

Cell-to-cell transmission of HIV-1 from provirus-activated cells to resting naïve and memory human primary CD4 T cells is highly efficient and requires CD4 and F-actin but not chemokine receptors

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Abstract

Latently infected cells harboring replication-competent proviruses represent a major barrier to HIV-1 cure. One major effort to purge these cells has focused on developing the “shock and kill” approach for forcing provirus reactivation to induce cell killing by viral cytopathic effects, host immune responses, or both. We conducted kinetic and mechanistic studies of HIV-1 protein expression, virion production, and cell-to-cell virus transmission during provirus reactivation. Provirus-activated ACH-2 cells stimulated with romidepsin (RMD) or PMA produced Nef early, and then Env and Gag in parallel with the appearance of virions. Env on the surface of provirus-activated cells and cellular F-actin were critical in the formation of virological synapses to mediate cell-to-cell transmission of HIV-1 from provirus-activated cells to uninfected cells. This HIV-1 cell-to-cell transmission was substantially more efficient than transmission seen via cell-free virus spread and required F-actin remodeling and CD4, but not chemokine receptors. Resting human primary CD4⁺ T cells including naïve and memory subpopulations and, especially the memory CD4⁺ T cells, were highly susceptible to HIV-1 infection via cell-to-cell transmission. Cell-to-cell transmission of HIV-1 from provirus-activated cells was profoundly decreased by protease inhibitors (PIs) and neutralizing antibodies (nAbs) that recognize the CD4-binding site (CD4bs) such as VRC01, but not by reverse transcriptase (RT) inhibitor Emtricitabine (FTC). Therefore, our results suggest that PIs with potent blocking abilities should be used in clinical application of the “shock and kill” approach, most likely in combination with CD4bs nAbs, to prevent new HIV-1 infections.

KEYWORDS

antiretroviral drugs, HIV-1, latency, neutralizing antibodies, provirus reactivation, virus transmission

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1 | INTRODUCTION

The HIV-1 pandemic has claimed over 36 million lives, with 37.7 million people worldwide living with HIV-1 (WHO Fact Sheet updated 2020, <https://www.unaids.org/en/resources/fact-sheet>), and will continue to contribute to human morbidity and mortality as there is no vaccine or cure available. Currently, the only effective treatment available for HIV-1 patients is combination antiretroviral therapy (ART) that can successfully suppress plasma HIV-1 RNA levels from an average of 30,000 copies/ml to undetectable levels (<50 copies/ml) by standard diagnostic assays. However, most patients on effective ART for years still have residual viremia measurable by ultrasensitive assays (≥ 1 copies/ml), and many of them also experience transient viral increases (viral blips) between 51 and 200 copies/ml of plasma viral RNA with an overall frequency of three episodes per year.^{1–3} This persistent viremia mainly arises from the latently HIV-1-infected cells harboring replication-competent proviruses. These latently infected cells have a long lifespan and slowly divide several times within a year, suggesting that they persist indefinitely. Studies have suggested that these latently HIV-1-infected cells are the major reservoirs for the rapid rebound of plasma viremia in patients after interruption of ART.⁴ Thus, latently HIV-1-infected cells represent a major barrier to viral eradication in infected subjects.

Considerable efforts to purge latently HIV-1-infected cells have focused on developing the “shock and kill” approach for forcing provirus reactivation to induce cell killing by viral cytopathic effects (CPEs), host immune responses, or both. Several latency-reversing agents (LRAs) including IL-7,^{5–8} prostratin,⁹ and histone deacetylase inhibitors (HDACi) such as valproic acid (VPA),^{10–12} suberoylanilide hydroxamic acid (SAHA or vorinostat), and romedepsin (RMD)^{12,13} have been explored to force reactivation of proviruses in latently HIV-1-infected cells. Clinical trials of SAHA in HIV-1 patients in whom viremia has been fully suppressed by ART and in vitro studies of RMD have demonstrated that these LRAs are capable of disrupting HIV-1 latency as they induce viral RNA synthesis, viral protein expression, and production of new infectious virions.^{14–17} These results have prompted researchers to screen a variety of small molecular compound libraries to identify novel agents that have more potent activity and specificity in reactivation of proviruses. Another strategy to enhance LRA potency in provirus reactivation is to combine two or more LRAs, as prostratin and SAHA have demonstrated a synergistic effect on provirus reactivation,^{18–21} illustrating that a combination of multiple LRAs may represent an exciting strategy to increase their potency in provirus reactivation. Undoubtedly, more potent LRAs will stimulate latently HIV-1-infected cells to produce more cell-free and cell-associated infectious virions, which would cause new rounds of infections. Thus, LRAs must be used in combination with ART to prevent new rounds of infections from provirus-activated latently HIV-1-infected cells. However, it is unclear whether ART is able to completely block new rounds of infections from provirus-

activated latently HIV-1-infected cells to uninfected cells. It is also unclear which ART regimen is more effective in prevention of cell-free and cell-associated HIV-1 transmission from provirus-activated cells. In addition, highly potent broadly neutralizing antibodies (nAbs) represent a promising approach to combating HIV-1 infection. These broadly nAbs may also be employed to block cell-free and cell-associated HIV-1 transmission from provirus-activated cells.

In the current work, we conducted kinetic and mechanistic studies of HIV-1 protein expression, virion production, and virus transmission upon provirus reactivation. We then evaluated the blocking efficacy of antiretroviral drugs and broadly nAbs against new infections via cell-free and cell-to-cell transmission of HIV-1 from provirus-activated latently infected cells to an uninfected T cell line and resting human primary CD4⁺ T cells. Our data demonstrated that HIV-1 cell-to-cell transmission was considerably more efficient than that via cell-free virus spread and required F-actin remodeling and CD4, but not chemokine receptors. Certain PIs and HIV-1 broadly nAbs against the CD4-binding site (CD4bs) effectively blocked new rounds of HIV-1 infections from provirus-activated latently infected cells and should be used in clinical application of the “shock and kill” approach.

2 | MATERIALS AND METHODS

2.1 | Cell lines and human primary CD4⁺ T cells

The ACH-2, A3.01, and parental GHOST(3) cell lines were obtained through the NIH AIDS Reagent Program, NIH. ACH-2 is a human T cell line latently infected with HIV-1_{LAV} strain, whereas A3.01 is the parental uninfected cell line. The parental GHOST(3) cell line was derived from a clone of human osteosarcoma cells that stably express human CD4, but neither CCR5 nor CXCR4.²² The GHOST(3) cells were stably transfected with an HIV-2 LTR-GFP construct, which can be turned on to express GFP upon HIV-1 entry and infection. The parental GHOST(3) cell line and its derivatives have been employed for quantitative evaluation of HIV-1 coreceptor use.²³ Cells were cultured in either RPMI 1640 or DMEM medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS), L-glutamine, penicillin, and streptomycin. For GHOST(3) cells, culture medium contains G418 at 500 μ g/ml and puromycin at 1 μ g/ml (Sigma-Aldrich).

Resting human primary CD4⁺ T cells were isolated from peripheral blood mononuclear cells (PBMCs) from healthy blood donors as previously described.¹⁴ Briefly, PBMCs from healthy blood donors were purchased from the Indiana Blood Center for isolation of primary CD4⁺ T cells using the negative selection EasySep reagents (StemCell Technologies). The isolated CD4⁺ T cells were stained with antibodies (Abs) against the human T cell activation markers of CD25 (eBioscience), CD69 (BioLegend), and HLA-DR (BioLegend), followed by flow cytometric analysis to verify that the resting CD4⁺ T cells were activation marker negative as previously described.¹⁴ The purity

of resting human primary CD4⁺ T cells was >97% by flow cytometric analysis.

2.2 | Antiretroviral drugs and broadly neutralizing antibodies against HIV-1

Antiretroviral drugs including a reverse transcriptase (RT) inhibitor (Emtricitabine or FTC), fusion inhibitor (T20), and eight protease inhibitors (PIs) were used to evaluate their blocking activities against HIV-1 cell-to-cell transmission or cell-free infection. T20 was obtained from the NIH AIDS Reagent Program, NIH. FTC was purchased from Selleckchem. Eight PIs were used including Amprenavir (APV; Sigma-Aldrich), Atazanavir (ATV; APExBio), Darunavir (DRV; APExBio), Indinavir (IDV; Sigma-Aldrich), Lopinavir (LPV; Selleckchem), Nelfinavir (NFV; Tocris, Avonmouth), Ritonavir (RTV; Sigma-Aldrich), and Saquinavir (SQV; Tocris, Avonmouth). All antiretroviral drugs were used at concentrations equivalent to their plasma concentrations in HIV-1 patients (Table 1).

Anti-HIV-1 broadly nAbs and non-nAbs were obtained through the NIH AIDS Reagent Program, NIH. Numerous monoclonal Abs (mAbs) with potent neutralizing activity against a broad range of HIV-1 strains across HIV-1 clades have been obtained from HIV-1-infected individuals. Among these broadly nAbs, VRC01, 2G12, PGT121, and 4E10 are four of the best-characterized IgG1 isotype (kappa light chain) mAbs isolated from individuals chronically infected with HIV-1 subtype (or clade) B strains. VRC01 and 2G12 are two nAbs that recognize the conserved gp120 neutralization epitopes of the CD4-binding site (CD4bs) and carbohydrate-rich outer domain region, respectively.^{24–31} PGT121 recognizes the N332 glycan-V3 loop of HIV-1 gp120,²⁵ while 4E10 recognizes adjacent but distinct epitopes in the well-defined cluster of the membrane-proximal external region (MPER) of gp41 ectodomain.³² A32 (IgG1, lambda light chain),³³ a well-characterized non-nAb binding to a highly conserved epitope of HIV-1 gp120, was also used in this study as a control Ab.

TABLE 1 Antiretroviral drugs and their concentrations used

	Name	Concentration
Protease Inhibitor	Amprenavir (APV)	5 μM
	Atazanavir (ATV)	8 nM
	Darunavir (DRV)	9 nM
	Indinavir (IDV)	4 μM
	Lopinavir (LPV)	2 μM
	Nelfinavir (NFV)	5 μM
	Ritonavir (RTV)	5 μM
	Saquinavir (SQV)	3 μg/ml
RT inhibitor	Emtricitabine (FTC)	1 μM
Fusion inhibitor	T20	5 μg/ml

Note: RT inhibitor, reverse-transcriptase inhibitor.

2.3 | Provirus reactivation in latently HIV-1-infected cells

Provirus reactivation in ACH-2 cells was conducted as previously reported.¹⁴ As demonstrated in this report, RMD at an optimum dose of 6 nM can effectively activate proviruses in ACH-2 cells without inducing cell death during a short period of cell culture (up to 24 h poststimulation).¹⁴ RMD at 6 nM was used in this study. Briefly, ACH-2 cells were treated or not treated with RMD at 6 nM in the presence or absence of antiretroviral drugs or nAbs at various concentrations. Stimulated ACH-2 cells were subjected to immunostaining to determine expression of Env on the cell surface, intracellular staining (ICS) of p24 to assess HIV-1-positive cells, and western blot to detect viral protein expression, whereas the supernatant was subjected to measurement of HIV-1 p24 using the p24 Ag ELISA kit (XpressBio) to determine levels of HIV-1 virion production in response to various provirus stimulants.

2.4 | Blockade of cell-free and cell-to-cell transmission of HIV-1 from provirus-activated cells

Cell-free transmission of HIV-1 from provirus-activated ACH-2 cells to A3.01 cells or resting human primary CD4⁺ T cells was carried out using an in vitro transwell coculture system or by directly adding cell-free virions or culture supernatant from RMD- or PMA-treated ACH-2 cells. In 12-well transwell plates, ACH-2 cells (2×10^5 /well) were seeded in the upper chambers, while A3.01 cells or human primary CD4⁺ T cells (0.2×10^5 , 2×10^5 , or 20×10^5 cells per well) were seeded in the lower chambers. The transwell co-cultures were treated with RMD (6 nM), PMA (10 ng/ml)/ionomycin (100 nM), or dimethyl sulfoxide (DMSO). Alternatively, cell-free HIV-1 virions or culture supernatant from RMD- or PMA-treated ACH-2 cells were directly added into A3.01 cell or human primary CD4⁺ T cell cultures. Cells were cultured at 37°C in a 5% CO₂ incubator for up to 24 h. Cells and supernatant were harvested at different time points (0 h, 1 h, 3 h, 6 h, 12 h, and 24 h). Harvested cells were subjected to p24 ICS to determine the percentage of HIV-1-positive A3.01 cells or CD4⁺ T cells representing the efficacy of cell-free transmission of HIV-1, while supernatants were directly subjected to p24 ELISA (XpressBio) to determine levels of HIV-1 virions.

For cell-to-cell transmission of HIV-1, ACH-2 cells were used as HIV-1 donor cells, while A3.01 cells or resting human primary CD4⁺ T cells were used as target cells. ACH-2 cells were labeled with 0.5 μM carboxyfluorescein diacetate succinimidyl ester (CFSE) (Sigma-Aldrich) or TFL-4 (1: 2000 dilution) (Oncolmmunin) for 10 min at room temperature or 45 min at 37°C, respectively. After washing with phosphate-buffered saline (PBS) containing 2% heat-inactivated FBS, labeled ACH-2 cells were cocultured with A3.01 cells or resting human primary CD4⁺ T cells at ratios (donor cells to target cells) of 10:1, 1:1, and 1:10 in the presence or absence of RMD (6 nM), phorbol 12-myristate 13-acetate (PMA, 10 ng/ml)/ionomycin (100 nM), or DMSO. Cells were cultured at 37°C in a 5% CO₂ incubator for up to 24 h. Cells were harvested at different time points (0 h, 1 h, 3 h, 6 h, 12 h, and 24 h) for analysis of target cell infection.

To test the blocking activity of various antiretroviral drugs and nAbs against cell-free or cell-to-cell transmission of HIV-1 from provirus-activated cells to uninfected cells, the cell-free and cell-to-cell transmission systems mentioned above were treated or not treated with individual antiretroviral drugs (RT inhibitor, T20, or PIs), nAbs (VRC01, 2G12, PGT121, or 4E10), or non-nAb A32. Pharmacological antagonists of CCR5 (MVC, NIH AIDS Reagent Program) and CXCR4 (JM2987, NIH AIDS Reagent Program) and blocking Abs against human CD4 (RPA-T4, BioLegend), CXCR4 (12G5 and 44717, NIH AIDS Reagent Program, and α 4 β 7 integrin (Act-1, NIH AIDS Reagent Program) were also used to study their involvements in cell-to-cell transmission of HIV-1 from provirus-activated cells to uninfected cells. CK-548 (Sigma-Aldrich), an inhibitor of actin assembly, was also used to block cell-to-cell transmission of HIV-1 from provirus-activated cells to uninfected cells, as studies have demonstrated that actin in productive HIV-1-infected T cells is required for conservation of viral assembly and budding platforms and for cell-to-cell spread across a virological synapse.^{34,35}

2.5 | Flow cytometric analysis

ACH-2 cells, A3.01 cells, resting human primary CD4⁺ T cells, or their co-cultures were treated or not treated with RMD (6 nM), PMA (10 ng/ml)/ionomycin (100 nM), or DMSO at 37°C for various time intervals ranging from 1 h to 24 h. Cells were subjected to ICS for HIV-1 Gag to determine the percentage of p24-positive cells. The BD Cytotfix/Cytoperm Plus Kit (BD Biosciences) was used for the ICS in accordance with the manufacturer's recommendations. Cells were stained with PE-labeled KC57 mAb (Beckman Coulter) that recognizes p55, p39, p33, and p24 proteins of the core antigens of HIV-1.

To analyze and compare the sensitivity of naïve CD4⁺ T cells (T_N) versus memory CD4⁺ T cells including central memory (T_{CM}) and effector memory (T_{EM}) cells to HIV-1 infection, the resting human primary CD4⁺ T cells cocultured with provirus-activated ACH-2 cells were subjected to surface staining using a panel of fluorochrome-conjugated Abs consisting of CD4^{APC}, CD45RA^{Alxexa Fluor 700}, and CCR7^{BV421} (BioLegend), followed by staining with Fixable Viability Dye (FVD) eFluor 780 (eBioscience) to exclude FVD-positive dead cells. After the cell surface staining, cells were subjected to p24 ICS and subsequently flow cytometric analysis. Cells were gated on live CD4⁺ T cells to determine the sensitivity of T_N (CD45RA⁺CCR7⁺), T_{CM} (CD45RA⁻CCR7⁺), and T_{EM} (CD45RA⁻CCR7⁻) to HIV-1 infection via cell-to-cell transmission from provirus-activated cells. Flow cytometric data of both cell-surface staining and ICS were analyzed using FlowJo V10 software (Tree Star).

2.6 | Confocal and fluorescent microscopy

ACH-2 cells or cocultured ACH-2 with A3.01 cells were treated with RMD (6 nM), PMA (10 ng/ml)/ionomycin (100 nM), or DMSO at 37°C for 24 h. Cells were fixed with 2% paraformaldehyde (PFA) and

subsequently subjected to immunostaining for confocal microscopy analysis of viral protein expression and colocalization of Env with ganglioside M1 (GM1) lipid rafts on the cell surface. Cells were incubated with cholera toxin subunit B (CTB) conjugated with Alexa Fluor 488 (Life Technologies) at 4°C for 20 min to stain GM1 lipid rafts as previously described.³⁶ 2G12, an anti-HIV-1 gp120 mAb cloned from an HIV-1-positive individual, was used as a primary Ab to stain Env. After washing, cells were stained with a secondary Ab of goat anti-human IgG conjugated with DyLight 650 (Abcam). Both primary- and secondary-Ab incubations were carried out for 30 min at 4°C with 2% FBS/PBS. After staining, cells were adhered to poly-L-lysine-coated coverslips and mounted onto glass slides using the ProLong Gold Antifade reagent (Life Technologies) containing 4',6-diamidino-2-phenylindole (DAPI) dye for fluorescent staining of DNA content and nuclei. Cells were analyzed using an Olympus FV1000-MPE confocal/multiphoton microscope fitted with a 60X objective lens. Images were processed and analyzed using the FV10-ASW 3.0 Viewer software (Olympus America Inc.).

To observe the virological synapse, CFSE-labeled A3.01 cells were cocultured with ACH-2 cells in the presence of RMD (6 nM) or PMA (10 ng/ml)/ionomycin (100 nM) at 37°C for 24 h. After incubation, cells were subjected to staining of lipid rafts, Env, and nuclei as described above. Cells were analyzed using Zeiss observer Z1 with ApoTome microscope fitted with a 60X objective lens. Images were processed and analyzed using the axiovision SE64 Rel.4.9.1 (Zeiss).

2.7 | Western blot

ACH-2 cells were treated or not treated with RMD (6 nM), PMA (10 ng/ml)/ionomycin (100 nM), or DMSO for various time intervals ranging from 1 h to 24 h. Cells were harvested and lysed in 1X cell lysis buffer (Cell Signaling Technology) plus 1X Protease Inhibitor Cocktail (Sigma-Aldrich). Cellular lysates were cleared by centrifugation at 13,000rpm for 10 min at 4°C and subjected to NuPAGE Novex high-performance electrophoresis (Invitrogen). Proteins were blotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore). After blocking with 5% nonfat milk, the blot was incubated overnight at 4°C with anti-Gag polyclonal Abs at 1 μ g/ml, anti-Nef mAb (EH1) at 1 μ g/ml, anti-Vif mAb (#319) at 2.4 μ g/ml, and anti-gp120 mAb (2G12) at 1 μ g/ml. Anti-Gag polyclonal Abs were purchased from ViroLabs, while mAbs against Nef, Vif, and gp120 were obtained through the NIH AIDS Reagent Program. After washing, the blot was visualized with the appropriate horseradish peroxidase (HRP)-conjugated secondary Ab and an enhanced chemiluminescence (ECL) detection system (Pierce). To ensure that equal amounts of cellular proteins were analyzed, the same blot was used for the detection of human β -actin protein expression after stripping, blocking, and incubation with mouse anti-human β -actin protein mAb (Abcam) followed by incubation with HRP-conjugated anti-mouse IgG (Jackson Immuno-Research Laboratories) and ECL detection system.

2.8 | Statistical analysis

Data were compared using Student's *t* test. Group data comparisons were analyzed using one-way analysis of variance with the Bonferroni *post hoc* test. A *p* value < 0.05 was considered statistically significant.

3 | RESULTS

3.1 | Kinetic analysis of HIV-1 protein expression and virion production during provirus reactivation

Latently HIV-1-infected cells are phenotypically indistinguishable from uninfected cells and functionally invisible to the immune system as viral protein expression is silenced. However, latently HIV-1-infected cells harbor replication-competent proviruses that can be reactivated *in vitro* and *in vivo* by LRAs such as HDACi.^{14,16} To monitor viral protein expression and virion production from latently HIV-1-infected cells in response to stimulation by LRAs, ACH-2 cells were treated with RMD for various time intervals ranging from 1 h to 24 h. PMA and DMSO were used as positive and negative stimulation controls, respectively. Figure 1 illustrates the kinetic analysis of viral protein expression and virion production during provirus reactivation. HIV-1 Nef protein was detected at 12 h and 3 h, while other viral proteins including Vif, Gag, and Env (gp120) were detected at 24 h and 6 h posttreatment with RMD and PMA, respectively (Figure 1A). Notably, Nef protein was the first viral protein to accumulate to detectable levels in ACH-2 cells treated with either RMD or PMA (Figure 1A). It is well known that Nef is produced as an early protein in productive HIV-1 infection.³⁷⁻³⁹ We found that Nef was detected earlier than other viral proteins in provirus-activated cells, suggesting that Nef functions as an early gene during provirus reactivation. Following Nef protein detection, viral structure proteins including Gag and gp120 were detected in parallel with the appearance of virus particles, as virion-associated p24 was detected in the supernatant of provirus-activated ACH-2 cells (Figure 1B). On the surface of productively infected cells, Env is detected and strongly associated with lipid rafts.⁴⁰ We found a similar association between Env and lipid rafts on the surface of provirus-activated cells (Figure 1C). We also observed multiclustered Env distributions on the surface of provirus-activated ACH-2 cells (Figure 1C, gp120 panel), which exclusively colocalized with cell membrane lipid rafts (Figure 1C).

3.2 | Rapid and efficient cell-to-cell transmission of HIV-1 from provirus-activated latently infected cells to uninfected cells

Cell-to-cell transmission and cell-free spread are two modes of HIV-1 infection. Studies have revealed that cell-to-cell transmission is more efficient than cell-free spread during productive HIV-1 infection.⁴¹

We cocultured provirus-activated ACH-2 cells with A3.01 cells to study cell-to-cell transmission of HIV-1 in a latently HIV-1-infected model. To determine the time course for cell-to-cell transmission of HIV-1, CFSE-labeled ACH-2 cells were cocultured with A3.01 cells in the presence or absence of RMD or PMA. Intracellular p24 in ACH-2 and A3.01 cells was measured at various time points using flow cytometry to determine provirus reactivation and the kinetic transmission of HIV-1 via cell-to-cell contacts. As shown in Figure 2A, intracellular p24 became detectable in CFSE-labeled ACH-2 cells at 3 h posttreatment with RMD and PMA and increased over time. At 24 h posttreatment, p24-positive ACH-2 cells reached approximately 87% and 99% in RMD and PMA conditions, respectively (Figure 2A). Overt cell-to-cell transmission was observed in CFSE-negative A3.01 cells starting at 12 h post-RMD or -PMA treatment with higher efficiency in the PMA-stimulated cultures (Figure 2A,B). At 24 h posttreatment, p24-positive A3.01 cells reached approximately 15.6% and 20.6% in RMD and PMA conditions, respectively (Figure 2A,B). The efficacy of cell-to-cell transmission of HIV-1 was affected by the ratio of donor (HIV-1-positive cells) to target cells (HIV-1-negative cells). We tested 3 different donor-to-target cell ratios (1:10, 1:1, and 10:1) of ACH-2 to A3.01 cells. As shown in Figure 2C, the more donor cells added into the coculture system, the higher cell-to-cell transmission of HIV-1 took place.

In productive HIV-1 infection, virological synapses appear to be the dominant structure involved in cell-to-cell transmission of HIV-1. HIV-1 virological synapses assemble at the site of contact between an infected (donor) and receptor-expressing (target) cell. The assembly of these virological synapses relies on engagement of the viral receptors by Env and a functional actin cytoskeleton in the target cell.³⁵ We found that virological synapses were formed between provirus-activated ACH-2 and A3.01 cells, as Env clusters on the surface of ACH-2 cells were fused with the cytoplasm member of target cells at the site of cell-cell contact (Figure 2D). We also found that CK-548, an inhibitor of actin polymerization that can block cell-free HIV-1 infection of CD4 T cells,⁴² reduced cell-to-cell transmission of HIV-1 from provirus-activated cells to uninfected cells (Figure 2E), suggesting that actin is also involved in the formation of virological synapses to mediate cell-to-cell transmission of HIV-1 from provirus-activated cells to uninfected cells.

In comparison to cell-to-cell transmission, cell-free HIV-1 infection was substantially less efficient. As shown in Figure 2F,G, cell-free virus directly added to A3.01 cells at titers of less than 25 ng/ml of p24 resulted in low or undetectable levels of infection within a 24 h culture. Increased inputs of cell-free virus were able to infect more A3.01 cells. However, cell-free virus at a high titer of 125 ng/ml of p24 resulted in less than 3.5% of p24-positive A3.01 cells, while direct addition of supernatants from RMD- or PMA-treated ACH-2 cells 24 h posttreatment only infected approximately 4% and 6% of A3.01 cells, respectively (Figure 2H). Thus, our results demonstrate that cell-to-cell transmission is a critical mode of HIV-1 dissemination upon provirus reactivation in latently infected cells.

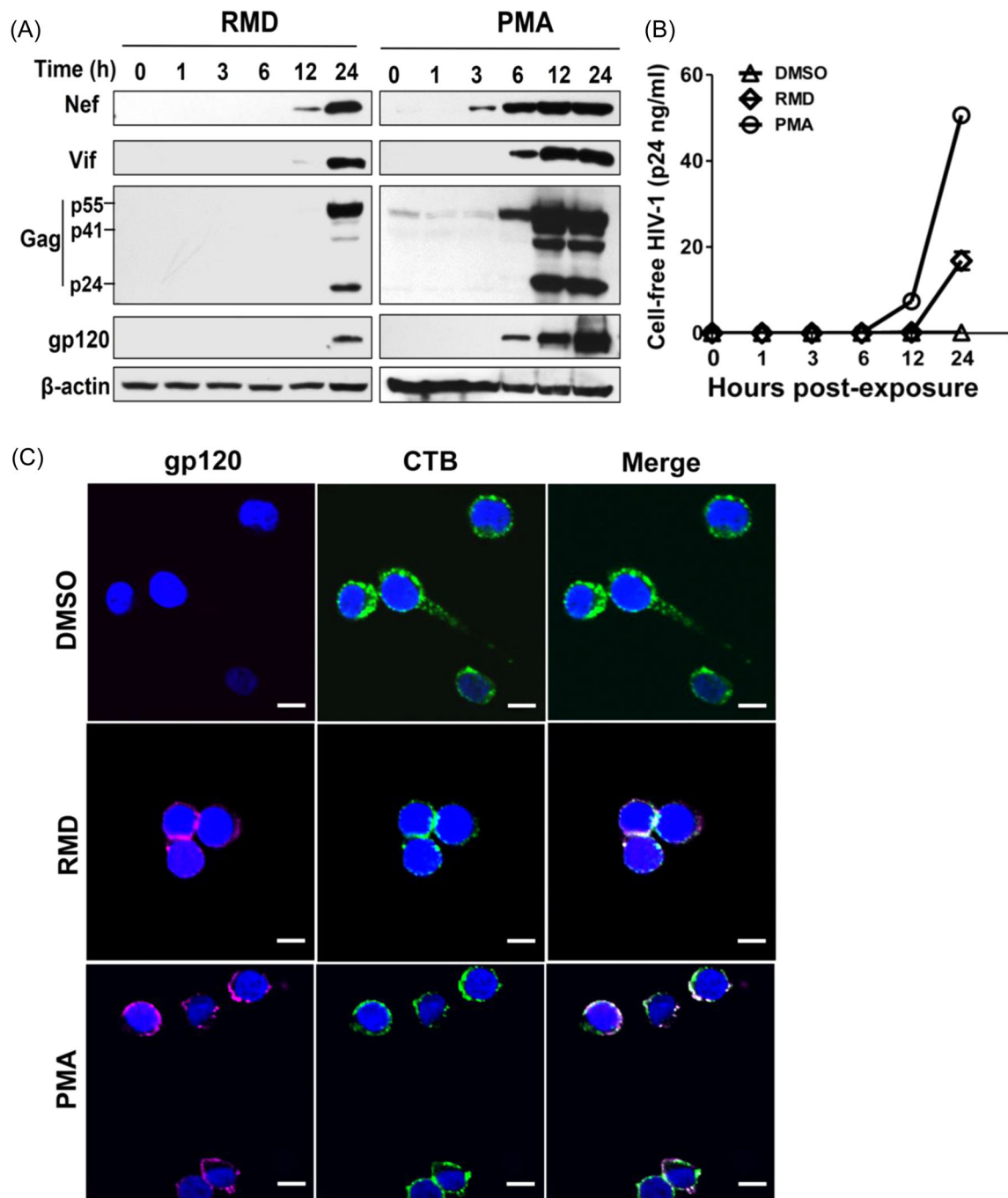


FIGURE 1 Kinetic analysis of HIV-1 protein expression and virion production during provirus reactivation. ACH-2 cells were treated with RMD (6 nM), PMA (10 ng/ml)/ionomycin (100 nM), or DMSO for up to 24 h. Cells and supernatants were harvested at different time points (0 h, 1 h, 3 h, 6 h, 12 h, and 24 h) for analysis of provirus reactivation and production of cell-free virions, respectively. (A) Western blot analysis of HIV-1 protein expression at different time points of provirus reactivation. (B) HIV-1 p24 ELISA titration of cell-free virus particles in the supernatants of provirus-activated ACH-2 cells at different time points. (C) Confocal microscopy analysis of HIV-1 Env expression and colocalization with lipid rafts on the surface of ACH-2 cells upon provirus reactivation. ACH-2 cells were treated or not treated with RMD (6 nM), PMA (10 ng/ml) plus ionomycin (100 nM), or DMSO for 24 h and then subjected to confocal microscopy analysis of Env expression on the cell surface. White color in the Merge panel indicates the colocalization of HIV-1 Env (red) with lipid rafts (green) on the surface of provirus-activated ACH-2 cells. Cellular DNA content and nuclei were stained with DAPI (blue). CTB is a marker for lipid rafts, while gp120 indicates surface staining with 2G12, a mAb against HIV-1 gp120. Scale bars, 5 μ m. The data represent the results from at least three independent experiments. CTB, cholera toxin subunit B; DAPI, 4',6'-diamidino-2-phenylindole; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; RMD, romidepsin.

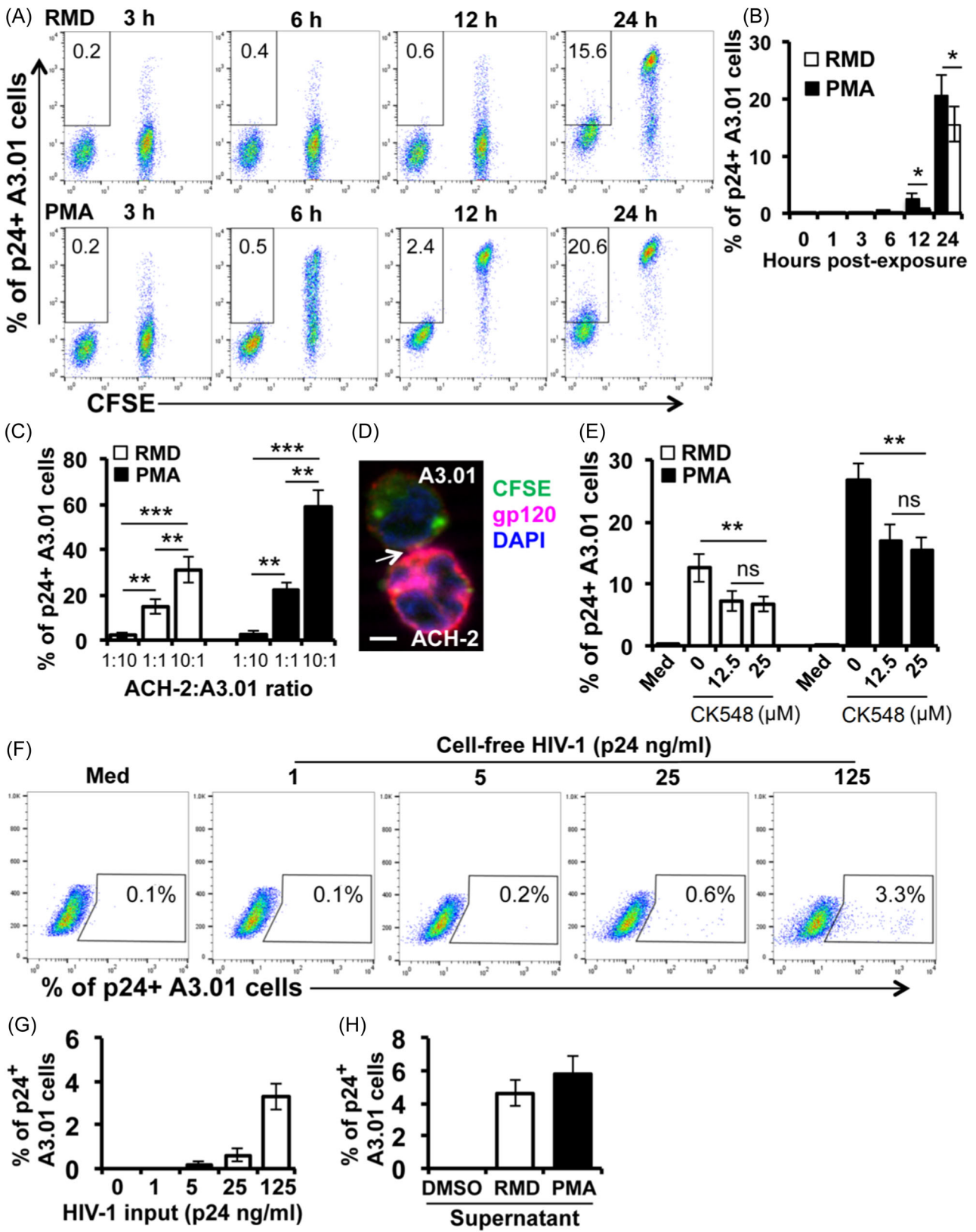


FIGURE 2 (See caption on next page)

3.3 | Blockade of cell-to-cell transmission of HIV-1 from provirus-activated latently infected cells to uninfected cells by antiretroviral drugs and nAbs

Considerable efforts to purge latently HIV-1-infected cells have focused on developing the "shock and kill" approach to force reactivation of the proviruses in these cells. This approach has to be used in combination with antiretroviral drugs to prevent new infections. We evaluated the blocking activities of antiretroviral drugs including an RT inhibitor (FTC), fusion inhibitor (T20), and eight PIs (APV, IDV, SQV, RTV, NFV, LPV, ATV, and DRV) against HIV-1 cell-to-cell transmission or cell-free infection. All antiretroviral drugs were used at concentrations equivalent to their plasma concentrations in HIV-1 patients (Table 1). As shown in Figure 3, PIs and T20 were able to block HIV-1 cell-to-cell transmission, albeit to different degrees. In contrast, RT inhibitor FTC failed to protect A3.01 cells from HIV-1 cell-to-cell transmission (Figure 3A). According to the observed preventive activities against HIV-1 cell-to-cell transmission, the eight PIs tested could be classed into two groups. APV, IDV, SQV, RTV, NFV, and LPV were the best PIs that dramatically inhibited cell-to-cell transmission, and all of these PIs suppressed *de novo* infection to about 1% or less in both RMD and PMA stimulations (Figure 3A). ATV and DRV only partially blocked HIV-1 cell-to-cell transmission, reducing *de novo* infection of A3.01 cells from 15.6% to 7.2% and 6.3% in the RMD condition and 26.9% to 8.7% and 13.2% in the PMA condition (Figure 3A), respectively. The anti-fusion reagent T20 also blocked HIV-1 cell-to-cell spread in a dose-dependent manner (Figure 3B). However, T20 was not as potent as PIs in protecting uninfected cells from HIV-1 infection via cell-to-cell transmission in comparison to the most effective PIs. Taken together, our results suggest that antiretroviral drugs, especially the most effective PIs, can be used in the "shock and kill" approach to prevent new infections of uninfected cells from provirus-activated cells via cell-to-cell contacts.

We also tested whether broadly nAbs including VRC01, 2G12, PGT121, and 4E10 were able to block HIV-1 cell-to-cell transmission from provirus-activated ACH-2 to uninfected A3.01 cells. A32, a non-nAb, was used in parallel as a control Ab. As predicted, non-nAb A32 did not inhibit HIV-1 cell-to-cell dissemination, even at a high concentration of 20 $\mu\text{g/ml}$ (Figure 3C). In contrast, the majority of the nAbs significantly blocked *de novo* infection from cell-to-cell transmission, albeit to different degrees (Figure 3C). VRC01 demonstrated the greatest blocking effect, where 20 $\mu\text{g/ml}$ of VRC01 suppressed *de novo* infection from 12.6% to 0.8% in the presence of RMD stimulation (Figure 3C). Under PMA stimulation, cell-to-cell transmission was reduced from 21.4% to less than 5% (Figure 3C). Compared to VRC01, 2G12 had a weaker ability of inhibition under RMD and PMA stimulation (Figure 3C). Compared with these two nAbs, 4E10 moderately reduced HIV-1 cell-to-cell transmission in both RMD and PMA stimulations, while PGT121 had no effect (Figure 3C). Our data demonstrate that certain nAbs, especially nAbs that recognize the CD4bs of Env such as VRC01, but not non-nAbs, were able to protect uninfected cells from cell-to-cell transmission of HIV-1 from the provirus-activated cells.

We also used the same blocking strategy to evaluate the blocking activities of antiretroviral drugs and nAbs against cell-free virus spread from provirus-activated ACH-2 cells. We found that the antiretroviral drugs and nAbs mentioned above effectively blocked or suppressed new infection as p24-positive A3.01 cells were barely detected (data not shown).

3.4 | Resting human primary CD4⁺ T cells were highly susceptible to cell-to-cell transmission of HIV-1 from provirus-activated cells

Next, we evaluated cell-to-cell transmission of HIV-1 from provirus-activated latently infected cells to resting human primary CD4⁺

FIGURE 2 Cell-to-cell and cell-free transmission of HIV-1 from provirus-activated cells to uninfected cells. (A) Time course for cell-to-cell transmission of HIV-1 from provirus-activated ACH-2 cells to A3.01 cells. CFSE-labeled ACH-2 cells were cocultured with A3.01 cells in the presence or absence of RMD (6 nM), PMA (10 ng/ml)/ionomycin (100 nM), or DMSO for up to 24 h. Intracellular p24 in ACH-2 cells and A3.01 cells was measured at various time points using p24 ICS and flow cytometry to determine provirus reactivation and the kinetic transmission of HIV-1 via cell-to-cell contacts. The ratio of ACH-2 cells to A3.01 cells was 1:1. (B) Pooled data of HIV-1 cell-to-cell transmission from RMD- or PMA/ionomycin-treated ACH-2 cells to A3.01 cells at the donor to target cell ratio of 1:1. The experiments were repeated three times for each test. (C) Pooled data of HIV-1 cell-to-cell transmission from RMD- or PMA/ionomycin-treated ACH-2 cells to A3.01 cells at the donor to target cell ratios of 1:10, 1:1, and 10:1. The experiments were repeated three times for each test. (D) Fluorescent microscopy analysis of the virological synapse between a provirus-activated ACH-2 cell and A3.01 cell. Green, CFSE-labeled A3.01 cells (target cells); Red, provirus-activated ACH-2 cells expressing Env (stained with 2G12); Blue, cellular DNA content and nuclei stained with DAPI (blue). The data represent the results from at least three independent experiments. Scale bars, 5 μm . (E) Inhibition of actin polymerization prevented HIV-1 cell-to-cell transmission from provirus-activated ACH-2 cells to A3.01 cells. RMD- or PMA-treated CFSE-labeled ACH-2 cells were cocultured with A3.01 cells at a ratio of 1:1 in the presence or absence of the actin assembly inhibitor CK548 at various concentrations for 24 h. Cells were subjected to p24 ICS, and subsequently flow cytometric analysis. After gating on CFSE-negative A3.01 cells, p24-positive A3.01 cells were determined. The data were obtained from at least three independent experiments. (F) A representative cell-free virus infection of A3.01 cells. A3.01 cells were infected with cell-free HIV-1 at various concentrations for up to 24 h. Cells were subjected to ICS of p24 to determine the efficacy of cell-free virus infection. (G) Pooled data of cell-free virus infection of A3.01 cells from at least three independent experiments. (H) Pooled data of cell-free virus infection of A3.01 cells by culture supernatant of provirus-activated ACH-2 cells from at least three independent experiments. The results are represented by means \pm SD. Statistical significance (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) is indicated by asterisks. ns, not significant. CFSE, carboxyfluorescein diacetate succinimidyl ester; DAPI, 4',6'-diamidino-2-phenylindole; RMD, romidepsin; SD, standard deviation.

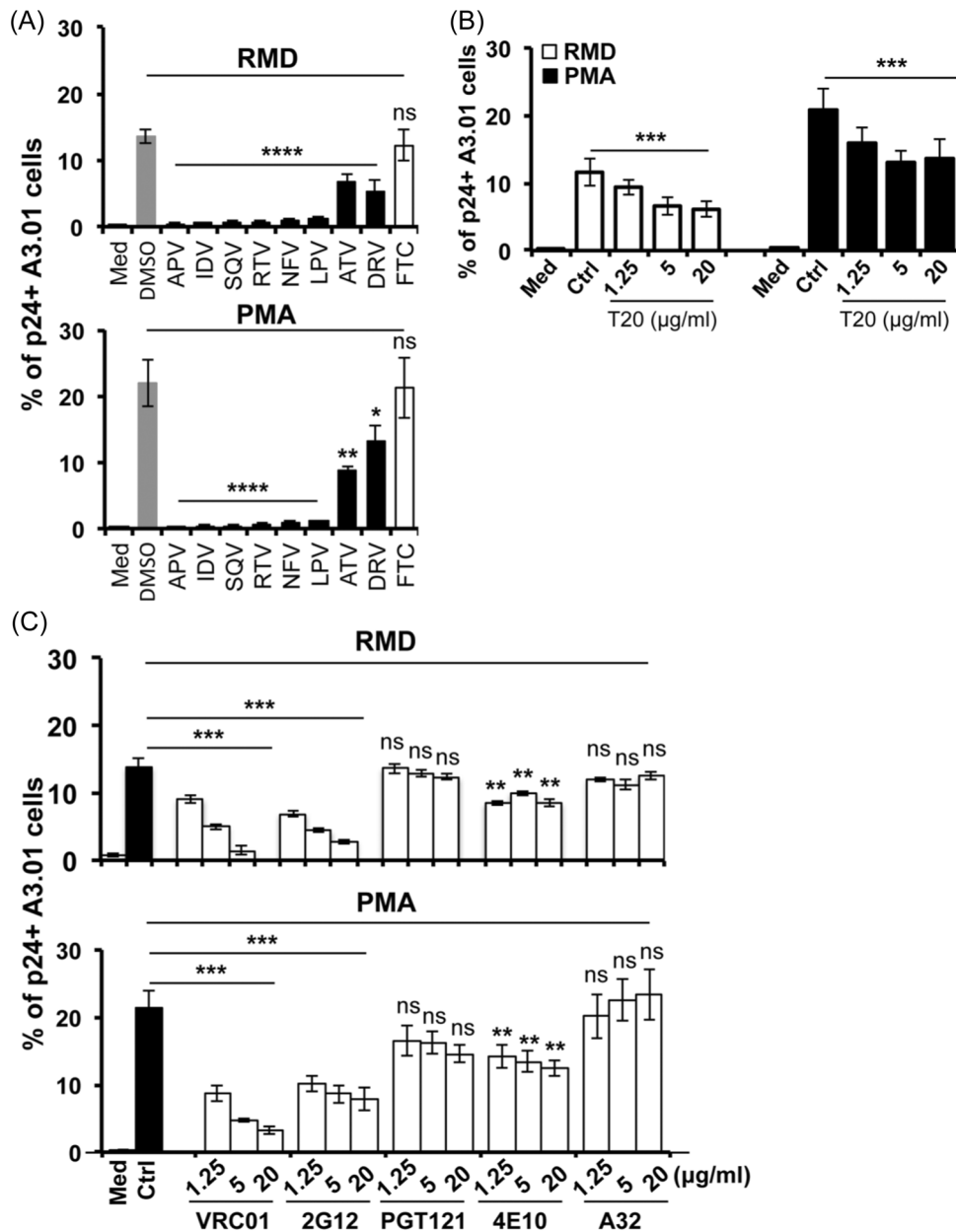


FIGURE 3 Blockade of cell-to-cell transmission of HIV-1 from provirus-activated latently infected cells to uninfected cells by antiretroviral drugs and nAbs. (A) Antiretroviral drugs including RT inhibitor FTC and eight PIs were tested for their activities against HIV-1 cell-to-cell transmission or cell-free infection. FTC and PIs were used at concentrations equivalent to their plasma concentrations in HIV-1 patients (Table 1). Reduction of p24-positive A3.01 cells was used to determine the blocking activities of these antiretroviral drugs. (B) T20 was tested at 0–20 µg/ml. (C) Four nAbs including VRC01, 2G12, PGT121, and 4E10 and one non-nAb (A32) were used at 1.25–20 µg/ml to test their blocking activities against cell-to-cell transmission of HIV-1 from provirus-activated ACH-2 cells to A3.01 cells. Data of infected A3.01 cells were obtained from at least three independent experiments. Statistical significance (** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$) is indicated by asterisks. nAbs, neutralizing antibodies; ns, not significant; RT, reverse transcriptase.

T cells. Surprisingly, we found that resting human primary CD4⁺ T cells were highly susceptible to cell-to-cell transmission of HIV-1 from provirus-activated latently infected cells (Figure 4). As shown in Figure 4A, 33.8% of CD4⁺ T cells became p24-positive 24 h after coculture with RMD-treated ACH-2 cells, after gating on TFL4-negative live cells. The rate of cell-to-cell transmission was significantly higher from provirus-activated ACH-2 cells to resting

human primary CD4⁺ T cells in comparison to A3.01 cells (Figure 4B). Peripheral blood CD4⁺ T cells are phenotypically heterogeneous and include naïve and memory compartments in accordance to differential expression of CD45RA and CCR7. As shown in Figure 4C, three subpopulations of resting CD4⁺ T cells including naïve (T_N, CD45RA⁺CCR7⁺), central memory (T_{CM}, CD45RA⁻CCR7⁺), and effector memory (T_{EM}, CD45RA⁻CCR7⁻) were observed. Analysis of

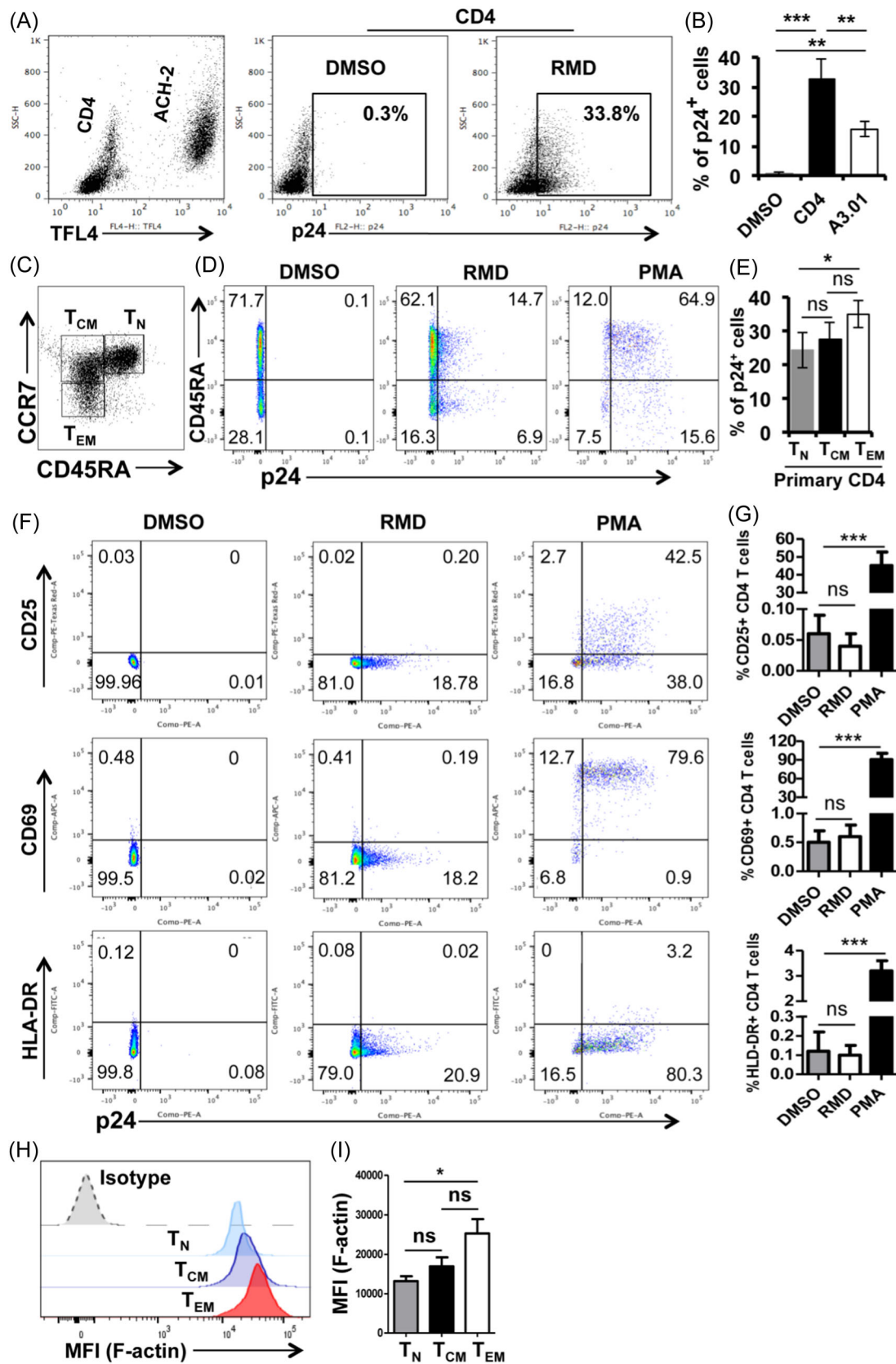


FIGURE 4 (See caption on next page)

the rates of cell-to-cell transmission of HIV-1 from provirus-activated ACH-2 cells to naïve ($CD4^+CD45RA^+$) versus memory ($CD4^+CD45RA^-$) $CD4^+$ T cells revealed that both naïve and memory $CD4^+$ T cells were highly sensitive to HIV-1 infection (Figure 4D). Compared to naïve $CD4^+$ T cells, memory $CD4^+$ T cells were more sensitive to cell-to-cell transmission of HIV-1 from provirus-activated latently infected cells (Figure 4D,E). These results recapitulate the observations that the memory $CD4^+$ T cells in ART-treated or -naïve patients comprises the largest subpopulation of the latently-infected $CD4^+$ T cells,^{43–45} as the memory $CD4^+$ T cells contain high amounts of HIV-1 DNA.⁴⁶

To better understand the mechanisms underlying the high levels of HIV-1 transmitted from provirus-activated ACH-2 cells to human primary $CD4^+$ T cells, we analyzed the activation markers on the surface of human primary $CD4^+$ T cells. Compared with DMSO, RMD did not activate human primary $CD4^+$ T cells in the coculture system, as CD25, CD69, and HLA-DR were at extremely low levels that were not different from DMSO-treated cells (Figure 4F,G). Interestingly, p24-positive cells were exclusively observed within the resting cell population that was negative of CD25, CD69, and HLA-DR (Figure 4F), indicating that resting human primary $CD4^+$ T cells can be directly and robustly infected by HIV-1 through cell-to-cell transmission. In contrast to RMD, PMA drastically activated human primary $CD4^+$ T cells as the levels of CD25, CD69, and HLA-DR were greatly increased (Figure 4F,G). In addition, p24-positive cells were found in both activated and nonactivated $CD4^+$ cell populations in the PMA condition (Figure 4F), further demonstrating that resting human primary $CD4^+$ T cells including naïve and memory subsets can also be directly and robustly infected by HIV-1 through cell-to-cell transmission.

Since HIV-1 cell-to-cell transmission is mainly mediated by Env-induced, actin-dependent virological synapses as we demonstrated in the latently infected cell line model, we hypothesized that human memory $CD4^+$ T cells might have higher levels of F-actin than the naïve $CD4^+$ T cell compartment, which renders memory $CD4^+$ T cells more sensitive to HIV-1 infection via cell-to-cell transmission. To test this hypothesis, we did surface staining of $CD4^+$ T cells with

antibodies against human CD45RA and CCR7, followed by ICS of F-actin. As shown in Figure 4H,I, memory $CD4^+$ T cells, especially T_{EM} subset, had higher levels of cellular F-actin than T_N subset. This finding is consistent with a study which showed higher F-actin contents in memory $CD4^+$ T cells.⁴⁷ Therefore, the high sensitivity of memory $CD4^+$ T cells to HIV-1 infection via cell-to-cell transmission is likely related to their high levels of F-actin expression.

3.5 | Blockade of cell-to-cell transmission of HIV-1 from provirus-activated latently infected cells to resting human primary $CD4^+$ T cells

We used the same blocking strategy described above to evaluate the blocking activities of antiretroviral drugs and nAbs against HIV-1 transmission from provirus-activated ACH-2 cells to primary $CD4^+$ T cells. As shown in Figure 5, six PIs including APV, IDV, SQV, RTV, NFV, and LPV were the best PIs that dramatically inhibited HIV-1 cell-to-cell transmission, while ATV and DRV only exhibited a partial blockade. As expected, RT inhibitor FTC failed to protect primary $CD4^+$ T cells from new infection of HIV-1 (Figure 5). These results were similar to those observed in the prevention of new infection of HIV-1 from provirus-activated ACH-2 cells into A3.01 cells via cell-to-cell contacts. Surprisingly, T20 did not demonstrate any protective activity even at 20 $\mu\text{g/ml}$ (Figure 5), a concentration that protected A3.01 cells from HIV-1 cell-to-cell transmission from provirus-activated ACH-2 cells. We also found that only VRC01 was able to protect primary $CD4^+$ T cells, but not as effectively as in the blockage of HIV-1 transmission from provirus-activated ACH-2 cells into A3.01 cells (Figure 5). Except VRC01, other nAbs including 2G12 and 4E10 exhibited weak, but not significant blocking activities (Figure 5). Thus, PIs including APV, IDV, SQV, RTV, NFV, and LPV persistently exhibit blocking abilities against new infection of A3.01 cells and primary $CD4^+$ T cells from HIV-1 cell-to-cell transmission, while other antiretroviral drugs and nAbs demonstrate variable blocking abilities to different types of target cells.

FIGURE 4 Resting human primary $CD4^+$ T cells were highly susceptible to HIV-1 infection via cell-to-cell transmission. (A) A representative dot plot of flow cytometric data illustrated the sensitivity of resting human primary $CD4^+$ T cells to HIV-1 infection via cell-to-cell transmission. (B) Pooled data demonstrated the sensitivities of resting human primary $CD4^+$ T cells versus A3.01 cells to HIV-1 infection via cell-to-cell transmission from provirus-activated ACH-2 cells. (C) Three subpopulations of resting human primary $CD4^+$ T cells including naïve (T_N , $CD4^+CD45RA^+CCR7^+$), central memory (T_{CM} , $CD4^+CD45RA^-CCR7^+$), and effector memory (T_{EM} , $CD4^+CD45RA^-CCR7^-$) were observed. (D) Both naïve ($CD45RA$ -positive) and memory ($CD45RA$ -negative) human primary $CD4^+$ T cell subsets were highly susceptible to HIV-1 infection via cell-to-cell transmission. (E) Pooled data illustrated that memory $CD4^+$ T cells, especially effector memory (T_{EM}) $CD4^+$ T cells, were more sensitive to cell-to-cell transmission of HIV-1 from provirus-activated latently infected cells. (F) A representative set of flow cytometric dot plots illustrated that resting human primary $CD4^+$ T cells were not activated in RMD-treated co-cultures, but activated in PMA-treated co-cultures. (G) Pooled data showed the activation of resting human primary $CD4^+$ T cells in RMD-treated versus PMA-treated co-cultures. (H) A representative histogram of flow cytometric data illustrated the expression of F-actin in T_N , T_{CM} , and T_{EM} cells. (I) Pooled data demonstrated the expression of F-actin in T_N , T_{CM} , and T_{EM} cells. The experiments were performed with resting human primary $CD4^+$ T cells isolated from six blood donor samples. The number in each dot plot indicates % of cell subpopulation of $CD4^+$ T cells. The results are represented by means \pm SD. Statistical significance ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$) is indicated by asterisks. DMSO, dimethyl sulfoxide; ns, not significant; RMD, romidepsin; SD, standard deviation.

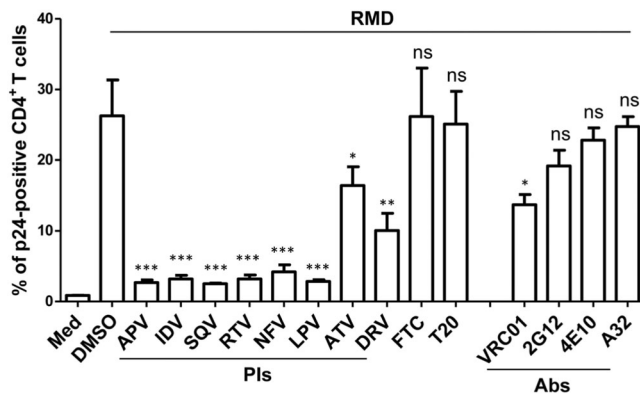


FIGURE 5 Blockage of cell-to-cell transmission of HIV-1 from provirus-activated latently infected cells to resting human primary CD4⁺ T cells by antiretroviral drugs and nAbs. Antiretroviral drugs including eight PIs, RT inhibitor FTC, and T20 were tested for their activities against HIV-1 cell-to-cell transmission. FTC and PIs were used at the concentrations equivalent to their plasma concentrations in HIV-1 patients (Table 1), while T20 was used at 20 μ g/ml. Three nAbs including VRC01, 2G12, and 4E10 and one non-nAb (A32) were used at 20 μ g/ml. Reduction of p24-positive CD4⁺ T cells was used to determine the blocking activities of these antiretroviral drugs and Abs. The results are represented by means \pm SD. Statistical significance (* p < 0.05; ** p < 0.01; *** p < 0.001 vs. DMSO) is indicated by asterisks. DMSO, dimethyl sulfoxide; ns, not significant; RMD, romidepsin; RT, reverse transcriptase; SD, standard deviation

3.6 | Cell-to-cell transmission of HIV-1 required CD4 but not chemokine receptors

HIV-1 utilizes CD4 as the primary receptor together with several members of the chemokine receptor family such as CCR5 and CXCR4 as co-receptors to initiate virus binding and entry. During productive infection, HIV-1 spread between infected and uninfected CD4⁺ T cells is dependent on Env binding to CD4 but independent of coreceptor engagement.⁴⁸ We found that anti-human CD4 Ab blocked cell-to-cell transmission of HIV-1 from provirus-activated ACH-2 cells to primary CD4⁺ T cells in a dose-dependent manner and almost completely blocked this transmission when the Ab was used at 20 μ g/ml (Figure 6A). In contrast, anti-CXCR4 Abs (12G5 and 44717), CXCR4 pharmacological antagonist (JM2987), CCR5 pharmacological antagonist (MVC), and Ab against human α 4 β 7 integrin had no effect on this cell-to-cell infection (Figure 6A). To further validate the results of CD4-dependent but chemokine receptor-independent HIV-1 transmission, we cultured RMD-treated ACH-2 cells with parental GHOST(3) cells, a clone of human osteosarcoma cells that stably express human CD4, but neither CCR5 nor CXCR4.²² We found that GHOST(3) cells were still susceptible to HIV-1 infection via cell-to-cell transmission (Figure 6B,C), and this cell-to-cell infection was profoundly blocked by anti-human CD4 Ab (Figure 6B,C). Thus, cell-to-cell transmission of HIV-1 from provirus-activated cells to uninfected cells requires CD4, but not chemokine receptors.

4 | DISCUSSION

One of the most significant achievements of modern biomedical research has been the discovery and widespread use of ART for the treatment of HIV-1 patients. Since the introduction of ART in 1996, ART has saved millions of lives and dramatically increased the life expectancy of HIV-1 patients. However, ART alone cannot eradicate HIV-1 infection. The major barrier to HIV-1 eradication is the tremendously stable latent reservoir of HIV-1 in resting CD4⁺ T cells harboring replication-competent proviruses.^{44,49} These cells are phenotypically indistinguishable from uninfected cells and functionally invisible to the immune system as viral protein expression is silenced. To purge these cells, several strategies such as the “shock and kill” approach have been developed.^{14,50–52} The “shock and kill” approach employs LRAs to force provirus reactivation to induce cell killing by viral CPEs, host immune responses, or both. In this study, we carried out kinetic and mechanistic studies of HIV-1 protein expression, virion production, and virus transmission and blockade during provirus reactivation in the “shock and kill” approach. We used RMD as an LRA in our study, as RMD exhibits potent activity in activation of proviruses in latently HIV-1-infected cells in vivo and in vitro without causing significant cytotoxicity.^{14,53} We found that provirus-activated ACH-2 cells in response to stimulation with RMD produced Nef early, followed by detection of HIV-1 structural proteins (Gag and Env) that are in parallel with the appearance of virions (Figure 1A,B). These results suggest that Nef functions as an early gene during provirus reactivation. Confocal microscopy analysis revealed that Env presented as multiclustered distributions on the surface of provirus-activated ACH-2 cells, which exclusively colocalized with cell membrane lipid rafts (Figure 1C). Since Env molecules on the surface of productively HIV-1-infected cells interact with CD4 molecules on the target cells to initiate the formation of virological synapses to mediate cell-to-cell transmission of HIV-1, Env on the surface of provirus-activated cells likely plays a similar role. In fact, Env on the surface of provirus-activated ACH-2 cells together with cellular F-actin was involved in cell-to-cell transmission via virological synapses, as the fusion of Env on the surface of provirus-activated cells with target cell cytoplasm membrane was observed (Figure 2D). In addition, Abs against CD4 and the CD4-binding site of Env and an F-actin blocker effectively blocked cell-to-cell transmission of HIV-1 from provirus-activated cells to uninfected cells (Figures 2E, 3, and 6). Thus, the “shock and kill” approach rapidly generates cell-free and cell-associated virions that must be blocked from provirus-activated cells to uninfected cells by the ongoing ART regimen.

Studies have revealed that CD4⁺ T cells harboring replication-competent proviruses represent a major latent HIV-1 reservoir in both the peripheral blood and lymphatic organs such as lymph nodes and mucosa-associated lymphoid tissue (MALT).⁵⁴ Given that the vast majority of lymphocytes are distributed in lymphatic organs where most HIV-1 infection takes place in infected patients and that only 2% of the total amount of lymphocytes are in the peripheral circulation,⁵⁵ the frequency of latently infected cells is substantially more in lymphatic organs than in the peripheral circulation. In fact,

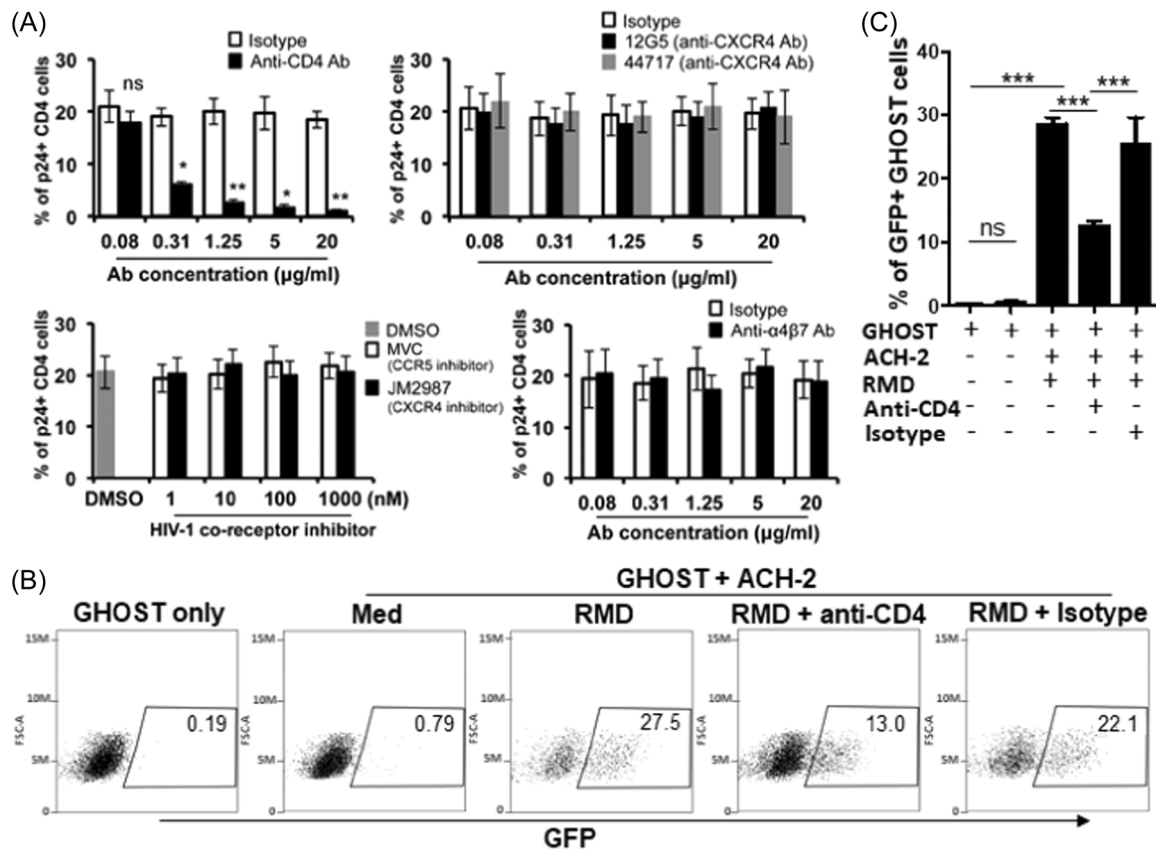


FIGURE 6 HIV-1 cell-to-cell transmission requires CD4 but not chemokine co-receptors. (A) Abs against human CD4, CXCR4, and $\alpha 4\beta 7$ integrin and pharmacological antagonists of CXCR4 (JM2987) and CCR5 (MVC) were tested for their inhibitory activities against HIV-1 cell-to-cell transmission from provirus-activated ACH-2 cells to resting human primary CD4⁺ T cells. Each Ab was tested at concentrations ranging from 0.08 to 20 µg/ml. Antagonists of CXCR4 (JM2987) and CCR5 (MVC) were tested at the concentrations ranging from 1 to 1000 nM. (B) A representative set of flow cytometric data illustrated the susceptibility of GHOST(3) cells that were stably transfected with an HIV-2 LTR-GFP construct, which can be turned on to express GFP upon HIV-1 entry and infection, to HIV-1 infection via cell-to-cell transmission. GHOST(3) cells stably transfected with an HIV-2 LTR-GFP construct were cocultured with RMD-treated ACH-2 cells (RMD at 6 nM for 24 h) for 24 h, followed by flow cytometric analysis. The number in each flow cytometric dot plot indicated % of GFP-positive GHOST(3) cells. (C) Data were summarized from three separated experiments. The results are represented by means \pm SD. Statistical significance ($*p < 0.05$; $**p < 0.01$; $***p < 0.001$) is indicated by asterisks. ns, not significant; RMD, romidepsin; SD, standard deviation

single-cell sequencing analysis of paired lymph node and peripheral blood samples from HIV-1 patients has revealed that the frequency of latently infected memory CD4⁺ T cells from lymph node tissue is up to 17 times higher than that in the memory CD4⁺ T cells from peripheral blood.⁵⁶ Since the vast majority of latently infected cells reside in lymphatic organs where cells are tightly packed, cell-to-cell transmission of HIV-1 from latently infected cells upon provirus reactivation is likely more significant than the via cell-free virus spread. We found that overt HIV-1 cell-to-cell transmission was observed 12 h poststimulation with RMD or PMA and increased over time of co-cultures (Figure 2A,B). At 24 h posttreatment, p24-positive A3.01 cells reached approximately 15.6% and 20.6% in RMD and PMA conditions, respectively (Figure 2A,B). Surprisingly, resting human primary CD4⁺ T cells were substantially more susceptible to cell-to-cell transmission of HIV-1 from provirus-activated cells (Figure 4A,B), increasing from $15.6 \pm 1.6\%$ ($n = 6$) of p24-positive A3.01 cells to $32.8 \pm 6.3\%$ ($n = 6$) of p24-positive CD4⁺ T cells 24 h

after coculture with RMD-treated ACH-2 cells (Figure 4). In contrast, the efficacy of cell-free virus transmission from provirus-activated ACH-2 to A3.01 cells was low (Figure 2F-H) and less than 1% to resting primary human CD4⁺ T cells (data not shown). These results suggest that cell-to-cell transmission is a more efficient and rapid means of viral spread and the predominant mode of HIV-1 transmission in provirus-activated cells in the “shock and kill” application.

The high sensitivity of human primary CD4⁺ T cells to HIV-1 infection via cell-to-cell transmission from provirus-activated cells is not due to activation of these cells, as RMD did not increase cell activation markers such as CD25, CD69, and HLA-DR on the cell surface (Figure 4F,G). In addition, p24-positive cells in the RMD-treated group were exclusively observed within the resting cell population that was negative of CD25, CD69, and HLA-DR (Figure 4F). These data indicate that resting human primary CD4⁺ T cells can be directly and robustly infected by HIV-1 through

cell-to-cell transmission from provirus-activated cells. These resting human primary CD4⁺ T cells are phenotypically heterogeneous and include naïve and memory compartments in according to differential expression of CD45RA and CCR7. As shown in Figure 4C, three subpopulations of CD4⁺ T cells including naïve (T_N, CD4⁺CD45RA⁺CCR7⁺), central memory (T_{CM}, CD4⁺CD45RA⁻CCR7⁺), and effector memory (T_{EM}, CD4⁺CD45RA⁻CCR7⁻) were observed. Analysis of the rates of cell-to-cell transmission of HIV-1 from provirus-activated ACH-2 cells to naïve (CD4⁺CD45RA⁺) versus memory (CD4⁺CD45RA⁻) CD4⁺ T cells revealed that both naïve and memory CD4⁺ T cells were highly sensitive to HIV-1 infection (Figure 4D). Compared to naïve CD4⁺ T cells, memory CD4⁺ T cells, especially effector memory (T_{EM}) CD4⁺ T cells, were more sensitive to cell-to-cell transmission of HIV-1 from provirus-activated latently infected cells (Figure 4D,E). Since HIV-1 cell-to-cell transmission is mainly mediated by Env-induced, actin-dependent virological synapses as we demonstrated in the latently infected cell line model, we hypothesized that human memory CD4⁺ T cells might have higher levels of F-actin than the naïve CD4⁺ T cell compartment, which renders memory CD4⁺ T cells more sensitive to HIV-1 infection via cell-to-cell transmission. To test this hypothesis, we did surface staining of CD4⁺ T cells with antibodies against human CD45RA and CCR7, followed by ICS of F-actin. As shown in Figure 4H,I, memory CD4⁺ T cells, especially T_{EM} subset, had higher levels of cellular F-actin than T_N subset (Figure 4H,I). Therefore, the high sensitivity of memory CD4⁺ T cells to HIV-1 infection via cell-to-cell transmission is likely related to their high levels of F-actin expression. These findings infer the mechanisms by which memory CD4⁺ T cells in HIV-1 patients, treated or untreated with ART, comprises the largest subpopulation of the latently-infected CD4⁺ T cells,^{43–45} as these memory CD4⁺ T cells contain high amounts of HIV-1 DNA.⁴⁶ Taken together, both naïve and memory CD4⁺ T cells are highly susceptible to HIV-1 infection via cell-to-cell spread and prevention of HIV-1 cell-to-cell transmission is crucial in clinical practice of the “shock and kill” approach.

Antiretroviral drugs, especially PIs, and broadly nAbs can effectively block cell-free and cell-to-cell transmission of HIV-1 from productively infected cells.^{57,58} We evaluated the blocking activities of eight PIs (APV, NFV, RTV, SQV, LPV, IDV, ATV, and DRV) and four nAbs (VRC01, 2G12, 4E10, and/or PGT121) against cell-free and cell-to-cell transmission of HIV-1 from provirus-activated ACH-2 to A3.01 cells or resting primary human CD4⁺ T cells. The fusion inhibitor T20 and RT inhibitor FTC were also included in the blocking study. We found that all antiretroviral drugs (PIs, T20, and FTC) and nAbs effectively blocked or suppressed new infection via cell-free virus spread (data not shown). In contrast, only six PIs including APV, IDV, SQV, RTV, NFV, and LPV dramatically inhibited cell-to-cell transmission of HIV-1 from provirus-activated cells to A3.01 or resting primary human CD4⁺ T cells (Figures 3 and 5), while the other two PIs (ATV and DRV) exhibited partial blockage of HIV-1 cell-to-cell transmission (Figures 3 and 5).

The anti-fusion reagent T20 moderately protected A3.01 cells, but not primary human CD4⁺ T cells, from HIV-1 cell-to-cell spread

from provirus-activated cells to A3.01 cells (Figures 3 and 5). This discrepancy might be due to the differences of expression level of CD4 in primary cells and immortalized cells. One study showed that although the three main actin network architectures in immortalized T cells (Jurkat T cells) are similar to that in primary T cells, there are differences in the organization and molecular mechanisms underlying these networks.⁵⁹ This might be another reason why T20 worked differently in primary cells and cell lines. The RT inhibitor FTC failed to protect either A3.01 cells or primary human CD4⁺ T cells from HIV-1 cell-to-cell transmission (Figures 3 and 5). Our data suggest that the RT inhibitors do not prevent virion release from capsid and transferring from the donor cells to new target cells. However, they likely prevent *de novo* virion production from the newly infected target cells. Chemokine coreceptors might be more critical for primary HIV infection than established infection. Therefore, CCR5 or CXCR4 antagonist would not be effective at blocking cell-associated HIV-1 transmission from provirus-activated cells.

VRC01 effectively blocked *de novo* infection of both the A3.01 cell line and primary human CD4⁺ T cells from HIV-1 cell-to-cell transmission (Figures 3 and 5), whereas other nAbs including 2G12, PGT121, and 4E10 moderately blocked HIV-1 cell-to-cell transmission from provirus-activated ACH-2 cells to the A3.01 cell line, but not primary human CD4⁺ T cells (Figures 3 and 5). Our data imply that certain nAbs, especially nAbs that recognize the CD4bs of Env such as VRC01 are able to protect uninfected cells from cell-to-cell transmission of HIV-1 from the provirus-activated cells. Our results are consistent with the notion that virological synapse-dependent viral transmission requires CD4-Env interaction. Therefore, PIs including APV, IDV, SQV, RTV, NFV, and LPV persistently exhibit blocking activities against new infection of A3.01 cells and primary CD4⁺ T cells from HIV-1 cell-to-cell transmission, while other antiretroviral drugs and nAbs demonstrate variable blocking activities to different types of target cells.

There are currently more than 25 antiretroviral drugs licensed and used for the treatment of HIV-1 infections, and these drugs are organized into six major classes by how they interfere with steps of the HIV-1 life cycle (<http://www.niaid.nih.gov/topics/hivaids/understanding/treatment/Pages/arvDrugClasses.aspx>). The major classes include (1) fusion inhibitors that interfere with the virus ability to fuse with a cellular membrane, thereby preventing HIV-1 from entering a cell; (2) two types of RT inhibitors (nucleoside reverse-transcriptase inhibitors or NRTI and non-nucleoside reverse-transcriptase inhibitor or NNRTI) that prevent the HIV-1 enzyme RT from converting single-stranded viral genome RNA into double-stranded viral DNA; and (3) PIs that bind the viral protease active site with high affinity to inhibit cleavage of viral polypeptides and subsequent maturation of the virion from infected cells. The additional three antiretroviral drug classes are the entry inhibitors, maturation inhibitors, and integrase inhibitors. Antiretroviral drugs are usually used in combinations of three or more drugs from more than one class to increase therapy efficacy, overcome problems of tolerance, and decrease emergence of viral resistance. According to the World Health Organization (WHO) recommendations, the

first-line ART regimen for patients chronically infected with HIV-1 should consist of two NRTIs such as tenofovir (TDF) and lamivudine (3TC) or emtricitabine (FTC) plus one NNRTI, efavirenz (EFV) or nevirapine (NVP).⁶⁰ This regimen or similar treatment plans can also be used as pre-exposure prophylaxis (PrEP) to prevent HIV-1 infection,^{61–63} although prevention efficacy does not reach 100%.

Our data demonstrate that CD4bs nAb and PIs, but not fusion inhibitor T20 or RT inhibitor FTC, effectively block cell-to-cell of HIV-1 from provirus-activated latently infected cells. The first-line ART regimen containing NRTI/NNRTI effectively suppresses virus replication in productively infected cells by inhibiting RT activity, but affect neither the production of new virions nor viral transmissions from provirus-activated latently infected cells as these events take place after the reverse transcription step of the HIV-1 life cycle in latently infected cells. Therefore, the ART regimen to be used together with the “shock and kill” approach should be optimized with RTs plus PIs to simultaneously prevent and inhibit new infection from provirus-activated cells.

AUTHOR CONTRIBUTIONS

Qigui Yu conceived and designed the work and contributed to manuscript writing. Jie Lan and Wei Li performed data processing work and contributed to manuscript writing. Richard Yu and Fahim Syed contributed to data analysis and manuscript writing. All authors contributed to the data interpretation, revision of the manuscript and approved the final manuscript version.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request. All data created during this study are included in this article. Further enquiries can be directed to the corresponding author.

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