toward HIV-1 reservoir cells, offering a phenotypic proof-of-concept and a starting point for future "Shock and Kill" strategies.

2 | Materials and Methods

2.1 | Cell Lines and Cell Culture Reagents

THP-1, Jurkat, THP-1 Nluc #225 cells (#225) [15], J-Lat 10.6 cells (10.6), J-Lat A2 cells (A2), and J-Lat A72 cells (A72) [16] were cultured in RPMI-1640 (Nacalai Tesque) supplemented with 10% heat-inactivated FCS (Sigma Aldrich) and Penicillin/Streptomycin (Nacalai Tesque). For primary cell culture, heat-inactivated human type AB serum without HIV was used instead of FCS.

2.2 | Chemical Compounds

2-hydrido-2,2′-spirobi(1,3,2-benzodioxaphosphole), JQ1, PEP-005, Prostratin and PMA were obtained from Princeton Bio-Molecular Research, Adooq Biosciences, Cayman Chemical Company, Sigma and Wako Pure Chemical, respectively. In addition to the concentration, moles of compounds per cell are indicated in figures to enhance the scalability of the results [17].

2.3 | Small Molecule Library Screening

Screening was conducted as previously described [14]. Briefly, 2×10^4 of #225 cells/well were seeded into 96-well plates together with 10 μM of compounds for 24 h. The Nano-Glo Luciferase Assay System (Promega) was then used according to the manufacturer's protocol to determine NanoLuc activity. Compounds that caused more than two-times higher induction of NanoLuc activity compared with unstimulated controls were selected for further analysis. NanoLuc activity was measured using the GloMax Multi-detection system (Promega).

2.4 | Measurement of Cell Viability

Briefly, 2×10^4 cells/well were seeded into 96-well plates together with the test compounds for 24 h. Viral ToxGlo (Promega) was then used according to the manufacturer's protocol to determine the cellular ATP content using the GloMax Multi-detection system. For primary cells, concentrations were set to 4×10^4 cells/well.

2.5 | Reporter Protein Analysis

For #225 cells, 2×10^4 cells/well were seeded into 96-well plates together with the compounds for 24 h. NanoLuc activity was determined as described above. For 10.6, A2 and A72 cells, flow cytometry (FCM) analysis was conducted using CytoFLEX SRT (Beckman Coulter) as previously described [14].

2.6 | Quantitative RT-PCR

Total RNA was extracted using RNeasy mini kits (QIAGEN). Quantification of the doubly-spliced form of HIV-1 $NL_{4\text{-}3}$-associated RNA by quantitative RT-PCR (RT-qPCR) was performed as previously described using the StepOnePlus Real-Time PCR system (Applied Biosystems) [14].

2.7 | Live Cell Labelling

Cells were resuspended in complete RPMI-1640 medium at a density of 1×10^6 cells/mL and incubated with 1 μM CellTracker Deep Red dye (Invitrogen) for 30 min at 37°C. The labelled cells were then washed twice with PBS(-) and resuspended in fresh complete RPMI-1640 and utilized for experiments.

2.8 | Cleaved Caspase-3 Analysis

1×10^6 cells/well were seeded into 24-well plates together with the compounds for 24 h and cleaved Caspase-3 was stained using the Cleaved Caspase-3 Staining Kit (Red) (Abcam) according to the manufacturer's protocol. Cells were then fixed with 0.8% PFA and analysed by FCM using CytoFLEX SRT.

2.9 | FCM of Cell Surface Markers

PBMCs from donors without HIV alone or in a 1:1 mixture with CellTracker-stained 10.6 cells were treated with compounds for 24 h. Then, anti-human CD3 PE-Cy5/CD4 PE/CD8 FITC Cocktail, BV605 anti-human CD4 mAb (OKT4), BV421 anti-human CD25 mAb (BC96), APC/Fire 750 anti-human CD69

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mAb (FN50), Spark Red 718 anti-human CD107a mAb (H4A3), and BV605 anti-human CCR7 mAb (G043H7) (all from BioLegend) were used to stain CD3, CD4, CD8, CD25, CD69, CD107a and CCR7 cell-surface markers according to the manufacturer's protocol to investigate the immunomodulatory effect of the compounds. Expression levels of each marker were analysed by FCM using CytoFLEX SRT.

2.10 | Enzyme-Linked Immune Sorbent Assay (ELISA) of HIV-1 p24 Protein

5×10^6 10.6 cells were seeded into a T25 flask together with the compounds for 24 h. Viable cells were sorted using CytoFLEX SRT, and production of HIV-1 p24 was measured by ELISA using the RETROTEK HIV Type 1 p24 Antigen ELISA (ZeptoMetrix) according to the manufacturer's protocol. The measured amount of p24 was normalised to the number of sorted cells.

2.11 | Isolation of Peripheral Blood Mononuclear Cells (PBMCs) From Donors Without HIV

PBMCs were isolated from the whole blood of donors without HIV by density-gradient centrifugation using Ficoll-Paque PLUS (cytiva). $CD4^+$ T-cells were enriched using the human $CD4^+$ T-cell Isolation Kit (Miltenyi Biotec) according to the manufacturer's instructions. Freshly isolated PBMCs or $CD4^+$ T cells were utilized immediately for downstream experiments.

2.12 | Primary $CD4^+$ T-Cell Isolation From PLWH Samples and In Vitro Reactivation

The isolation of primary cells from PLWHs and the in vitro reactivation experiments were conducted as described previously [18]. In brief, whole PBMCs were isolated from PLWH who had been receiving combined ART for at least 3 years (Table 1) by density-gradient centrifugation with Lymphoprep (STEMCELL Technologies), and $CD4^+$ T-cells were purified using the MojoSort human CD4 T-cell isolation kit (BioLegend) according to the manufacturer's protocol. All PLWH maintained a low viral load (< 20 copies/mL) during therapy. $CD4^+$ T-cell counts in peripheral blood samples ranged from 324 to 670 cells/mm^3 (average, 514 cells/mm^3), and plasma viral loads were < 20 copies/mL as measured by qPCR (Roche Applied Science, COBAS AmpliPrep/COBAS TaqMan HIV-1 Test version 2.0) at the time of study enrolment. Written informed consent was obtained from all PLWH. The Ethics Committee at Kagoshima University and the Japan Institute for Health Security approved this study (NCGM-S-002259-08). This study abided by the principles of the Declaration of Helsinki. Purified $CD4^+$ T-cells were plated at a density of 2.5×10^6 cells/mL and treated with LRAs or 100 nM PMA plus 2 μM ionomycin for 24 h. Cells were then harvested, and total RNA was extracted using RNeasy mini kits, following the manufacturer's protocols. RT-qPCR analyses for intracellular HIV RNA were conducted using One Step PrimeScript III RT-qPCR Mix (TaKaRa Bio). The primer pair 5'-TGTGTGCCCGTCTGTTGTGT-3', and 5'-GAGTCCTGCGTCGAGAGAGC-3' was used for HIV RNA detection. HIV RNA copy numbers were normalized to RNA

TABLE 1 | Clinical characteristics of participants living with HIV included in this study.

Participant ID	Sex	Age	VL[a] (copies/mL)	CD4 count[a] (cells/mm^3)	cART	Duration of therapy (years)
Participant 1	M	39	< 20	634	BIC/TAF/FTC	6
Participant 2	M	56	< 20	324	BIC/TAF/FTC	20
Participant 3	M	50	< 20	428	BIC/TAF/FTC	5
Participant 4	M	45	< 20	670	BIC/TAF/FTC	3

Abbreviations: BIC, bictegravir; DTG, dolutegravir; FTC, emtricitabine; TAF, tenofovir alafenamide fumarate.
[a]Plasma HIV-1 RNA level and $CD4^+$ T-cell count were measured at the time of the study.

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input. The number of HIV–RNA copies was calculated using a standard curve obtained from serially-diluted HIV plasmids.

2.13 | Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 10. Means from at least three independent experiments with error bars indicating standard deviation (SD) are presented in the Results. Statistical significance between means was determined using the unpaired two-tailed Student's *t*-test or analysis of variance (ANOVA) and Tukey's post-hoc multiple comparison test. For multiple *t*-test, Benjamini, Krieger and Yekutieli's method was used to adjust *p*-values [19]. p-values < 0.05 were considered significant.

3 | Results

3.1 | 2-hydrido-2,2′-spirobi(1,3,2-benzodioxaphosphole) Reactivates HIV-1 in Both a Latent HIV-1 Cell-Line Model and ART-Suppressed Cells From PLWH

We identified 2-hydrido-2,2'-spirobi(1,3,2-benzodioxaphosphole), here designated "2-HSB", as a novel LRA candidate through small molecule library screening using monocytic line #225 cells (Figure 1A). To further investigate its reactivation parameters, #225 as well as the T-cell-derived reservoir cell line 10.6 were treated with this compound. In the *nef* region of the HIV-1 provirus, #225 cells possess NanoLuc protein [15], and 10.6 cells possess EGFP [16], such that their expression indicates the reactivation of the provirus. Before testing reactivation, we calculated the 50% inhibitory concentration (IC50) of 2-HSB on these model cell lines. The IC50 of 2-HSB on #225 and 10.6 cells was 5.222 and 7.571 μM (13.05 and 18.93 fmol/cell), respectively. Notably, 2-HSB had little effect on their parental cell lines, THP-1 and Jurkat (IC50 > 40 μM/100 fmol/cell for both), suggesting that the cytopathic effect may be contingent upon the presence of the HIV-1 provirus (Figure 1B). Next, HIV-1 reactivation was assessed by monitoring reporter protein expression. PEP-005 (PKC agonist) [20] and JQ1 (BETi) [21] acted as controls. This revealed that 2-HSB increased reporter activity in both #225 cells and 10.6 cells (Supporting Information S1: Figure 1A,B) in a dose-dependent manner. This reactivation was further validated at the transcriptional level via RT-qPCR for HIV-1 tat mRNA, which demonstrated a significant induction of viral transcription following 2-HSB treatment, consistent with the reporter assays (Figure 1C).

Validating the efficacy of LRAs in primary cells is essential for clinical translation. To assess the translational potential of 2-HSB, we investigated its efficacy and safety in primary $CD4^+$ T cells from donors without HIV. As shown in Figure 1D, the IC50 value of 2-HSB was found to be 55.23 μM (69.04 fmol/cell) (Figure 1D). The test concentration of 2-HSB on primary $CD4^+$ T cells from ART-suppressed PLWH was set at 50 μM (20 fmol/cell), a dose at which cell viability remained largely unaffected. While the reactivation of latent HIV by conventional LRAs (Prostratin and JQ1) failed in several PLWH samples, both the positive control (PMA/Ionomycin) and 2-HSB successfully reactivated HIV-1 across all tested samples (Figure 1E). Especially, 2-HSB alone mediated a statistically significant induction of viral expression compared with the untreated control, supporting its further mechanistic and ex vivo evaluation as a latency-reversing candidate.

3.2 | 2-HSB Selectively Induces Caspase-3-dependent Apoptosis in HIV-1 Reservoir Cell Lines and Depletes Them From the Population

The ultimate success of the "Shock and Kill" depends not only on the "Shock" (viral reactivation) but also on the effective "Kill" component—the subsequent elimination of reactivated reservoir cells [22, 23]. Therefore, we next investigated whether the preferential cytopathic effects of 2-HSB observed in Figure 1B would reduce the latent reservoir size. To evaluate the efficacy of 2-HSB in a more physiologically complex environment, we utilized heterogeneous cell populations created by mixing #225 or 10.6 cells with their parental cells. This resulted in the preferential induction of cell death being conserved in a heterogeneous population of HIV-1-positive and negative cells, with the percentage of HIV-1-positive cells and the level of cytopathic effect showing a positive correlation (Figure 2A).

To rigorously evaluate the selectivity of 2-HSB-induced apoptosis in mixed populations, we measured the levels of cleaved Caspase-3, which is known to be involved in the apoptotic pathway [24]. THP-1 or Jurkat cells were labelled with a fluorescent probe and mixed with unlabelled #225 or 10.6 cells. They were then treated with 2-HSB for 24 h, and the amount of cleaved Caspase-3 was measured in unlabelled/labelled cells. This resulted in 2-HSB treatment causing a significant induction of cleaved Caspase-3 exclusively within the HIV-1-positive population, while no significant increase was observed in the HIV-1-negative population (Figure 2B and Supporting Information S1: Figure 2). Furthermore, this led to a specific depletion of HIV-1-positive cells from the co-culture over time (Figure 2C and Supporting Information S1: Table 1). Taken together, these results indicate that 2-HSB preferentially induces Caspase-3-dependent apoptosis in provirus-bearing cells within the tested mixed-population models, without detectable bystander cytotoxicity in the provirus-negative fraction. Thus, in these cell-line reservoir models, 2-HSB exhibits a dual-action phenotype, coupling latency reversal with the preferential elimination of HIV-1-harbouring cells.

3.3 | HIV-1 Tat Protein Is a Crucial Viral Factor for the Action of 2-HSB

Given that specific HIV-1 accessory proteins are intricately involved in maintaining viral latency and modulating host cell viability [25, 26], we sought to identify the viral determinant required for 2-HSB activity. Due to its dual effects, HIV-1 reactivation and selective killing of HIV-1 provirus-positive cells, we hypothesised that HIV-1 Tat was a possible viral factor targeted by 2-HSB, which is a crucial protein for both HIV-1 transcription and HIV-1-induced cell death [27]. We compared the effects of 2-HSB on J-Lat A2 and J-Lat A72 cells to determine the importance of HIV-1 Tat. While both cell lines are

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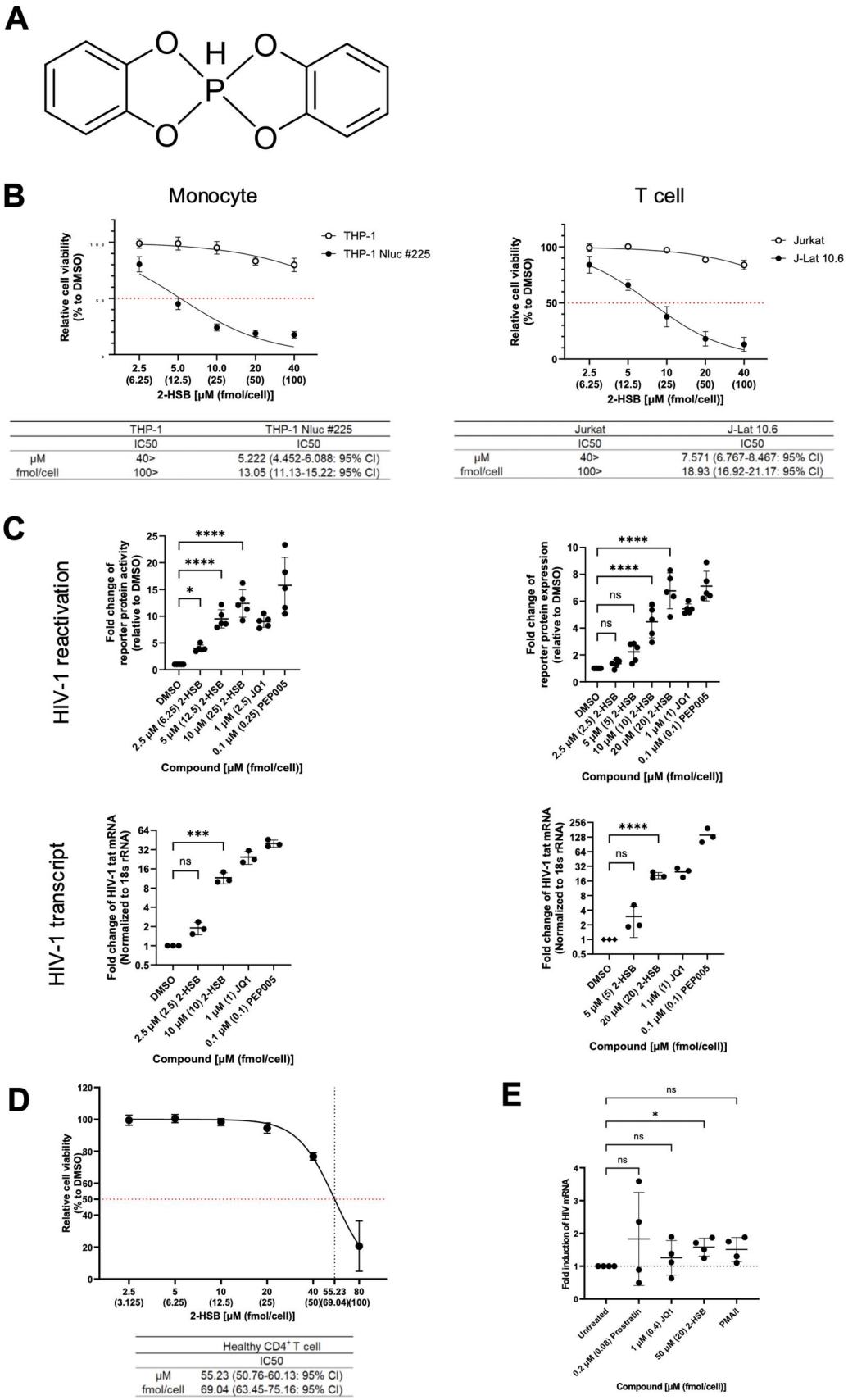

	THP-1	THP-1 Nluc #225
	IC50	IC50
μM	40>	5.222 (4.452-6.088: 95% CI)
fmol/cell	100>	13.05 (11.13-15.22: 95% CI)

	Jurkat	J-Lat 10.6
	IC50	IC50
μM	40>	7.571 (6.767-8.467: 95% CI)
fmol/cell	100>	18.93 (16.92-21.17: 95% CI)

	Healthy CD4$^+$ T cell
	IC50
μM	55.23 (50.76-60.13: 95% CI)
fmol/cell	69.04 (63.45-75.16: 95% CI)

FIGURE 1 | Legend on next page.

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Jurkat-derived (similar to 10.6 cells), they harbour truncated HIV-1 provirus that differs specifically in the presence or absence of the *tat* gene (Figure 3A). We conducted reactivation and cytotoxicity assays in the same manner as for 10.6 cells. While 2-HSB mediated significant reactivation in A2 cells, it had no effect in A72 cells (Figure 3B). This pattern was reproduced with the cytopathic effect as well; thus, A2 and 10.6 cells were similarly susceptible, but A72 cells were more resistant (albeit the effect was still significantly stronger than on Jurkat) (Figure 3C). These results indicate that HIV-1 Tat makes a substantial contribution to the activity of 2-HSB in our reporter-cell systems, although the incomplete loss of cytopathic activity in Tat-deficient A72 cells suggests that additional determinants, potentially including the 5′-LTR, also participate. The viral-factor dependence of 2-HSB is consistent with a degree of HIV selectivity that distinguishes it from non-specific cytotoxic agents, and justifies further mechanistic investigation into how Tat and LTR-associated elements shape its dual-action profile.

3.4 | Only One or Other of the Dual Effects of 2-HSB Is Seen in HIV-1 Reservoir Cells

Since activated HIV-1 may cause apoptosis [28] and reciprocally the induction of apoptosis may reactivate latent viral reservoirs [29], we elected to investigate whether the dual *Shock* and *Kill* effects of 2-HSB were sequential or simultaneous. Briefly, 10.6 cells were exposed to a high concentration of 2-HSB for 24 h, followed by staining of cleaved Caspase-3. The cells were analysed by FCM to determine whether 2-HSB was causing only one outcome or both simultaneously at the individual cell level (Figure 4A). We compared these features with JQ1 (Figure 4B), for which the IC50 reported previously (9.51 μM/23.78 fmol/cell, calculated from Kitamura et al. [14]) was similar to 2-HSB (Figure 1B). The proportions of three populations were compared, namely, only EGFP-positive (Shock-only), only cleaved Caspase-3-positive (Kill-only), and both EGFP and cleaved Caspase-3-positive (Shock and Kill). While there were no differences between 2-HSB and JQ1 on the properties of the Shock-only population, the percentages of Kill-only and Shock and Kill cells were significantly higher with JQ1 than with 2-HSB (Figure 4C). Furthermore, the percentage of the Shock and Kill population within the cleaved Caspase-3-positive population was significantly lower with 2-HSB; in fact, there was no significant difference from the DMSO control (Figure 4D). This indicates that 2-HSB caused neither HIV-1-induced cell death nor cell damage-induced activation, or a "random" cytopathic effect. Hence, 2-HSB induced either reactivation alone or cell death in individual cells, suggesting the presence of precise cellular regulators of these two effects.

3.5 | 2-HSB Modulates the Immune Activation Profile in PBMCs

Based on our observation that a "shock-only" cell population may persist without undergoing 2-HSB-induced cytolysis, we hypothesized that these reactivated cells could serve as a stimulus for broader immune induction. To test this, we investigated whether 2-HSB influences the activation status of HIV-1-uninfected bystander cells. PBMCs from donors without HIV, cultured either alone or in the presence of CellTracker-stained 10.6 cells, were treated with 2-HSB for 24 h, followed by FCM analysis of cell-surface immune markers. Monocytes and lymphocytes were gated based on their forward scatter and side scatter profiles (Figure 5A, Supporting Information S1: Figure 3). In PBMCs cultured alone, treatment with the PKC agonist PEP-005 significantly upregulated the early and late lymphocyte activation markers CD69 and CD25 [30]. In contrast, 2-HSB treatment did not significantly alter their expression, although a marginal trend toward increased CD69 expression was observed (Figure 5B). Notably, however, 2-HSB significantly elevated the expression of CCR7 on monocytes, suggesting a potential enhancement of their migratory capacity [31] (Figure 5C). These phenotypic changes induced by 2-HSB were consistently observed in the PBMC/10.6 cell co-culture model (Figure 5D). We further evaluated the impact of 2-HSB on effector functions by measuring CD107a expression, a surrogate marker of degranulation [32], on $CD3^+CD8^+$ T cells. While 2-HSB induced CD107a expression in both the presence and absence of 10.6 cells, the induction was significantly more pronounced in the co-cultures (Figure 5E). Given that 2-HSB treatment of 10.6 cells leads to a significant increase in HIV-1

FIGURE 1 | 2-HSB reactivates latent HIV-1 in both a latent HIV-1 cell-line model and primary $CD4^+$ T cells from ART-suppressed PLWH. (A) Chemical structure of 2-HSB. (B) #225, THP-1 (left-hand panels), 10.6 and Jurkat (right-hand panels) cells were treated for 24 h with twofold serial dilutions of 2-HSB starting at 40 μM (100 fmol/cell). The amount of intracellular ATP was measured as an indication of cell viability. Viability of DMSO-treated cells was set at 100% for calculating relative viability. IC50 values, the concentration required to reduce the viability of DMSO-treated cells by 50%, were calculated. (C) HIV-1 reactivation (upper panels) and HIV-1 transcripts (lower panels) following treatment with 2-HSB for 24 h. #225 cells were lysed in the plates after treatment, and NanoLuc reporter activity was measured (upper left-hand panel). Reporter activity was normalised to intracellular ATP. The fold-change of normalised reporter activity relative to DMSO-treated cells is shown as the mean and SD calculated from five independent experiments. The number of GFP-positive 10.6 cells in surviving Via-Probe unstained cells was measured by FCM (upper right-hand panel). The fold-change of GFP-positive cells relative to DMSO-treated cells is shown as the mean and SD calculated from five independent experiments. Total cell RNA was extracted using RNeasy mini kits after treatment. HIV-1 transcripts (HIV-1 *tat* mRNA) were measured by RT-qPCR. Transcription levels were calculated by the ΔΔCt method using 18S ribosomal RNA as an internal reference. The means and SD calculated from three independent experiments are shown (lower panels). (D) Primary $CD4^+$ T cells isolated from three independent donors without HIV were treated for 24 h with twofold serial dilutions of 2-HSB starting at 80 μM (100 fmol/cell), and the IC50 was determined as in Figure 1B. (E) Primary $CD4^+$ T cells isolated from ART-suppressed PLWH were treated for 24 h with or without LRAs. Total cell RNA was collected after the treatment, and the amount of HIV-1 mRNA was measured by RT-qPCR. The fold-induction relative to untreated samples is plotted. The means and SD calculated from four independent samples are shown. Statistical significance was determined by one-way ANOVA and Tukey's post-hoc multiple comparison testing. ns., not significant ($p > 0.05$), *$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$.

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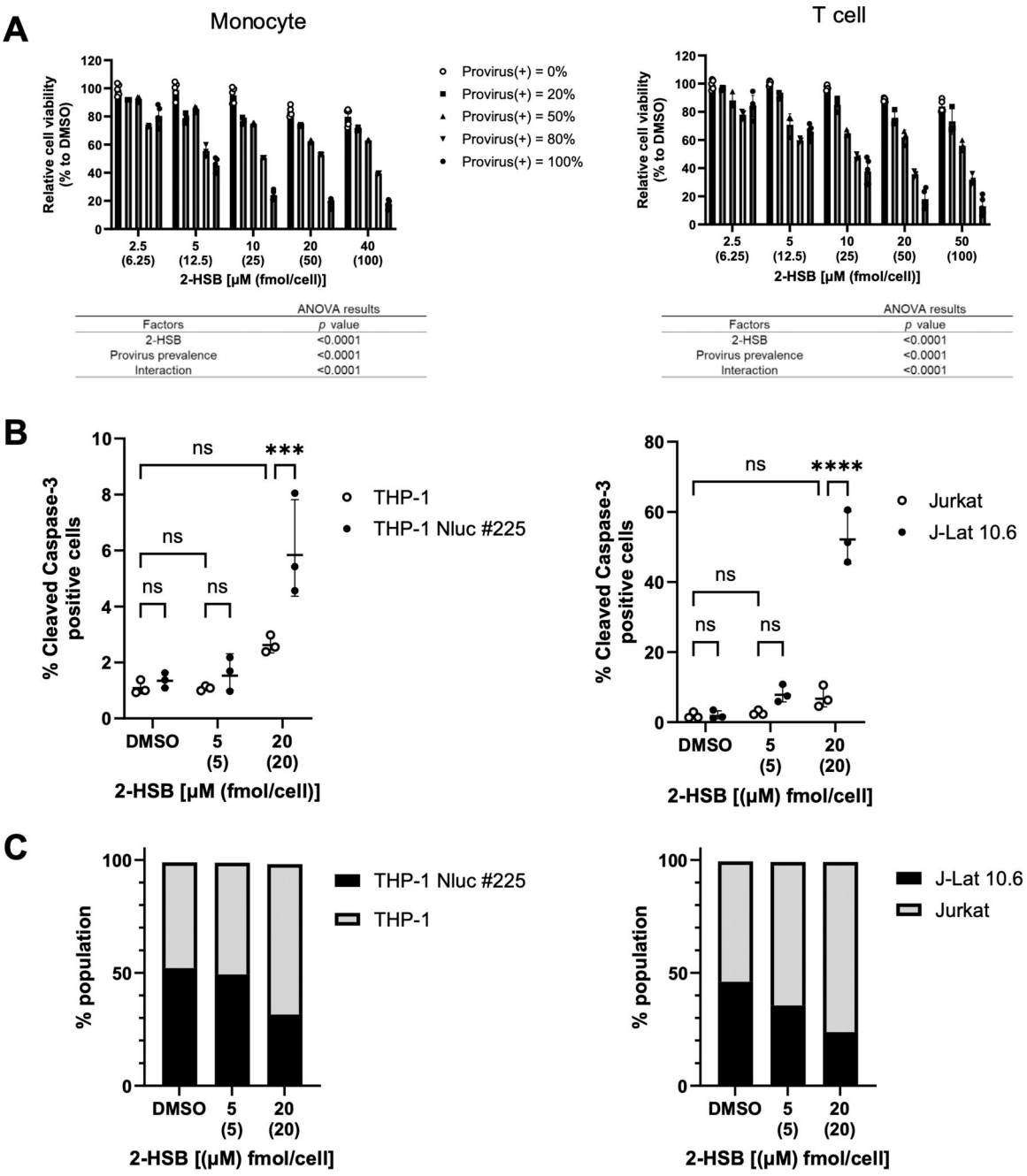

FIGURE 2 | Selective induction of Caspase-3-dependent apoptosis of HIV-1 reservoir cells. (A) THP-1 and #225 cells (left), Jurkat and 10.6 cells (right) were mixed to create a variable percentage of HIV-1 provirus-positive cells in the population, and treated with twofold serial dilutions of 2-HSB for 24 h. The amount of intracellular ATP was measured to indicate cell viability. Viability of DMSO-treated cells was set at 100% for calculating relative viability. The results are shown as mean values and standard deviation calculated from at least three independent experiments. The effects of the presence of the provirus and of 2-HSB were determined by two-way ANOVA. (B,C) CellTracker-labelled HIV-1-negative cells (THP-1 or Jurkat) were mixed 1:1 with unlabelled HIV-1-positive cells (#225 or 10.6) and treated with 2-HSB for 24 h. Apoptosis markers and population dynamics were analysed by flow cytometry. (B) The percentage of cleaved Caspase-3-positive cells was quantified within the labelled (parental) and unlabelled (HIV-1-positive) populations. Left: Monocytic lineage (THP-1 vs. #225). Right: T-lymphocytic lineage (Jurkat vs. 10.6). (C) Specific depletion of HIV-1-positive cells. The population distribution (% of total cells) of labelled and unlabelled cells following treatment. Left: Monocytic lineage. Right: T-lymphocytic lineage. The means and SD calculated from three independent experiments are shown. Statistical significance was determined by two-way ANOVA. ns., not significant ($p > 0.05$), ***$p < 0.001$, ****$p < 0.0001$.

p24 protein —a major source of Gag-derived cytotoxic T lymphocyte (CTL) epitopes [33]—these results may facilitate immune conditions permissive for HIV-1-directed cytolytic activity (Figure 5F). Furthermore, 2-HSB treatment resulted in an expansion of the $CD3^+CD8^+$ T-cell population without affecting the frequency of $CD3^+CD4^+$ T cells. This expansion appeared to be linked to the upregulation of CD3 expression on the $CD8^+$ subset (Figure 5G). As CD3 is a central component of the T-cell receptor (TCR) signalling complex [34], its upregulation may enhance the functional sensitivity of $CD3^+CD8^+$ T

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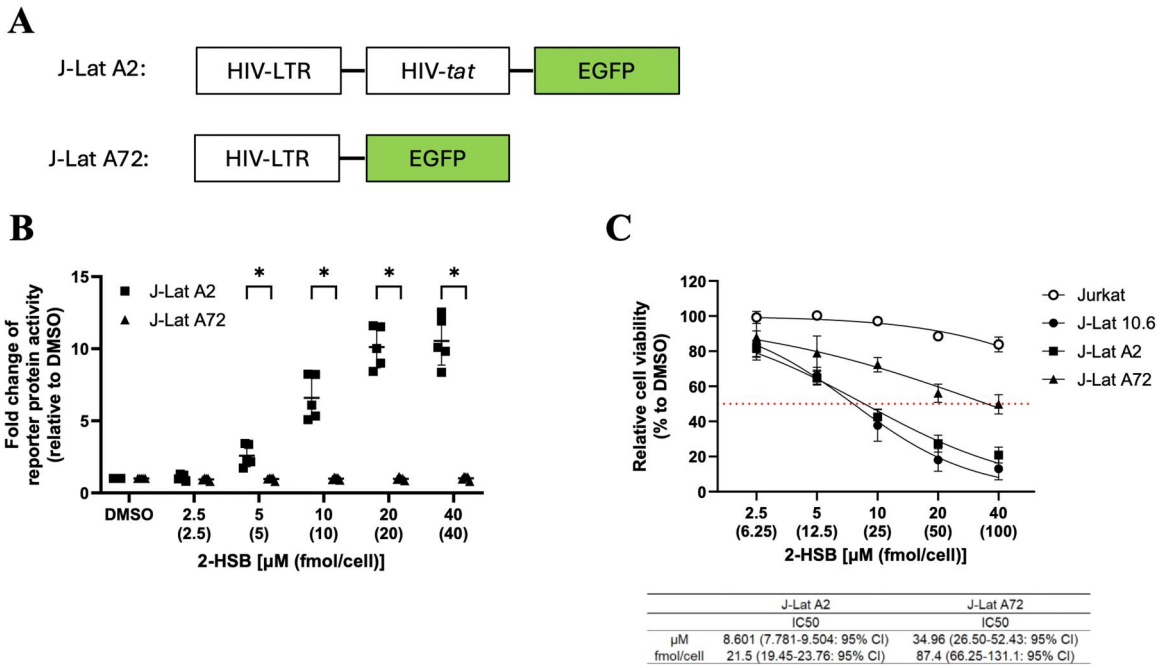

	J-Lat A2	J-Lat A72
	IC50	IC50
µM	8.601 (7.781-9.504: 95% CI)	34.96 (26.50-52.43: 95% CI)
fmol/cell	21.5 (19.45-23.76: 95% CI)	87.4 (66.25-131.1: 95% CI)

FIGURE 3 | The effects of 2-HSB are HIV-1 Tat-dependent. (A) Schematic diagrams of HIV-1 provirus in A2 and A72 cells. (B) Reactivation of HIV-1 in A2 and A72 cells was measured following 24 h of treatment with 2-HSB. The number of GFP-positive cells in surviving Via-Probe-unstained cells was measured by FCM. The fold- change of GFP-positive cells relative to DMSO-treated cells is shown as the mean and SD calculated from five independent experiments. (C) A2 and A72 cells were treated for 24 h with twofold serial dilutions of 2-HSB starting at 40 µM. The amount of intracellular ATP was measured to indicate cell viability. Viability of DMSO-treated cells was set at 100% for calculating relative viability. IC50, the concentration required to reduce the viability of DMSO-treated cells by 50%, was then determined. Results of Jurkat (Figures 2A) and 10.6 cells (Figure 1B) are also depicted. The means and SD calculated from five independent experiments are shown. Statistical significance was determined by two-tailed multiple *t*-test. $*p < 0.05$.

cells. Taken together, these data indicate that 2-HSB functions not only as a LRA but also as a modulator of the immune microenvironment, with a profile distinct from that of canonical PKC agonists.

4 | Discussion

Multiple LRAs have been developed in attempts to reactivate latent HIV from reservoirs impermeable to ART and to render the virus susceptible to destruction [9]. However, their promising in vitro reactivation capabilities failed to translate into a significant reduction of the viral reservoir in clinical trials [10, 11]. Thus, there is a need to develop new LRAs with novel modes of action. Here, we identified and evaluated a novel LRA candidate, 2-HSB (Figure 1A), which serves as a *Shock "AND" Kill* agent at the cell population level while acting as a *Shock "OR" Kill* agent at the single-cell level. It is capable of both reactivating HIV in latent reservoir cells and inducing their selective apoptosis without damaging uninfected bystander cells, achieving targeted depletion in both monocytic and T-cell populations (Figures 1C and 2B,C). Currently, T cells are considered to be the most important HIV-1 reservoir populations [35, 36]. More recently, a role of monocytic cell reservoirs has been increasingly implicated [37], and means to eradicate all types of reservoirs are being investigated [38]. Thus, these findings support further preclinical investigation of 2-HSB across distinct reservoir cell types.

The HIV-1 Tat protein is a crucial viral factor in HIV-1 biology, not only essential for efficient HIV-1 transcription and replication [39] but also for manipulating host gene expression to create favourable environments [40]. Targeting Tat as a therapeutic strategy might offer increased safety of treatments due to virus selectivity. The finding that 2-HSB essentially failed to induce Caspase-3-dependent apoptosis in HIV-1 provirus-negative cells (Figure 2A,B) reinforces this notion of safety, as its effects appeared Tat-dependent. This is illustrated by our findings that its abilities to reactivate HIV and to induce cell death were almost absent (Figure 3B) and nearly halved (Figure 3C) in Tat-negative model cells, respectively. While HIV reactivation by 2-HSB was heavily dependent on Tat, the HIV-1 long terminal repeat (LTR) itself also played a major role in its cytopathic effects. As it is well known that Tat-defective provirus can nonetheless produce viral progeny [41], even though Tat dysfunction might lead to latency [42], Tat-defective reservoirs are also important targets. Thus, understanding how 2-HSB induced cell death in Tat-defective cells, albeit at a modest level, is worth investigating. While the involvement of different viral proteins in the apoptosis of HIV-1-infected cells is well studied [25], little is known about the role of the HIV-1 LTR alone in apoptosis. As the provirus in A72 cells, which we used to demonstrate the effect of Tat deficits, consists only of the HIV-1 LTR and EGFP (Figure 3A), our results imply a novel role for the HIV-1 LTR itself in the pathogenicity of HIV-1 infection, although these findings require further clarification.

One of the most intriguing findings of this study is the mutual exclusivity of reactivation and apoptosis at the single-cell level.

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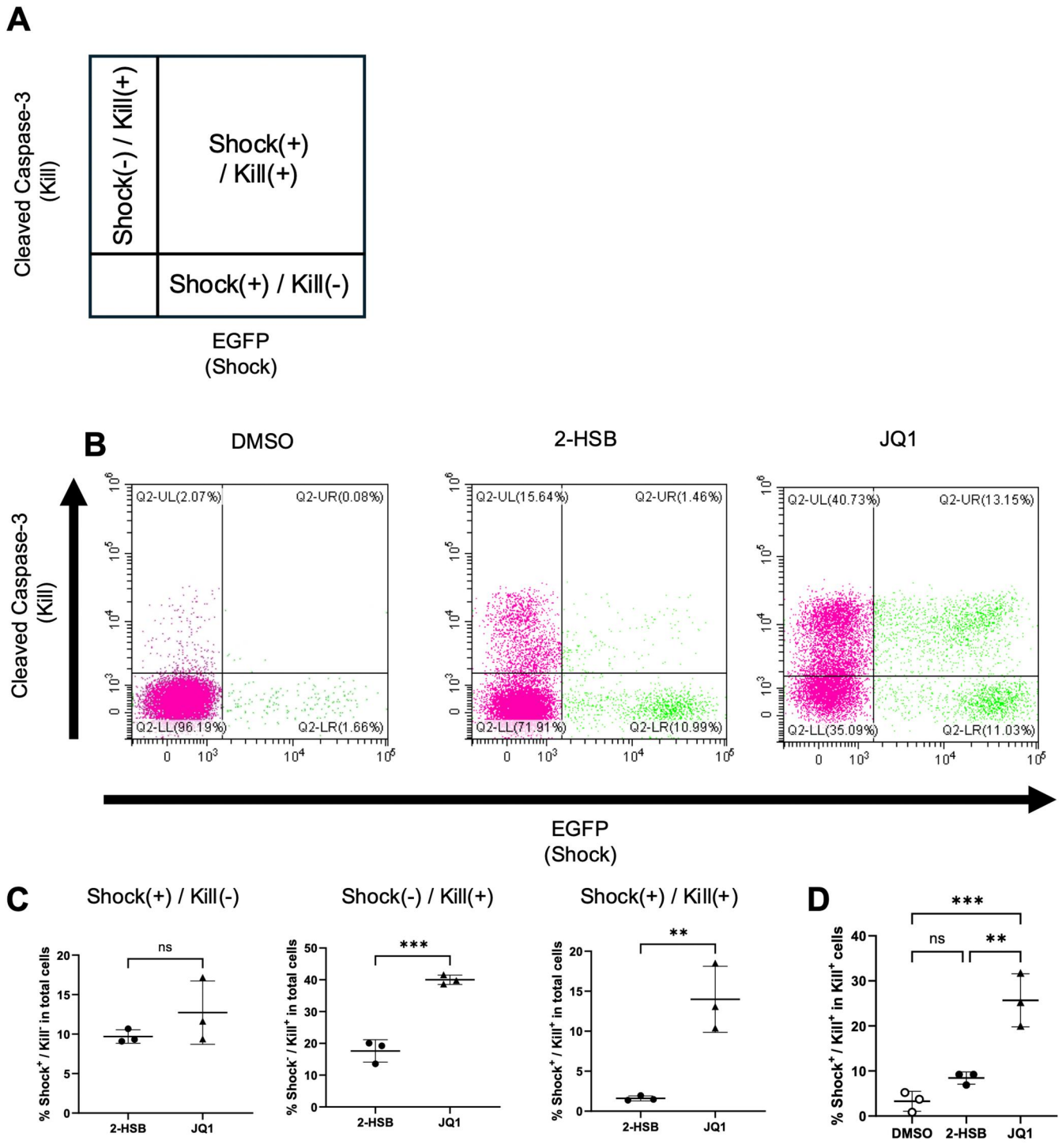

FIGURE 4 | The *Shock* and *Kill* effects of 2-HSB are independent of each other. (A) Schematic diagrams of flow cytometry analysis. (B) 10.6 cells were treated for 24 h with 2-HSB (left) or JQ1 (right) at 20 μM (20 fmol/cell) followed by staining for intracellular cleaved Caspase-3 using Cleaved Caspase-3 Staining Kits (red); the number of cleaved Caspase-3/EGFP-double-positive cells was measured by FCM. (C) The percentages of cells in Shock(+) (left)/Kill(-), Shock(-) (middle)/Kill(+) and Shock(+)/Kill(+) (right) fractions within the total cell population. (D) The percentage of Shock (+)/Kill(+) cells within the fraction of Kill(+) cells. The means and SD calculated from three independent experiments are shown. Statistical significance was determined by two-tailed Student's t-testing (C) or one-way ANOVA (D). ns., not significant ($p > 0.05$), $^{**}p < 0.01$, $^{***}p < 0.001$.

Despite the reactivation and cytopathic effects of 2-HSB being HIV-1 Tat-influenced, these were found to be independent of each other. We showed that even at a concentration that killed > 50% of HIV-1 reservoir cells, those cells in which HIV-1 reactivation was induced and those in which Caspase-3-dependent apoptosis was observed were clearly separate (Figure 4B,C). This bifurcated phenotype offers a useful conceptual framework for considering how a dual-action compound might behave in the shock-and-kill setting. On the one hand, because the cytopathic effect of 2-HSB was observed predominantly in cells without concomitant viral reactivation, it is conceivable that reservoir depletion could proceed with

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limited concomitant induction of viral antigen expression in the dying fraction, a profile that might be advantageous given that both active HIV replication and apoptosis can elicit inflammatory responses [43, 44]. On the other hand, the persistence of a reactivated but non-apoptotic population raises the possibility that such cells could remain available as targets for host immune clearance, although we do not yet know whether reservoirs reactivated by 2-HSB sustain virus production or become susceptible to immune-mediated elimination. These considerations remain speculative and are based solely on cell-line experiments; whether this bifurcation translates into a favourable balance between reservoir depletion, inflammatory

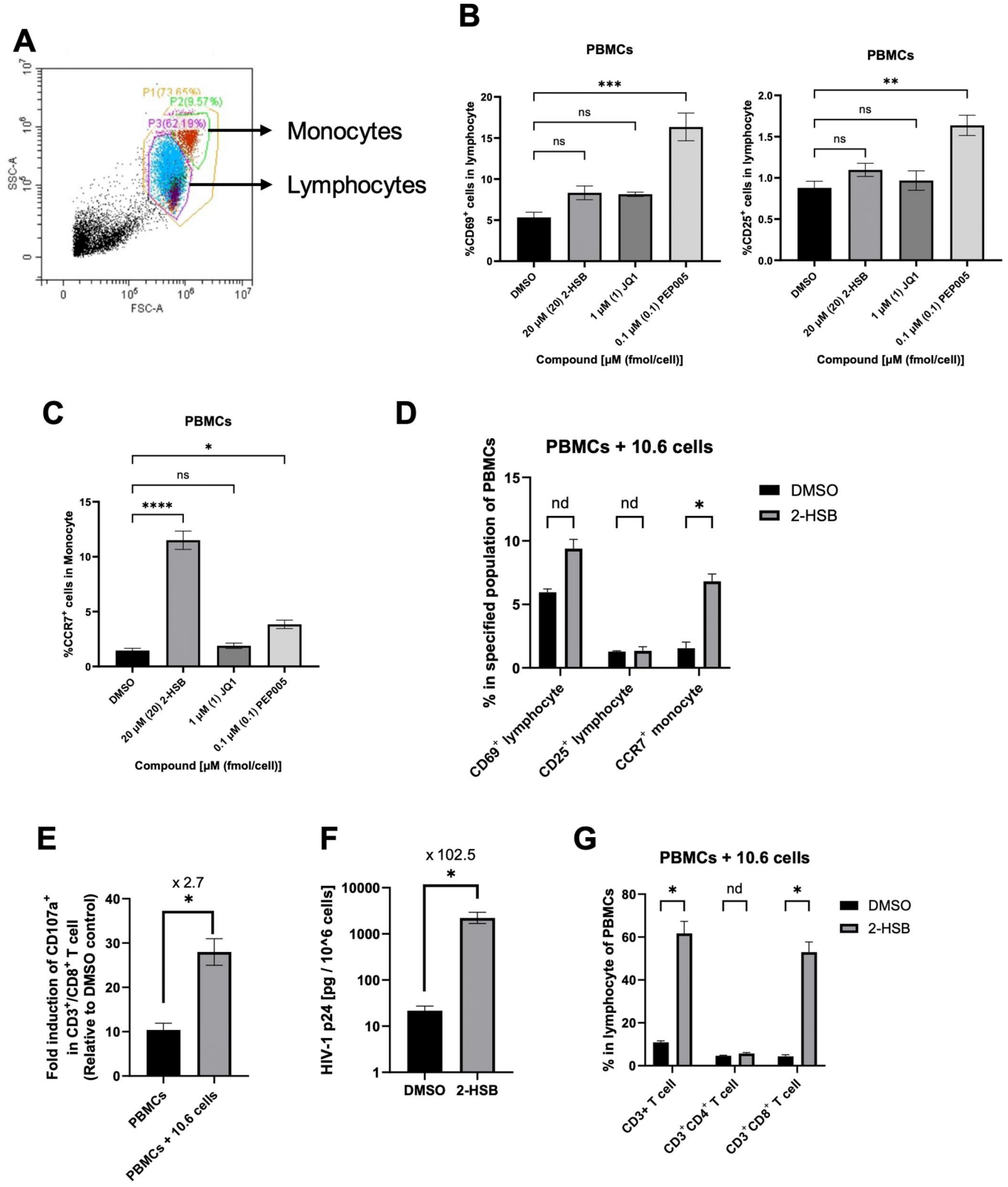

FIGURE 5 | Legend on next page.

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burden, and immune recognition in primary reservoir settings is an important question for future investigation.

Importantly, we have demonstrated the reactivation ability of 2-HSB in samples from ART-suppressed PLWH, which supports its further investigation in clinically relevant ex vivo settings (Figure 1E). Compared with model cell lines, which are clonal populations, HIV-1 reservoirs in PLWH are much more heterogeneous in several respects, such as integration sites [45, 46] and epigenetic modifications [47]. Hence, demonstrating HIV-1 reactivation ability on different clinical samples is a critical step for future studies. Furthermore, our data suggest that 2-HSB may function as an immunoregulatory agent. The significant increases in proportions of $CD3^+CD8^+$ T cells and the upregulation of CCR7 on monocytes (Figure 5C,G) are consistent with enhanced functional responsiveness of cytotoxic T cells and altered monocyte trafficking potential [31, 34]. In fact, 2-HSB treatment of PBMCs mixed with HIV-1 reservoir cells resulted in significantly higher induction of CD107a, a surrogate marker of degranulation [32], on $CD3^+CD8^+$ T cells (Figure 5E,F). Whether this degranulation response reflects HIV-1 antigen-specific recognition will require dedicated assays, such as peptide-stimulated or co-culture-based cytotoxicity measurements. Such a multifaceted profile—engaging both viral reactivation and host cytolytic activation—illustrates how a single compound might extend the conventional "Shock and Kill" framework, while underscoring that the immunological component of 2-HSB activity is at an early stage of characterisation.

Overall, our data show that 2-HSB exhibits latency-reversing activity in monocyte- and T-cell-derived reservoir models and induces viral transcription in ex vivo CD4 + T cells from ART-suppressed PLWH. While the precise mode of action of 2-HSB remains to be fully elucidated, our findings do not closely match the typical phenotypic profiles associated with canonical BET inhibitors or PKC agonists, although these possibilities cannot be formally excluded at this stage. First, the distinct separation of "Shock" and "Kill" at high concentrations in a cell-line model (Figure 4B) is less consistent with a BET-inhibitor-like mechanism, which would be expected to cause non-specific cytotoxicity as an anticancer effect rather than the selective cell death observed here. Second, the immunomodulatory profile shown in Figure 5B is not characteristic of a canonical PKC agonist: whereas PEP-005 significantly induced the lymphocyte activation markers CD69 and CD25, 2-HSB had little effect on these markers, and strong lymphocyte activation via PKC signalling is well characterised [48]. Nevertheless, additional approaches, such as phosphoproteomic profiling, will be required to delineate the precise signalling pathways involved. However, considering the use of 2-HSB in the clinic, further investigation of its mode of action is necessary to prevent unexpected side effects and enhance safety. An additional limitation is that, due to the limited amount of available samples from ART-suppressed PLWH, we were unable to confirm 2-HSB's cytotoxicity in vitro. Nevertheless, some of our findings are compatible with, but do not directly demonstrate, the possibility that 2-HSB may promote processes relevant to reservoir reduction in ex vivo PLWH-derived samples. To begin with, 2-HSB has shown an HIV-selective cytopathic effect in at least four cell lines: #225, 10.6, A2, and A72, whose provirus integration sites differ [49–51] (Figures 1B and 3C), suggesting that its phenotypic effects are not restricted to a single integration-site context, although this does not substitute for direct testing in polyclonal primary reservoir populations. Additionally, observation of Caspase-3 cleavage in treated HIV-positive cells (Figure 2B) suggests that the "Kill" effect of 2-HSB is cellular mechanism-mediated apoptosis rather than damage-mediated necrosis. As #225 and 10.6 cells are human cell lines, these findings raise the possibility that similar mechanisms operate in primary human cells; however, this remains to be determined experimentally. Moreover, although direct evidence of apoptosis induction in primary reservoirs has not been obtained, we were able to show reactivation of samples from ART-suppressed PLWH (Figure 1E) and enhanced degranulation of primary ex vivo $CD3^+CD8^+$ T cells in the co-culture setting (Figure 5E). As several clinical studies observed reservoir size reduction by combining LRAs and strengthened HIV-1-specific immunity by HIV vaccine [12, 13], these observations raise the possibility that 2-HSB could be incorporated into reservoir-reduction strategies, although this remains hypothetical and untested in primary reservoir-bearing cells. Overall, the present study identifies 2-HSB as a novel latency-reversing candidate with an unusual dual-action phenotype, combining HIV-1 reactivation with preferential elimination of reservoir cell lines. At the same time, the current findings should be interpreted with appropriate caution. Although HIV-1 reactivation was reproducibly observed in primary $CD4^+$ T cells from ART-suppressed PLWH, selective elimination of reservoir cells has thus far been demonstrated only in in vitro cell-line models. In addition, the molecular events upstream of Tat-associated activation and Caspase-3-dependent apoptosis remain to be fully defined. Therefore, the present work should be regarded as a proof-of-concept study that defines the phenotypic profile of 2-HSB and establishes priorities for subsequent mechanistic and ex vivo validation. Taken together, the present findings support further

FIGURE 5 | 2-HSB-mediated immunomodulation and enhancement of cytolytic potential in primary PBMCs. (A) Representative flow cytometric gating strategy for identifying viable monocyte and lymphocyte populations based on forward (FSC) and side scatter (SSC) profiles. (B, C) Primary PBMCs from donors without HIV were stimulated with the indicated LRAs for 24 h, followed by cell-surface marker analysis. (B) Expression levels of early activation marker CD69 (left) and the late activation marker CD25 (right) on the gated viable lymphocyte population. (C) Expression level of CCR7 on the viable monocyte population. (D, E, G) PBMCs were co-cultured with CellTracker-labeled 10.6 cells and treated with 2-HSB for 24 h. (D) Quantitative analysis of CD69, CD25, and CCR7 expression, consistent with the parameters in (B) and (C). (E) Fold-induction of the degranulation marker CD107a on $CD3^+CD8^+$ T cells in the presence or absence of 10.6 cells. (F) Intracellular HIV-1 p24 protein levels in 10.6 cells following 24 h treatment with DMSO or 2-HSB, as quantified by ELISA. (G) Proportional distribution of $CD3^+$ T-cell subsets within the viable lymphocyte population. The means and SD calculated from three independent experiments are shown. Statistical significance was determined by one-way ANOVA (B, C), unpaired multiple two-tailed Student's *t*-test (D, G) or unpaired two-tailed Student's *t*-test (E, F). ns., not significant ($p > 0.05$), nd., not a discovery (adjusted $p > 0.05$), *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

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investigation of 2-HSB as a mechanistically distinct lead compound for studies of dual-component HIV-1 cure strategies.

Author Contributions

Yuichiro Hara: writing – original draft, writing – review and editing, methodology, funding acquisition, investigation, formal analysis, visualisation, conceptualisation. **Haruki Kitamura:** investigation, formal analysis. **Kouki Matsuda:** writing – original draft, writing – review and editing, methodology, investigation, resources, formal analysis. **Chieko Fujisaki:** writing – original draft, writing – review and editing, methodology, investigation, resources, formal analysis. **Sayaka Sukegawa:** investigation. **Kosuke Tanimoto:** formal analysis. **Kenji Maeda:** writing – original draft, writing – review and editing, resources, supervision. **Hiroaki Takeuchi:** writing – review and editing, project administration, supervision, funding acquisition, resources, methodology, conceptualisation.

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Data Availability Statement

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section.

Figure S1: Flow cytometry of J-Lat 10.6 cells treated with LRAs. (A) Flow cytometry panel of 10.6 cells treated with DMSO, 2-HSB, JQ1 or PEP005 for 24 hours. The X-axis shows the FITC channel (GFP) and the Y-axis shows the PC5.5 channel (Via-Probe). (B) The absolute percentage of GFP-positive cells in 10.6 cells after treatment with various compounds. The fold-change relative to DMSO-treated cells is summarized in Figure 1 C.

Figure S2: Flow cytometric analysis of selective cytotoxicity in mixed cell populations treated with 2-HSB. Mixed populations of labelled (HIV-1-negative) and unlabelled (HIV-1-positive) cells were treated with DMSO or 2-HSB (5 or 20 μM/5 or 20 fmol/cell) for 24 hours. (A) Representative histograms showing the population distribution of labelled vs unlabelled cells. (Upper panels) Co-culture of THP-1

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(labelled) and #225 (unlabelled). (Lower panels) Co-culture of Jurkat (labelled) and 10.6 (unlabelled). (B–C) Histograms displaying the percentage of cleaved Caspase-3-positive cells within each population defined in (A). (B) Analysis of monocytic cells: THP-1 (upper) and #225 (lower). (C) Analysis of T-lymphocytic cells: Jurkat (upper) and 10.6 (lower). Representative data from three independent experiments are shown.

Figure S3: Gating strategy for flow cytometric analysis of the immunomodulatory effect of 2-HSB on primary PBMCs. PBMCs derived from donors without HIV, either cultured alone or co-cultured with CellTracker-labeled 10.6 cells, were treated with the indicated LRAs for 24 hours prior to flow cytometric analysis. (A) Identification of primary monocyte and lymphocyte populations. Viable cells were initially gated based on forward scatter (FSC) and side scatter (SSC) profiles (left-hand panels). Within the viable gate, PBMCs were distinguished from 10.6 cells by gating on CellTracker-negative population (middle panels). Monocyte and lymphocyte fractions were further delineated from the PBMC population based on their light-scattering characteristics (right-hand panels). (B) Gating of $CCR7^+$ monocytes. Representative plots showing the identification of $CCR7^+$ cells within the gated viable monocyte population. (C) Gating of lymphocyte activation markers. Identification of $CD69^+$ (left) and $CD25^+$ (right) populations with the gated viable lymphocytes. (D) Analysis of T-cell subsets and effector function. The $CD3^+$ T-cell population was identified within the lymphocyte gate (left), followed by the delineation of $CD4^+$ and $CD8^+$ T-cell subsets (middle). Antigen-specific degranulation was assessed by gating on $CD107a^+$ cells within the $CD3^+CD8^+$ T-cell fraction (right).

Supporting File: jmv71131-sup-0004-Revised_Supplementary_Table_Hiroaki_Takeuchi.xlsx.

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