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## Provirus activation plus CD59 blockage triggers antibody-dependent complement-mediated lysis of latently HIV-1-infected cells

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### Abstract

Latently HIV-1-infected cells are recognized as the last barrier towards viral eradication and cure. To purge these cells, we combined a provirus stimulant with a blocker of human CD59, a key member of the regulators of complement activation (RCA), to trigger antibody-dependent complement-mediated lysis (ADCML). Provirus stimulants including prostratin and histone deacetylase inhibitors (HDACi) such as romidepsin (RMD) and suberoylanilide hydroxamic acid (SAHA) activated proviruses in the latently HIV-1-infected T cell line ACH-2 as virion production and viral protein expression on the cell surface were induced. RMD was the most attractive provirus stimulant as it effectively activated proviruses at nanomolar concentrations that can be achieved clinically. Antiretroviral drugs including two protease inhibitors (atazanavir and darunavir) and a reverse transcriptase inhibitor (emtricitabine) did not affect the activity of provirus stimulants in the activation of proviruses. However, saquinavir (a protease inhibitor) markedly suppressed virus production, although did not affect the percentage of cells expressing viral Env on the cell surface. Provirus-activated ACH-2 cells expressed HIV-1 Env that colocalized with CD59 in lipid rafts on the cell surface, facilitating direct interaction between them. Blockage of CD59 rendered provirus-activated ACH-2 cells and primary human CD4<sup>+</sup> T cells that were latently infected with HIV-1 sensitive to ADCML by anti-HIV-1 polyclonal antibodies or plasma from HIV-1-infected patients. Therefore, a combination of provirus stimulants with RCA blockers represents a novel approach to eliminate HIV-1.

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## Keywords

HIV-1; latency; provirus; CD59; antibody; complement; cytotoxicity

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## Introduction

The eradication of human immunodeficiency virus type 1 (HIV-1) from infected individuals remains a major medical challenge. Antiretroviral therapy (ART) has led to a profound reduction in HIV-1-related morbidity and mortality. However, ART fails to eliminate the virus *in vivo*. Most HIV-1-infected patients on ART still have residual viremia measurable by ultrasensitive assays, and many of them also experience transient episodes of viremia (termed viral blips) above the detection limit of standard diagnostic assays (1–3). The persistent viremia mainly arises from replication-competent proviruses in long-lived latently infected cells, which represent a major obstacle for HIV-1 eradication (4–8).

Efforts to purge long-lived latently infected cells have focused on activation of the proviruses in these cells. It is presumed that these infected cells will be killed after activation of virus gene expression by viral cytopathic effects (CPEs), host immune responses or both. This has been known as the “shock and kill” approach (9, 10). Several stimulants including IL-7, prostratin, and histone deacetylase inhibitors (HDACi) such as valproic acid (VPA) and suberoylanilide hydroxamic acid (SAHA or Vorinostat) have been explored to force activation of proviruses in latently-infected resting CD4<sup>+</sup> T cells that constitute the major reservoir of HIV-1 *in vivo*. However, treatment with IL-7, VPA or SAHA in patients on ART has failed to reduce HIV-1 latency (11–14), suggesting that these stimulants alone are not sufficient to induce killing of latently infected cells. The underlying mechanisms of the failure to induce cell killing are unclear, but may include: (1) HIV-1 replication elicits little CPEs *in vivo* (15–19), (2) impaired host immunity in chronically HIV-1-infected patients is not fully restored with ART (20–24), and (3) these stimulants are not potent enough to fully activate proviruses in latently infected cells, and the cells can then survive long enough to revert back to a resting memory state if they are not killed (7, 25). A recent study has demonstrated that cytolytic T lymphocytes (CTLs) from HIV-1-infected patients on ART require extra antigen-specific stimulations in order to kill latently-infected CD4<sup>+</sup> T cells after provirus activation (26). Therefore, there is an urgent need to improve this “shock and kill” approach by identifying more potent proviral stimulants in conjunction with agents that enable anti-HIV-1 immunity to specifically kill latently infected cells after provirus activation.

Both HIV-1-infected cells and virions use their surface regulators of complement activation (RCA) to resist antibody-dependent complement-mediated lysis (ADCML) (27–31). In HIV-1-infected patients, the virus-specific antibody (Ab) response is vigorous at all stages of infection. Within a few weeks of infection, Abs against the viral envelope (Env or gp120 plus gp41), core (Gag), and matrix (p17) become detectable in the plasma of HIV-1-positive individuals (32–37). Ab levels mount in response to the gradual increase in viral load and appear to be maintained at high levels throughout the disease (38, 39). However, the vigorous and sustained Ab response has a limited effect on controlling virus proliferation or

on protecting the patients from developing AIDS (38, 40–42). Recent studies have demonstrated that Abs present in the circulation of HIV-1-infected patients are able to specifically recognize and bind both autologous and heterologous primary HIV-1 isolates (43), but fail to complete ADCML against virions and infected cells due to the presence of RCA in the HIV-1 Env or on the membrane of the infected cells (27–29). Abrogation of the biological function of RCA members, particularly CD59 (a key RCA member that controls formation of the membrane attack complex at the terminal stage of the complement activation cascades via all three activation pathways), has rendered both HIV-1-infected cells and virions sensitive to ADCML (27–31, 44). Thus, a combination of provirus stimulants and RCA blockers would allow anti-HIV-1 Abs naturally present in HIV-1-infected individuals to trigger ADCML of latently infected T cells upon provirus activation. This would represent a novel approach to promote specific killing of latently HIV-1-infected cells towards an HIV-1 cure.

Using ACH-2 cells (a human T cell line latently infected with HIV-1<sub>LAV</sub> strain) as an *in vitro* latency model, we evaluated the biological effects of prostratin and HDACi including romedepsin (RMD) and SAHA on provirus activation. We then evaluated ADCML efficacy of provirus-activated cells that were latently infected with HIV-1 in the presence of a blocker of human CD59 (hCD59) plus anti-HIV-1 Abs or plasma from HIV-1-infected patients who were on ART or ART-naïve. Our data demonstrated that prostratin and HDACi, particularly RMD, activated provirus to induce both virion production and viral protein expression on the cell surface. Blockage of CD59 rendered provirus-activated cells that were latently infected with HIV-1 including ACH-2 cells and primary human CD4<sup>+</sup> T cells sensitive to ADCML by anti-HIV-1 Abs or plasma from HIV-1-infected patients.

## Materials and Methods

### Blood samples from HIV-1-infected and uninfected donors

Peripheral blood was collected in BD Vacutainer tubes (containing 158 USP units of sodium heparin per 10-ml tube, BD, Franklin Lakes, NJ) from 6 ART-naïve HIV-1-infected patients, 9 virologically suppressed HIV-1-infected patients on ART with viral RNA less than 50 copies/ml of plasma for 6 months or more (Table 1), and 5 HIV-1-negative (healthy) blood donors. Plasma was separated from blood cells by centrifugation and then stored at -80°C until use. Blood cells were subjected to isolation of red blood cells (RBCs) and peripheral blood mononuclear cells (PBMCs) using the Ficoll-Hypaque density gradient centrifugation (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Fresh RBCs isolated from healthy blood donors were subjected to a hemolytic assay to determine biological activity of complement in plasma from HIV-1-infected patients and the optimal concentration of BRIC229, a mouse monoclonal Ab (mAb) widely used to block hCD59 without activating complement activity (IBGRL, Bristol, UK), for abrogation of hCD59 function (45). Written informed consent was provided by each participant. All investigational protocols were approved by the Indiana University Institutional Review Board (Indianapolis, IN).

## Hemolytic assay

Fresh RBCs isolated from healthy blood donors were washed four times with phosphate buffered saline (PBS), and then were used for a hemolytic assay as previously described (44). Briefly, RBCs (5,000 cells) were incubated with BRIC229 at various concentrations ranging from 1–20  $\mu\text{g}/\text{ml}$  at 37°C for 30 min to abrogate hCD59 function. Cells were then incubated with rabbit anti-human RBCs polyclonal Abs (pAbs, MyBioSource, San Diego, CA) plus serially diluted plasma from HIV-1-infected patients or healthy blood donors. Every experiment included heat-inactivated plasma as a control of complement inactivation to obtain background hemolysis, while total hemolysis was obtained by adding pure water to the RBCs pellet. All experiments were carried out in triplicates. The amount of hemoglobin released from lysed RBCs was determined by the optical density (OD) of the supernatant at 414 nm, and the percent lysis was calculated as follows:  $(\text{experimental OD}_{414} - \text{background OD}_{414})/(\text{total lysis OD}_{414} - \text{background OD}_{414}) \times 100$ .

## Latently HIV-1-infected cells and provirus activation

J-Lat GFP Clone A72, ACH-2 and A3.01 cells were obtained through the NIH AIDS Reagent Program (Germantown, MD). The J-Lat GFP Clone A72 cell line contains integrated but transcriptionally latent HIV-1 proviruses. The cell line was generated by transduction of Jurkat cells with the HIV-1-based vector consisting of Tat and enhanced green fluorescent protein (GFP) under the control of the HIV-1 LTR (5'-LTR-Tat-IRES-GFP-LTR-3') (46, 47). J-Lat GFP Clone A72 cells are GFP-negative under basal conditions and become GFP-positive after activation of HIV-1 latency (46, 47). ACH-2 is a human T cell line latently infected with HIV-1<sub>LAV</sub> strain, while A3.01 is the parental uninfected cell line. Cells were grown in the complete RPMI 1640 medium (RPMI 1640 medium supplemented with 10% FBS, 5% penicillin-streptomycin, and 2 mM glutamine). Cells were untreated or treated for 24 - 72 h with RMD, SAHA, or prostratin at various concentrations in the presence or absence of antiretroviral drugs at various concentrations. Reverse transcriptase (RT) inhibitor such as emtricitabine (FTC, Selleckchem, Houston, TX) or protease inhibitor (PIs) such as saquinavir (SQV, Tocris, Bristol, UK), atazanavir (ATV, ApexBio, Houston, TX) or darunavir (DRV, ApexBio, Houston, TX) was used. These stimulants were purchased from Sigma-Aldrich (St. Louis, MO). Phorbol myristyl acetate (PMA at 10 ng/ml; Sigma, St. Louis, MO) plus ionomycin (1  $\mu\text{g}/\text{ml}$ ; Sigma, St. Louis, MO) and DMSO were used as positive and negative stimulation controls, respectively. Stimulated J-Lat GFP Clone A72 cells were directly subjected to fluorescence microscopy analysis using Evo FL Auto Cell Imaging System (Life Technologies, Grand Island, NY). Stimulated ACH-2 cells or A3.01 cells were subjected to immunostaining to determine expression of HIV-1 Env on the cell surface, while the supernatant was subjected to measurement of HIV-1 p24 using the p24 Antigen ELISA kit (PerkinElmer, Santa Clara, CA) to determine levels of HIV-1 production in response to various provirus stimulants.

## ADCML of ACH-2 cells by anti-HIV-1 Env pAbs

Antibody-dependent complement-mediated lysis (ADCML) of latently HIV-1-infected ACH-2 cells was quantified using the calcein release assay. Briefly, ACH-2 or A3.01 cells untreated or treated with RMD, PMA/ionomycin, or DMSO in the presence or absence of

antiretroviral drugs were labeled at 37°C for 40 min with 15 µM calcein-AM dye (Invitrogen, Carlsbad, CA) in 200 µl of PBS. The cells were washed three times with PBS to remove the free calcein-AM dye, and resuspended in the complete RPMI 1640 medium at a concentration of  $1 \times 10^6$  cells per ml. Cells were dispensed into round-bottom 96-well microplates (100 µl containing 10,000 cells per well). Cells were pre-incubated for 30 min at 37°C with or without BRIC229 (20 µg/ml) in the presence of anti-gp120/gp160 pAbs at various concentrations (Abcam, Cambridge, MA). After the pre-incubation, 50 µl of the pooled sera from three healthy blood donors were added as a source of complement. The microplates were incubated at 37°C for 1 h, and followed by centrifugation at 130 g for 5 min. After the centrifugation, 100 µl of supernatant from each well was transferred into fresh 96-well microplates for measuring the fluorescence intensity using an automated fluorescence plate reader (Molecular Devices, Sunnyvale, CA). To measure maximum fluorescence intensity, a sample of calcein-labeled cells was lysed with 200 µl of Triton X-100 (5% in PBS). To obtain baseline or spontaneous cytolysis data, parallel ADCML assays were run with heat-inactivated sera from these healthy blood donors, culture medium alone, or irrelevant pAbs. All experiments were carried out in triplicates. The percent cytolysis of ADCML was determined as follows: % cytolysis = (experimental cytolysis - spontaneous cytolysis)/(maximum cytolysis - spontaneous cytolysis) X 100.

#### **ADCML of ACH-2 cells by anti-HIV-1 Abs in plasma of HIV-1-infected patients**

The calcein release assay was also used to quantitate ADCML of latently HIV-1-infected ACH-2 cells by anti-HIV-1 Abs in plasma samples from HIV-1-infected patients. ACH-2 or A3.01 cells untreated or treated with RMD, PMA/ionomycin, or DMSO were labeled at 37°C for 40 min with 15 µM calcein-AM (Invitrogen, Carlsbad, CA) in 200 µl of PBS. After washing, cells were pre-incubated for 30 min at 37°C with BRIC229 (20 µg/ml) to block hCD59 function. After pre-incubation, heat-inactivated plasma (56°C for 1 h) from either HIV-1-infected or healthy individuals (1:20 at final dilution) were individually added as a source of endogenous Abs or Ab control, followed by the exposure to either complement-competent or heat-inactivated sera from healthy blood donors. Triton X-100 was used for determining the total cytolysis. All experiments were carried out in triplicates and percent cytolysis was calculated as described above.

#### **Establishment of HIV-1 latency in primary resting CD4<sup>+</sup> T cells**

Establishment of HIV-1 latency in primary resting CD4<sup>+</sup> T cells was conducted using the spinoculation-infection approach as previously described (48). Briefly, leukapheresis products from 3 HIV-1-negative blood donors were purchased from the ALLCells (Emeryville, CA) for isolation of primary CD4<sup>+</sup> T cells using the negative selection RosetteSep reagents (StemCell Technologies, Vancouver, BC, Canada). The isolated CD4<sup>+</sup> T cells were stained with Abs against the human T cell activation markers of CD69, HLA-DR and CD25, and followed by fluorescence-activated cell sorting (FACS) to obtain resting CD4<sup>+</sup> T cells that were activation marker negative as previously described (48). The purity of primary resting CD4<sup>+</sup> T cells was >97% by FACS analysis. The primary resting CD4<sup>+</sup> T cells were subjected to spinoculation-infection of HIV-1 as previously described (48). Primary resting CD4<sup>+</sup> T cells at  $0.2 - 1.0 \times 10^6$  per well in flat-bottom 96-well microplates were mixed with HIV-1 strain of NL4-3 (NIH AIDS Reagent Program, Germantown, MD)

at 0.5 – 3.0  $\mu\text{g/ml}$  of p24<sup>Gag</sup>. The microplates were enclosed in plastic bags and centrifuged in microtiter plate carriers at 1,200 x g for 2 h at room temperature. The cells were washed five times with ice-cold RPMI 1640 medium to remove free HIV-1 particles, followed by resuspension in the complete RPMI1640 medium supplemented with 5  $\mu\text{M}$  saquinavir (SQV, a protease inhibitor or PI; Sigma-Aldrich, St. Louis, MO) to prevent infection by residual virus. The cells and supernatants were harvested immediately after spinoculation-infection and at multiple time points of culture for evaluation of HIV-1 latency. Cells were subjected to DNA extraction using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA) for determination of integrated HIV-1 DNA by the nested *Alu*-Gag qPCR assay, while the culture supernatants were subjected to p24 ELISA for measurement of virus production as described above. In principle, latently-infected resting CD4<sup>+</sup> T cells have high levels of integrated HIV-1 DNA in cells, but low or undetectable levels of viral p24 in culture supernatants.

### The nested *Alu*-Gag qPCR assay to quantify integrated HIV-1 DNA

DNA extracted from latently-infected primary resting CD4<sup>+</sup> T cells was subjected to the nested *Alu*-Gag qPCR assay to quantify integrated HIV-1 DNA as previously described (49). The nested *Alu*-Gag qPCR assay used two sequential sets of primers. The first set of primers was: genomic *Alu* forward, 5'-GCCTCCCAAAGTGCTGGGATTACAG-3'; and HIV-1 Gag reverse, 5'-GCTCTCGCACCCATCTCTCTCC-3' (49). This primer set was used for the first-round of PCR in the reaction conditions: 100 ng of extracted DNA, 1X Taq PCR Master Mix (QIAGEN, Valencia, CA), 100 nM *Alu* forward primer, and 600 nM Gag reverse primer. PCR cycling conditions were: a 120-sec hot start at 94°C, followed by 20 cycles of 93°C for 30 sec, 50°C for 60 sec, and 70°C for 100 sec. The first-round PCR products (12.5  $\mu\text{l}$ ) were subjected to qPCR containing the second set of primers (LTR forward, 5'-GCCTCAATAAAGCTTGCCTTGA-3'; and LTR reverse, 5'-TCCCACTGACTAAAAGGGTCTGA-3'), each at 250 nM plus 1X RT<sup>2</sup> SYBR Green Rox qPCR Mastermix (QIAGEN, Valencia, CA). The reactions were performed on a Prism 7700 Sequence Detection System running Sequence Detector version 1.6.3 software (Applied Biosystems, Foster City, CA). The thermal program was 120-sec hot start at 95°C, followed by 40 cycles of 95°C for 15 s and 60°C for 60 sec.

Plasmid pNL4-3 construct containing full-length of HIV-1 DNA was used to establish a standard curve in the qPCR performance for quantitative analysis of integrated HIV-1 DNA copy numbers. Plasmid pNL4-3 concentration was determined using a ND-1000 UV-vis Spectrophotometer (NanoDrop Technologies, Wilmington, DE). The number of pNL4-3 copies in the plasmid solution was calculated according to the plasmid size of 14,825 bp. A plasmid-based calibration curve was generated with 10-fold serial dilutions of pNL4-3; to control pipetting steps, three 10-fold serial dilutions were prepared, and concentrations were checked by qPCR. The quantification was linear over a range of 10 to 10<sup>7</sup> starting plasmid copy numbers, and the detection limit was ten copies per reaction. The human  $\beta$ -globin gene was used as an internal control for the normalization of the amount of viral DNA in each sample. In addition, DNA samples prepared from ACH-2 and A3.01 cells were used as positive and negative controls of HIV-1 DNA integration, respectively. The nested *Alu*-Gag qPCR detects only integrated proviruses because it relies on an initial amplification in which

one primer hybridizes with HIV-1 Gag sequence, while the other primer hybridizes with a conserved sequence in *Alu* elements that are most numerous repetitive elements in primate genomic DNA (49).

### **ADCML of latently HIV-1-infected primary resting CD4<sup>+</sup> T cells by anti-HIV-1 Env pAbs**

Spinoculation-infection approach can establish HIV-1 latency in approximately 4.5% of primary resting CD4<sup>+</sup> T cells that produce virus upon stimulation (48). The calcein release assay described above is not sensitive enough to determine ADCML efficacy of such a small percentage of latently HIV-1-infected primary resting CD4<sup>+</sup> T cells. We then used the nested *Alu*-Gag qPCR to measure the reduction of integrated HIV-1 DNA to indirectly determine ADCML efficacy of latently infected primary CD4<sup>+</sup> T cells. Briefly, latently infected primary CD4<sup>+</sup> T cells were untreated or treated with RMD, PMA/ionomycin, or DMSO for 24 h at 37°C. Cells were pre-incubated for 30 min at 37°C with or without BRIC229 (20 µg/ml) in the presence of anti-gp120/gp160 pAbs at various concentrations (Fitzgerald, Acton, MA). After the pre-incubation, 100 µl of the pooled sera from three healthy blood donors were added as a source of complement. Parallel tests were run with heat-inactivated sera from these healthy blood donors to obtain spontaneous cytolysis data. The microplates were incubated at 37°C for 1 h and followed by 3 washes with ice-cold PBS. Cells in all ADCML experimental conditions described above were treated with DNase enzymes to remove DNA released from lysed cells, and then subjected to DNA extraction for quantitating integrated HIV-1 DNA using the nested *Alu*-Gag qPCR as described above (49). ADCML efficacy of latently HIV-1-infected primary resting CD4<sup>+</sup> T cells by anti-HIV-1 Env pAbs was calculated as fold reductions of integrated HIV-1 DNA copies detected in ADCML experimental conditions (anti-gp120/gp160 pAbs plus competent complement or heat-inactivated complement) versus that detected in the ADCML control (anti-gp120/gp160 pAbs only).

### **Titration of plasma anti-HIV-1 Env Abs from HIV-1-infected patients**

Anti-HIV-1 Env Abs in plasma samples from infected patients on ART or ART-naïve were titrated using an ELISA Kit for Antibody to Human Immunodeficiency Virus 1&2 (BioChain, Hayward, CA). The 96-well microplates included in this kit were coated with the recombinant HIV antigens (gp120, gp36, and gp47), which specifically capture anti-HIV Env Abs. Test results for HIV-2 specific Abs, as measured by the FDA-licensed HIV-2 enzyme immunoassay, were negative in all subjects. Therefore, Abs measured by this ELISA kit were specifically against HIV-1 Env.

Each plasma sample was serially diluted fourfold starting from 1:800 in the Blocker Casein Buffer (Thermo Scientific, Rockford, IL). Each dilution (100 µl) was added to a well and incubated at 37 °C for 1 h. Five HIV-1-negative plasma samples were diluted in the same pattern and added as negative controls. After washing, 100 µl of 1:50000-diluted horseradish peroxidase (HRP)-conjugated anti-human whole IgG mAb (Millipore, Temecula, CA) was added per well and incubated at 37 °C for 1 h. Microplates were washed and substrate was added, followed by incubation at room temperature in darkness for 20 min. The reaction was stopped by 2 M sulfuric acid and the optical density of each well was read at 450 nm. All diluted samples were tested in duplicate. The cut-off was determined as the mean

absorbance value of five plasma samples from HIV-1-negative donors. The Ab titer was determined as the highest dilution with an absorbance value above the cut-off.

### Flow cytometric analysis (FACS)

ACH-2 or A3.01 cells were stimulated with RMD (1 - 9 nM), SAHA (0.2 - 0.8  $\mu$ M), or prostratin (0.2 - 5  $\mu$ M) for 24 - 72 h. PMA (10 ng/ml)/ionomycin (1  $\mu$ g/ml) and DMSO were included as positive and negative stimulation controls, respectively. Cells were subjected to surface staining with 2G12, an anti-HIV-1 gp120 mAb, to determine expression of HIV-1 Env on the cell surface. The percentage of latently infected primary resting CD4<sup>+</sup> T cells that produced virus upon stimulation was determined by intracellular staining (ICS) for HIV-1 Gag. The BD Cytotfix/Cytoperm Plus Kit (BD Biosciences, San Diego, CA) was used for the ICS in accordance with the manufacturer's recommendations. Cells were stained with phycoerythrin-labeled KC57 (Beckman Coulter, Fullerton, CA) mAb. This IgG1 mAb recognizes p55, p39, p33, and p24 proteins of the core antigens of HIV-1.

FACS data of both cell surface staining and ICS were analyzed using FlowJo software (Tree Star, San Carlos, CA).

### Confocal microscopy

ACH-2 cells were treated with RMD (3 nM), SAHA (0.8  $\mu$ M), or prostratin (1  $\mu$ M) for 24 h. PMA (10 ng/ml)/ionomycin (1  $\mu$ g/ml) and DMSO were included as positive and negative stimulation controls, respectively. Cells were fixed with 2% paraformaldehyde (PFA), and then subjected to immunostaining for confocal microscopy analysis of viral protein expression and colocalization of HIV-1 Env with CD59 and 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, Carlsbad, CA) at 4°C for 20 min to stain GM1 lipid rafts as previously described (50). Anti-HIV-1 gp120 mAb cloned from an HIV-1-positive individual (2G12, NIH AIDS Reagent Program, Germantown, MD) and BRIC229 were used as primary Abs to stain Env and CD59, respectively. After washing, cells were stained with the secondary Abs including goat anti-human IgG conjugated with Alexa Fluor 633 and donkey anti-mouse IgG conjugated with Alexa Fluor 546. Both primary- and secondary-Ab incubations were carried out for 30 min at room temperature 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, Carlsbad, CA) 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 60 objective lens. Images were processed and analyzed using the FV10-ASW 3.0 Viewer software (Olympus America Inc., Center Valley, PA).

### Western blot

ACH-2 cells untreated or treated with RMD (3 nM), SAHA (0.8  $\mu$ M), prostratin (1  $\mu$ M), PMA (10 ng/ml)/ionomycin (1  $\mu$ g/ml) or DMSO in the presence or absence of antiretroviral drugs for 24 h were lysed in 1 X cell lysis buffer (Cell Signaling Technology, Danvers, MA) plus 1 X Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO). Cellular lysates were



cleared by centrifugation at 13,800 x g for 15 min at 4 °C, and then subjected to NuPAGE Novex high-performance electrophoresis (Invitrogen, Carlsbad, CA). Proteins were blotted onto a polyvinylidene difluoride (PVDF) membrane (Millipore, Billerica, MA). After blocking with 5% of non-fat milk, the blot was incubated overnight at 4°C with mouse anti-Gag pAbs at 1 µg/ml. After washing, the blot was visualized with the appropriate HRP-conjugated secondary Ab and an enhanced chemiluminescence (ECL) detection system (Pierce, Rockford, IL). 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, Cambridge, MA) followed by incubation with HRP-conjugated anti-mouse IgG (Jackson Immuno-Research Laboratories, West Grove, PA) and ECL detection system.

### Statistical analysis

Data were compared using Tukey's Post hoc ANOVA test or the Student *t*-test. *p* < 0.05 was considered statistically significant.

## Results

### Expression of viral Env on the surface of latently HIV-1-infected cells upon provirus activation

Several reagents including prostratin and HDACi such as VPA and SAHA have been explored to force activation of provirus in latently HIV-1-infected cells *in vivo* and *in vitro* (14, 51–54). These reagents are able to activate provirus in latently HIV-1-infected primary cells and cell lines, inducing viral transcription, increasing viral RNA expression, or enhancing the acetylation of cellular histones (14, 51–54). It remains unclear whether and to what extent these reagents induce expression of HIV-1 proteins on the surface of latently-infected cells, which is critical for clearance of infected cells by immune responses. To clarify these issues, we used J-Lat GFP Clone A72 cells to determine the optimal concentrations of these reagents in provirus activation. We also included RMD in our experiments as it is a novel selective and potent HDACi recently approved by FDA for treatment of cutaneous T-cell lymphoma (CTCL). We found that all these reagents activated proviruses in J-Lat GFP Clone A72 cells in a dose-dependent manner (Fig. 1A). RMD, at nanomolar concentrations (3 nM or higher), effectively activated proviruses as GFP expression was greatly induced, whereas SAHA and prostratin were required at micromolar concentrations to trigger GFP expression (Fig. 1A). We then used ACH-2 cells to confirm the results described above and to study the effects of these reagents on HIV-1 protein expression on the cell surface. While Env-positive cells were barely detectable in medium or DMSO treatment, RMD, SAHA and prostratin triggered expression of HIV-1 Env on the surface of ACH-2 cells in a dose-dependent manner (Fig. 1B, 1C). RMD at a concentration as low as 3 nM strongly induced HIV-1 Env expression, as  $54.4 \pm 6.1\%$  of ACH-2 cells became gp120-positive 24 h post-stimulation. When RMD was used at 9 nM,  $88.9 \pm 7.5\%$  of ACH-2 cells became gp120-positive 24 h post-stimulation. In contrast, approximately 1 µM of prostratin was required to induce a level of Env expression similar to that by RMD at 9 nM (Fig. 1B, 1C), and SAHA only induced a moderate level of Env expression even at 0.8 µM or higher concentrations (Fig. 1B, 1C). It is pertinent to note that

prostratin is a phorbol ester that antagonizes HIV-1 latency by activating NF- $\kappa$ B (55), which may cause global cell activation leading to unacceptable side effects. Interestingly, the percentage of Env-positive cells was not correlated with levels of viral particles released into the culture supernatants (Fig. 1D). HIV-1 p24 in the supernatant of RMD-treated ACH-2 cells was almost 10-fold and 4-fold higher than that of SAHA- or prostratin-treated cells, respectively (Fig. 1D). The smaller fold increase seen in the prostratin-treated cells may be due to prostratin's anti-HIV-1 activity (56, 57). The highest doses of RMD (9.0 nM, equivalent to 1.62 mg/m<sup>2</sup> *in vivo*) and SAHA (0.8  $\mu$ M) in our experiments are within the tolerated doses of these drugs used in CTCL patients. RMD is used to treat CTCL patients at 7–14 mg/m<sup>2</sup> via intravenous infusion over a 4 h period (58), whereas SAHA is used at 400 mg once daily via oral administration (plasma concentration up to 400 – 600 ng/ml or 1.5 – 2.3  $\mu$ M) (59). Thus, prostratin and HDACi, particularly RMD, are able to activate provirus to induce both virion production and viral protein expression on the cell surface, which are expected to trigger viral CPEs, immune responses, or both to kill provirus-activated cells.

### Effects of ART on virus production and viral protein expression

The “shock and kill” approach has to be implemented in HIV-1-infected patients on ART to prevent new rounds of infections by virus released from the stimulated cells. It is unclear whether ART drugs interrupt the effects of provirus stimulants on virion production and viral protein expression, thereby attenuating the effects of these provirus stimulants on provirus activation. To clarify these issues, we treated ACH-2 cells with RMD (3 nM), SAHA (0.8  $\mu$ M), prostratin (1  $\mu$ M), PMA (10 ng/ml)/iononycin (1  $\mu$ g/ml), or DMSO in the presence or absence of reverse transcriptase (RT) inhibitors or PIs as they are among the most effective antiretroviral drugs. Supernatant and cells were then subjected to analysis of virion production and expression of viral Env on the cell surface, respectively. We found that emtricitabine (FTC, a RT inhibitor), atazanavir (ATV, a PI), and darunavir (DRV, a PI) had no effect on provirus activation by provirus stimulants, as virus production in the supernatants and percentage of Env-positive cells were not altered (Fig. 2A, 2B). Similar to ATV and DRV, saquinavir (SQV, a PI) did not impact the effects of provirus stimulants on expression of viral proteins on the cell surface, as percentage of Env-positive cells was not dramatically changed (Fig. 2B). However, SQV markedly attenuated the effects of provirus stimulants on virion production, as virions determined by p24 ELISA in the supernatant released from ACH-2 cells in response to every stimulation condition markedly decreased (Fig. 2A). Given that different PIs affect distinct steps in the viral life cycle, SQV probably inhibits multiple steps of HIV-1 replication, resulting in the formation of defective particles which contain precursor Gag (Pr55<sup>Gag</sup>) and little or no processed p24 (60). In fact, our western blot analysis supports this notion because it demonstrates that Gag p41 and p24 were significantly reduced by SQV, but not ATV or DRV (Fig. 2C). In contrast, the levels of Gag precursors (Pr55<sup>Gag</sup>) were comparable in the presence or absence of PIs (Fig. 2C). Both RT and PI are key components of common ART regimens. They inhibit distinct steps in the HIV-1 life cycle, resulting in the production of defective, noninfectious virions or reduction of viral products. As a consequence, ART drugs, particularly PIs, may attenuate the effects of provirus stimulants on viral CPEs in HIV-1-infected patients. Thus, the “shock and kill” approach for purging latently HIV-1-infected cells should rely on immune responses rather than viral CPEs.

## Colocalization of Env and hCD59 in lipid rafts on the surface of latently infected cells upon provirus activation

Productively HIV-1-infected cells use their surface RCA molecules, mainly CD59, to resist ADCML allowing them to escape the vigorous and sustained anti-HIV-1 Ab responses observed in almost all infected individuals (28). This suggests CD59 is located in close proximity to viral proteins such as Env on the cell surface, facilitating direct interaction between them. We used a confocal microscopy assay to analyze CD59 and Env colocalization on the surface of provirus-activated latently infected cells. The confocal microscopy analysis confirmed our FACS data that RMD, SAHA, and prostratin triggered provirus activation leading to Env expression on the cell surface (Fig. 3, gp120 panel). It is well known that Env on the surface of productively infected cells is made of a trimer consisting of three gp120/gp41 and that this trimeric Env is strongly associated with lipid rafts (61–63). We found a similar association between Env and lipid rafts on the surface of provirus-activated cells. We observed a multi-clustered Env distribution on the surface of provirus-activated cells (Fig. 3, gp120 panel), which was exclusively colocalized with cell membrane lipid rafts (Fig. 3, CTB panel). Importantly, CD59 was also strongly associated with cell membrane lipid rafts (Fig. 3, CD59 panel), and Env and CD59 were colocalized in lipid rafts on the surface of provirus-activated cells (Fig. 3, white in merge panel), suggesting that Env may directly interact with RCA molecules on the cell surface. There were no appreciable differences in the appearances of Env on the cell surface triggered by different stimulants including PMA/ionomycin, RMD, SAHA, and prostratin (Fig. 3), suggesting that expression of HIV-1 proteins on the cell surface can be triggered by different provirus stimulants through distinct signaling pathways.

## Anti-Env Abs-triggered ADCML of ACH-2 cells upon provirus activation

Next, we tested whether abrogation of CD59 function would render provirus-activated ACH-2 cells sensitive to ADCML. Initial experiments using a hemolysis assay were conducted to determine the most efficient blocking concentration of BRIC229 in abrogation of hCD59 function. We found that BRIC229 at 20  $\mu\text{g/ml}$  completely abrogated hCD59 function as ADCML of RBCs reached almost 100%, whereas RBCs were resistant to ADCML in the absence of BRIC229 (Fig. 4A). We then used this concentration of BRIC229 (20  $\mu\text{g/ml}$ ) to block hCD59 on the surface of provirus-activated cells to analyze the ADCML of latently HIV-1-infected cells. ACH-2 cells were stimulated with RMD (3 nM) in the presence or absence of antiretroviral drugs such as FTC, SQV, ATV, or DRV. Cells were treated with BRIC229 (20  $\mu\text{g/ml}$ ) plus purified rabbit anti-Env pAbs at various concentrations and complement. Cytolysis of ACH-2 cells was measured by the calcein release assay. We found that blockage of hCD59 with BRIC229 markedly increased cytolysis of ACH-2 cells after provirus activation by RMD (Fig. 4B). Anti-Env pAbs triggered cytolysis of provirus-activated ACH-2 cells in a dose-dependent manner (Fig. 4B). The lysis resulted from specific cell killing by anti-HIV-1 Env pAb-mediated ADCML because the lysis did not occur in the controls including: (1) no BRIC229, (2) irrelevant rabbit IgG pAbs in place of anti-HIV-1 Env pAbs, (3) heat-inactivated complement, (4) ACH-2 cells treated with DMSO, and (5) A3.01 cells that are ACH-2 parental cells prior to HIV-1-infection (Fig. 4C). Cytolysis was not affected by antiretroviral drugs as cytolysis

efficacy was not significantly altered in all experimental conditions in the presence or absence of antiretroviral drugs (Fig. 4D).

### **ADCML of latently HIV-1-infected cells by plasma from HIV-1-infected patients**

To evaluate whether provirus activation plus abrogation of hCD59 function renders latently infected cells sensitive to ADCML by naturally occurring Abs present in the circulation of HIV-1-infected patients, plasma samples from 15 chronically HIV-1-infected patients including 9 patients who are on virally-suppressive ART (R01 – R09) and 6 ART-naïve patients (G022, G023, G025, G026, G031, G040) (Table 1) were tested using the ADCML assay described above. All plasma samples contained high titers of Abs against HIV-1 Env (Table 1). Plasma samples from 5 HIV-1-negative blood donors were included as a cytolysis control. Summary data of cytolysis of provirus-activated ACH-2 cells from all 15 HIV-1-infected and 5 uninfected individuals are shown in Fig. 5A. In the presence of BRIC229, all 15 HIV-1 plasma samples contained Abs capable of mediating ADCML of provirus-activated ACH-2 cells although in varying degrees (Fig. 5A), whereas plasma samples from uninfected individuals did not produce significant ADCML. Specific ADCML of provirus-activated ACH-2 cells was observed in all HIV-1-positive plasma samples tested ranging from 19% (G022) to 40% (G025) with a mean value of 32% ( $\pm 6\%$ ,  $n=6$ ) in ART-naïve patients (Fig. 5A), and from 17% (R02) to 29% (R06) with a mean value of 25% ( $\pm 7\%$ ,  $n=9$ ) in patients on ART (Fig. 5A). HIV-1-positive plasma alone or plasma plus heat-inactivated complement showed a background spontaneous cytolysis of 3.4% and 3.0%, respectively (Fig. 5A, 5C). Plasma from uninfected individuals showed a background cytolysis of 5.3%, which is not significantly different from background seen with plasma alone or plasma plus heat-inactivated complement (Fig. 5A, 5C). The plasma samples did not have significant ADCML of HIV-1-negative A3.01 cells treated with RMD (Fig. 5B, 5C). These results indicate that the CD59 blocker (BRIC229) is able to specifically allow anti-HIV-1 Abs naturally present in infected individuals to trigger ADCML of latently infected T cells upon provirus activation.

### **ADCML of latently HIV-1-infected primary resting CD4<sup>+</sup> T cells by anti-HIV-1 Env pAbs**

Primary CD4<sup>+</sup> T cell models of HIV-1 latency closely recapitulate the biology of HIV-1 latency *in vivo*. These models have facilitated research on the mechanisms of HIV-1 latency and have also served as a platform for studying candidate compounds that have a potential to purge the latent reservoirs. We used the spinoculation-infection approach to establish HIV-1 latency in primary resting CD4<sup>+</sup> T cells without activation of these cells as previously reported (48). As shown in Fig. 6A, integrated HIV-1 DNA was measured by the nested *Alu*-qPCR in primary resting CD4<sup>+</sup> T cells 3 days after spinoculation-infection, while p24 in cell culture supernatants were at levels below detectable as determined by p24 ELISA (Data not shown). These results were consistent with those of other studies and indicate establishment of HIV-1 latency in primary resting CD4<sup>+</sup> T cells (48, 49, 64).

To determine the frequency of primary resting CD4<sup>+</sup> T cells that contained integrated viral DNA, we stimulated these cells 3 days after spinoculation-infection with RMD (3 nM) or anti-CD3/anti-CD28 beads for 24 h. Stimulated cells were subjected to intracellular staining with anti-p24<sup>Gag</sup> mAb (KC57). As shown in Fig. 6B, 5.0% of spinoculated-infected primary

resting CD4<sup>+</sup> T cells were viral Gag positive in response to stimulation with anti-CD3/anti-CD28 beads, whereas unstimulated cells produced minimal or no Gag (Fig. 6B). RMD induced Gag production in 3.1 % of latently infected primary resting CD4<sup>+</sup> T cells, which is 62% of maximal activation (anti-CD3/anti-CD28 beads). As a control, RMD induced Gag production in more than 65% of ACH-2 cells (Fig. 6B, 6C). Thus, approximately 5% of latently infected primary resting CD4<sup>+</sup> T cells contain replication-competent provirus (Fig. 6C), which is in agreement with that previously reported (48).

Next, we tested whether abrogation of CD59 function would render provirus-activated latently infected primary resting CD4<sup>+</sup> T cells sensitive to ADCML. Spinoculated-infected primary resting CD4<sup>+</sup> T cells were stimulated with RMD (3 nM), followed by incubation with BRIC229 (20 µg/ml) plus rabbit anti-Env pAbs at various concentrations and complement. Cytolysis of these primary cells was measured by reduction of integrated viral DNA. Cells in all ADCML experimental conditions were treated with DNase enzymes to remove DNA released from lysed cells, and then subjected to DNA extraction for quantitating integrated HIV-1 DNA using the nested *Alu*-Gag qPCR. The level of integrated HIV-1 DNA detected in the ADCML control (anti-gp120/gp160 pAbs only) was used to calculate relative levels of integrated HIV-1 DNA detected in ADCML experimental conditions (anti-gp120/gp160 pAbs plus competent complement or heat-inactivated complement). As shown in Fig. 6D, the level of integrated HIV-1 DNA detected in the ADCML experimental condition (RMD + BRIC229 + anti-HIV-1 Env + complement) was significantly reduced when compared with control conditions including RMD + BRIC229 + anti-HIV-1 Env + heat-inactivated complement, RMD + BRIC229 + irrelevant Abs or medium only + complement. Thus, blockage of hCD59 with BRIC229 markedly increased cytolysis of latently infected primary resting CD4<sup>+</sup> T cells after provirus activation by RMD. The lysis resulted from specific cell killing by anti-HIV-1 Env pAb-mediated ADCML because the lysis did not occur in the controls described above.

## Discussion

Efforts to eradicate long-lived latently HIV-1-infected cells have focused on activation of the proviruses in these cells. This is known as a “shock and kill” approach. It is presumed that these latently infected cells will be killed after provirus activation by virus-mediated CPEs, host immune responses or both. This approach has to be implemented in HIV-1-infected patients on ART to prevent new rounds of infections by virus released from the stimulated cells. A recent study has demonstrated that a single 400 mg oral dose of SAHA disrupts virus latency in HIV-1-infected patients in whom viremia has been fully suppressed by ART (26). This report provides proof-of-concept for use of provirus stimulants, particularly HDACi, as a therapeutic class of reagents with a tolerable safety profile while they force activation of HIV-1 proviruses *in vivo*. However, the use of SAHA in combination with suppressive ART in this study did not significantly reduce the number of latently infected resting CD4<sup>+</sup> T cells, suggesting that either viral CPEs or the immune response is not sufficient to clear HIV-1 reservoirs and that additional interventions are required. HIV-1 is cytopathic *in vitro* in cultured cells, but the viral replication elicits little CPEs *in vivo* (15–19). In addition, viral CPEs may be attenuated by ART due to suppression of production of infectious, mature virions. We found that provirus stimulants including

prostratin, RMD and SAHA activated proviruses in the latently HIV-1-infected T cell line ACH-2 as virion production and viral protein expression on the cell surface were induced (Fig. 1). We also noticed that SQV, a PI, attenuated the effects of provirus stimulants including RMD, SAHA, and prostratin on virion production from latently infected cells, as virions determined by p24 ELISA in the supernatant of provirus-activated ACH-2 cells decreased dramatically (Fig. 2). As a typical ART regimen combines three antiretroviral drugs, most often including PIs and RTs, these drugs should be carefully selected when used in combination with provirus stimulants. RTs and other PIs such as ATV and DRV should be given priority as they do not affect neither cells expressing viral proteins nor viral production (Fig. 2). In the SAHA clinical trial (26), PI-containing ART regimen(s) might have been used (personal communication with Dr. Margolis D. at UNC), although the exact ART regimens were not tracked. However, it is unlikely that the efficacy of the SAHA trial was substantially influenced by PI-attenuated CPEs, as CPEs do not play a significant role in the killing of HIV-1-infected cells *in vivo* (15–19).

HIV-1-infected patients are well known to mount a vigorous and sustained Ab response at all stages of viral infection (32–39). This response is also well known to have a limited effect on controlling virus proliferation or on protecting the patients from developing AIDS (38, 40–42). Recent studies have indicated that resistance of HIV-1 virions or infected cells to ADCML is dependent on RCA molecules including CD46, CD55 and CD59 (27–31). CD59 controls formation of the membrane attack complex (MAC) at the terminal stage of the complement activation cascades via all three activation pathways, whereas CD55 only prevents formation of the C3 and C5 convertases (65–67) and CD46 only acts as a cofactor for the cleavage of cell-bound C4b and C3b by the serum protease factor (68). Thus, CD59 plays the most critical role in restricting ADCML of HIV-1 virions and infected cells. Blockage of CD59 strongly enhances ADCML of HIV-1 virions, whereas blockage of CD46 or CD55 has a significantly weaker effect (28). We therefore focused on the abrogation of CD59 function to enhance ADCML of latently HIV-1-infected cells upon provirus activation. We showed that Env and CD59 were strongly colocalized in lipid rafts on the surface of provirus-activated cells, suggesting that Env may directly interact with RCA molecules on the cell surface (Fig. 3). ACH-2 cells post-activation of provirus became sensitive to ADCML in the presence of an hCD59 blocker plus anti-HIV-1 Env Abs (Fig. 4) or plasma from HIV-1-infected patients (Fig. 5). Abrogation of CD59 function can restore the functions of Abs in the plasma from both groups of patients who are on ART or ART-naïve to trigger ADCML of provirus-activated latently infected cells (Fig. 5). It seems that Abs present in ART-naïve patients have stronger cytotoxic activities than that in patients on ART (Fig. 5C). However, a larger sample size is necessary to analyze the statistical significance. The ADCML of provirus-activated cells resulted from specific cell killing by anti-HIV-1 Abs because ADCML did not occur using irrelevant Abs, HIV-1-negative plasma or inactivated complement (Fig. 4, Fig. 5). Therefore, a combination of provirus stimulants with RCA blockers represents a novel approach for purging HIV-1 latently infected cells. The remaining issues to be addressed include (1) whether ADCML efficacy is correlated to the titer, neutralization activity and affinity of anti-HIV-1 Abs naturally present in infected individuals, and (2) whether simultaneous abrogation of CD46, CD55 and CD59 can further enhance ADCML of latently HIV-1-infected cells upon provirus activation.

Primary CD4<sup>+</sup> T cell models of HIV-1 latency closely recapitulate the biology of HIV-1 latency *in vivo*. Several primary cell models of HIV-1 latency have been established (7, 69). These models have facilitated research on the mechanisms of HIV-1 latency and have also served as a platform for studying candidate compounds that have a potential to purge the latent reservoirs. Spinoculation of HIV-1 with primary CD4<sup>+</sup> T cells is a model that establishes HIV-1 latency without activation of CD4<sup>+</sup> T cells (7, 48, 70). Spinoculation-infection has the ability to establish latency in all populations of primary CD4<sup>+</sup> T cells including naïve and memory CD4<sup>+</sup> T cells, whereas other models predominantly establish HIV-1 latency in memory CD4<sup>+</sup> T cells. This model is also able to achieve a high percentage of cells harboring replication-competent provirus (48), thereby providing large numbers of latently-infected primary cells that can be used to study HIV-1 latency and the effects of agents on provirus activation. We used the spinoculation-infection approach to establish HIV-1 latency in primary resting CD4<sup>+</sup> T cells without activation of these cells as previously reported (48) (Fig. 6A). Approximately 5% of latently infected primary resting CD4<sup>+</sup> T cells contain replication-competent provirus, which is in agreement with that previously reported (48) (Fig. 6B, 6C). RMD effectively induced Gag production from latently infected primary resting CD4<sup>+</sup> T cells and ACH-2 cells (Fig. 6B, 6C). Blockage of hCD59 with BRIC229 markedly increased cytolysis of latently infected primary resting CD4<sup>+</sup> T cells after provirus activation by RMD (Fig. 6D). Thus, disarming RCA-protective shield allows anti-HIV-1 Env Abs to specifically trigger ADCML of latently infected primary cells and cell lines after provirus reactivation.

Because CD59 is widely expressed on almost all human cells and protects self-cells from damage caused by complement activation, potential side effects may be the main obstacle in clinical application of CD59 blockers. However, accumulating data suggest that short-term or transient abrogation of CD59 may be feasible and tolerable to HIV-1 patients: (1) intravenous (i.v.) injection of anti-rat CD59 Ab F(ab')<sub>2</sub> fragments completely inhibits rat CD59 function *in vivo* without causing any side effects in rats (71), (2) CD59 knockout mice do not develop severe illnesses (72), and (3) *in vivo* use of Abs against CD55 and CD59 increases the antitumor activity of Rituximab (anti-CD20 Ab) against lymphomas without causing hemolysis in a murine model (73). In addition, paroxysmal nocturnal hemoglobinuria (PNH) is a disease in humans due to deficiency of glycosylphosphatidylinositol-anchored proteins (GPI-APs) including CD59 (74–79). Patients with PNH usually have hemolytic anemia, but survive (74–79). Furthermore, we and others have demonstrated that the use of CD59 inhibitors *in vitro* to block CD59 does not cause nonspecific cytolytic effect of cell lines, human RBCs or PBMCs in the presence of sera/plasma from HIV-1-infected patients (28, 80). Finally, CD59 blockers are expected to be used short-term, not lifelong, thus making them easier to tolerate in HIV-1 patients.

In conclusion, HDACi (RMD and SAHA) and prostratin activate proviruses in the latently infected cells, resulting in virion production and viral protein expression on the cell surface. RMD was the most attractive provirus stimulant as it effectively activated proviruses at nanomolar concentrations that can be achieved clinically. Abrogation of CD59 function in conjunction with anti-HIV-1 Abs, either exogenous like the anti-HIV-1 Env Abs or endogenous such as those in plasma from HIV-1-infected patients, efficiently triggers

ADCML of provirus-activated ACH-2 cells in the presence or absence of antiretroviral drugs. Both ART-naïve patients and patients on ART have virus-specific Abs that are capable of mediating cytotoxicity of provirus-activated cell. This approach also effectively enhances ADCML of latently HIV-1-infected primary CD4<sup>+</sup> T cells. Therefore, a combination of provirus stimulants with RCA blockers helps restore function of naturally occurring Abs present in HIV-1-infected patients to trigger ADCML of latently infected cells, thereby representing a potentially novel approach to eliminate HIV-1.

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## Abbreviations

<b>RCA</b>	regulators of complement activation
<b>ADCML</b>	antibody-dependent complement-mediated lysis
<b>HDACi</b>	histone deacetylase inhibitor
<b>RMD</b>	romidepsin
<b>SAHA</b>	suberoylanilide hydroxamic acid
<b>ART</b>	antiretroviral therapy
<b>CPE</b>	cytopathic effect
<b>VPA</b>	valproic acid
<b>pAb</b>	polyclonal antibody
<b>CTB</b>	cholera toxin subunit B
<b>ICS</b>	intracellular staining
<b>RT</b>	reverse transcriptase
<b>PI</b>	protease inhibitor
<b>FTC</b>	emtricitabine
<b>SQV</b>	saquinavir
<b>ATV</b>	atazanavir
<b>DRV</b>	darunavir



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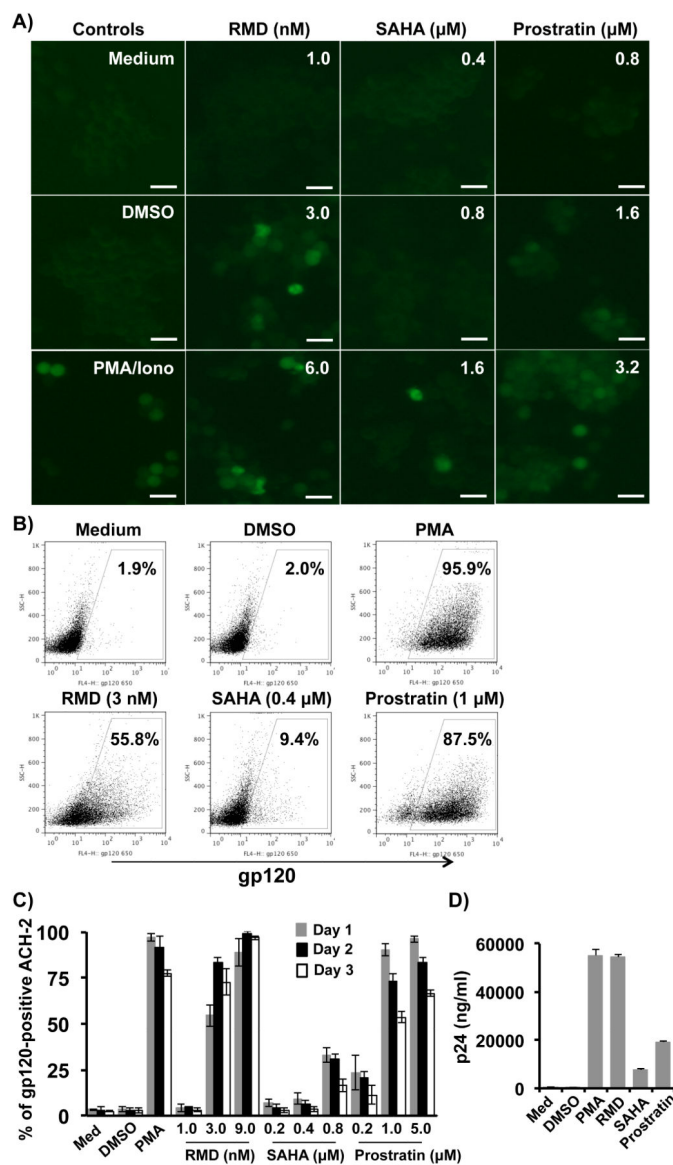
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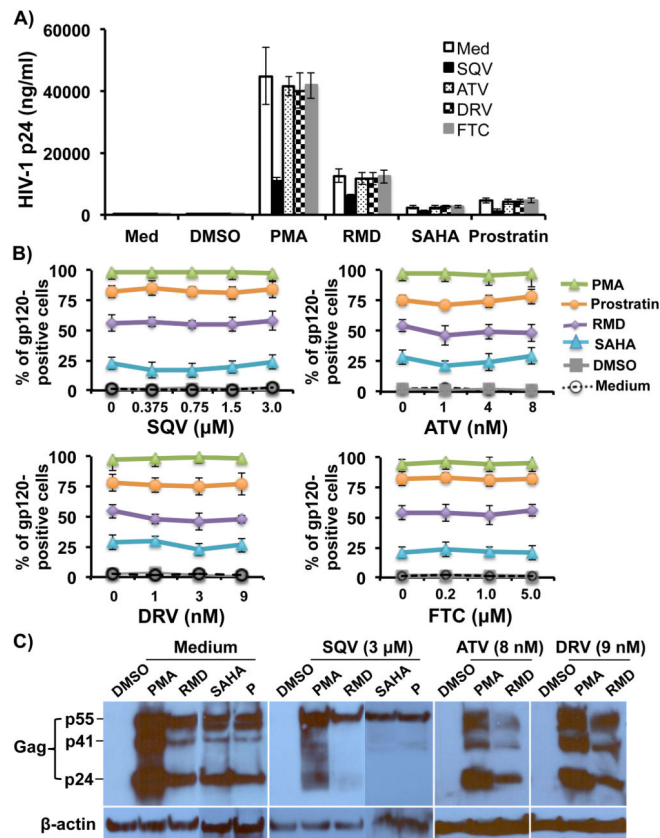
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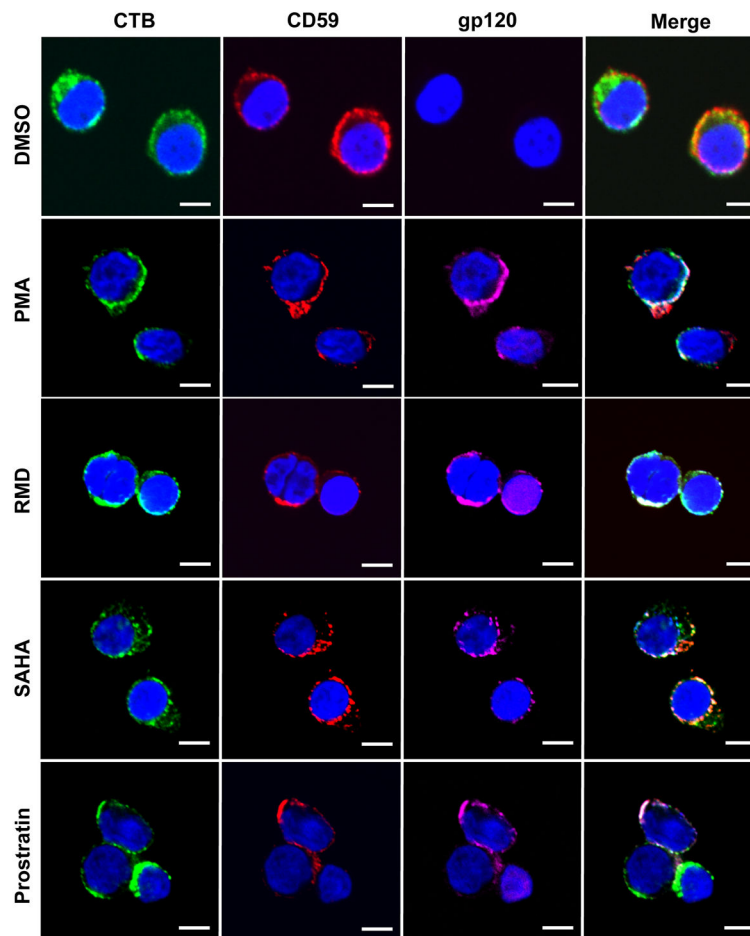
**Fig. 1. HIV-1 protein expression on the surface of latently-infected T cells upon provirus activation**  
 J-Lat GFP Clone A72 cells and ACH-2 cells were incubated with RMD, SAHA, and prostratin at various concentrations as indicated. DMSO and PMA (10 ng/ml) plus ionomycin (1  $\mu$ g/ml) were used as negative and positive stimulation controls, respectively. Stimulated J-Lat GFP Clone A72 cells were directly subjected to fluorescence microscopy analysis to determine provirus activation (GFP-positive). Stimulated ACH-2 cells were subjected to immunostaining with an anti-gp120 mAb (2G12) to determine expression of HIV-1 Env on the cell surface, and supernatant was subjected to p24<sup>Gag</sup> ELISA to determine levels of HIV-1 production in response to various provirus stimulants. A) A representative experiment of fluorescence microscopy analysis shows provirus activation in J-Lat GFP Clone A72 cells. B) Representative experiments of FACS analysis show the results of ACH-2 cells expressing gp120 on the cell surface 24 h post-stimulation with RMD (3 nM),

SAHA (0.4  $\mu\text{M}$ ), and prostratin (1  $\mu\text{M}$ ). C) Pooled data from three separate experiments of FACS analysis show the % of gp120-positive ACH-2 cells 24 - 72 h post-stimulation with RMD, SAHA, or prostratin at various concentrations as indicated. D) HIV-1 p24 levels in the supernatants from ACH-2 cells 24 h poststimulation with RMD (3 nM), SAHA (0.4  $\mu\text{M}$ ), or prostratin (1  $\mu\text{M}$ ). PMA, PMA (10 ng/mL) plus ionomycin (1  $\mu\text{g/mL}$ ). All experiments were repeated at least three times.



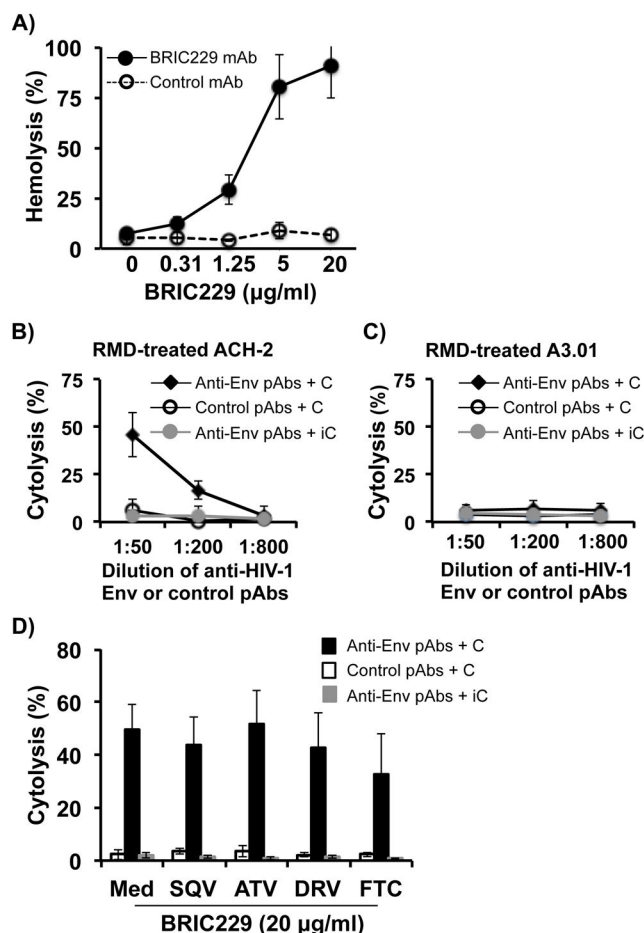
**Fig. 2. Effects of antiretroviral drugs on virus production and viral protein expression**  
 ACH-2 cells were untreated or treated with RMD (3 nM), SAHA (0.8  $\mu\text{M}$ ), prostratin (1  $\mu\text{M}$ ), DMSO, or PMA (10 ng/ml) plus ionomycin (1  $\mu\text{g}/\text{ml}$ ) in the presence or absence of a RT inhibitor such as FTC, or a PI such as SQV, ART, or DRV at various concentrations as indicated to study possible effects of antiretroviral drugs on provirus activation. A) and B) Supernatants and cells were collected 24 h posttreatment for p24 ELISA and FACS analysis to determine viral titer in the culture supernatants and % of gp120-positive cells, respectively. C) Cellular lysates were subjected to Western blot to analyze expression of Gag precursor and matured molecules. The data shows that SQV, but not other antiretroviral drugs, markedly inhibits cleavage of precursor Gag into mature capsid p24, resulting in little or no processed p24. The data represent the results from at least three independent experiments. FTC, emtricitabine; SQV, saquinavir; ATV, atazanavir; DRV, darunavir; Med, medium only; P, prostratin.





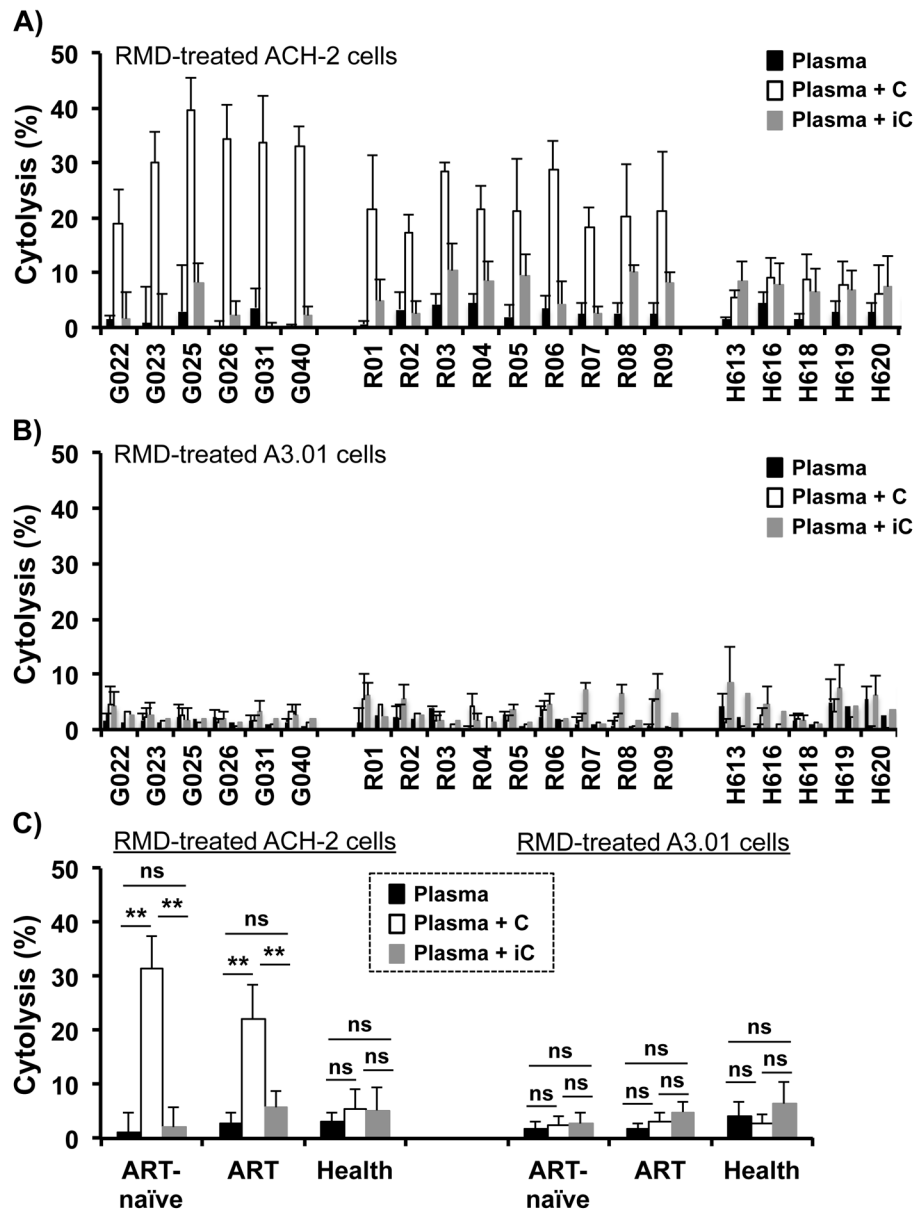
**Fig. 3. Colocalization of Env and hCD59 in lipid rafts on the surface of ACH-2 cells upon provirus activation**

ACH-2 cells were untreated or treated with RMD (3 nM), SAHA (0.8  $\mu$ M), prostratin (1  $\mu$ M), DMSO, or PMA (10 ng/ml) plus ionomycin (1  $\mu$ g/ml) for 24 h, and then subjected to confocal microscopy analysis of viral protein expression on the cell surface. White color in merged panel indicates the colocalization of HIV-1 Env (red) with lipid rafts (green) and CD59 (magenta) on the surface of provirus-activated ACH-2 cells. Cellular DNA content and nuclei were stained with DAPI (blue). Scale bars represent 5  $\mu$ m. The data represent the results from at least three independent experiments. CTB, cholera toxin subunit B as a marker for lipid rafts; gp120, surface staining with 2G12, a mAb against HIV-1 gp120.



**Fig. 4. Anti-Env Abs triggered ADCML of ACH-2 cells upon provirus activation**

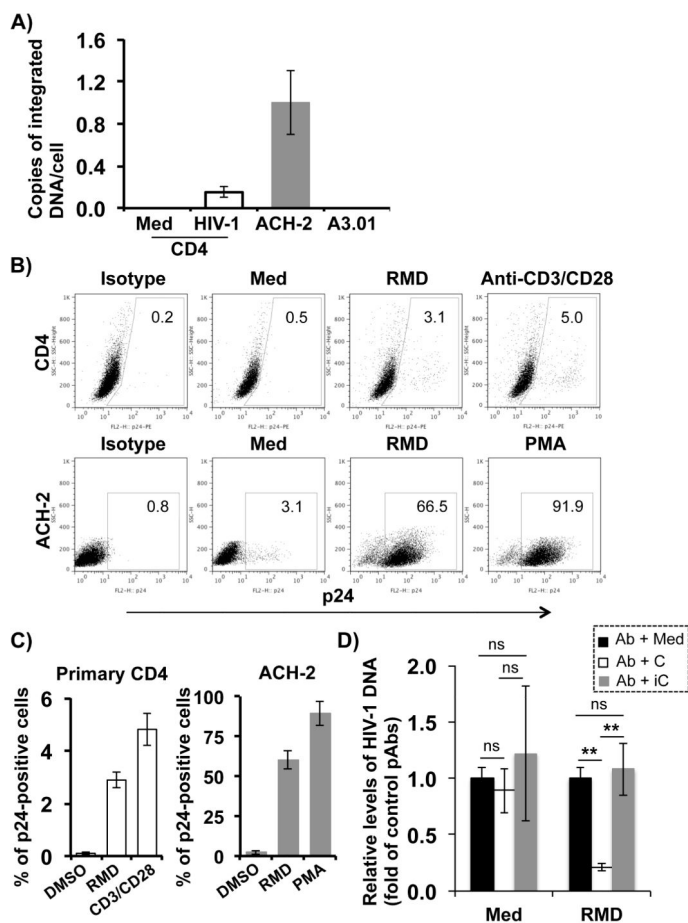
A) A hemolysis assay was conducted to determine the most efficient blocking concentration of BRIC229 in abrogation of hCD59 function. BRIC229 at 20 µg/ml completely abrogated hCD59 function as ADCML of human RBCs reached almost 100% (line with solid black circles), whereas RBCs resisted to ADCML in the absence of BRIC229 (dotted line with open circles). B) Anti-Env pAbs triggered ADCML of provirus-activated ACH-2 cells in a dose-dependent manner (line with solid black diamonds). Neither heat-inactivated complement (gray line with solid gray circles) nor irrelevant rabbit IgG pAbs (black line with open circles) triggered cytolysis of ACH-2 cells. C) A3.01 cells that are ACH-2 parental cells prior to HIV-1-infection were resistant ADCML. The cytolysis did not occur in any of the conditions mentioned above. D) Comparisons of ADCML efficacy of provirus-activated ACH-2 cells in the absence (Med condition) or presence of antiretroviral drugs (SQV, 5 µM; ATV, 8 nM; DRV, 9 nM; FTC, 5 µM). The data represent the results from at least three independent experiments. Black line with solid black diamonds: anti-Env pAbs plus complement; gray line with solid gray circles: anti-Env pAbs plus heat-inactivated complement; black line with open circles: irrelevant rabbit IgG pAbs plus complement. C, complement; iC, heat-inactivated complement.



**Fig. 5. Specific lysis of provirus-activated ACH-2 cells by anti-HIV-1 Abs present in plasma of infected patients**

Plasma samples from 15 chronically HIV-1-infected patients including 6 patients on virally-suppressive ART and 9 ART-naïve patients were tested for their activity in triggering ADCML of provirus-activated ACH-2 cells. Plasma samples from 5 uninfected individuals were included as controls. A) Cytolysis of provirus-activated ACH-2 cells by plasma samples from HIV-1-infected patients on ART or ART-naïve or plasma from uninfected individuals. B) A3.01 cells were resistant to ADCML by plasma samples from HIV-1-infected patients on ART or ART-naïve or from uninfected individuals. All plasma samples were heat-inactivated at 56°C for 30 min to destroy complement prior to performing the ADCML, and the final dilution of plasma was 1:10. C) Pooled data of cytolysis by plasma samples from HIV-1-infected patients on ART or ART-naïve or from uninfected

individuals. The experiments were repeated twice for each test. The results are represented by mean  $\pm$  SD. Black bars (negative controls), ACH-2 cells treated with BRIC229 (20  $\mu$ g/ml) + heat-inactivated plasma as a source of anti-HIV-1 Abs, but no complement was added; Open bars (experimental ADCML), ACH-2 cells treated with BRIC229 (20  $\mu$ g/ml) + heat-inactivated plasma as a source of anti-HIV-1 Abs + complement; Gray bars (complement controls), cells treated with BRIC229 (20  $\mu$ g/ml) + heat-inactivated plasma as a source of anti-HIV-1 Abs + heat-inactivated complement. Plasma, only plasma, no complement; Plasma + C, plasma plus complement; Plasma + iC: plasma plus heat-inactivated complement. Statistical significance (\*\* $p < 0.01$  versus no complement or heat-inactivated complement) is indicated by asterisks, while ns indicates no statistical difference.



**Fig. 6. Anti-Env Abs triggered ADCML of latently HIV-1-infected primary resting CD4<sup>+</sup> T cells**  
 The spinoculation-infection approach was used to establish HIV-1 latency in primary resting CD4<sup>+</sup> T cells without activation of these cells as previously reported (48). A) Integrated HIV-1 DNA was measured from DNA samples prepared from primary resting CD4<sup>+</sup> T cells 3 day after the spinoculation-infection using the nested *Alu*-Gag qPCR as previously described (49). DNA samples prepared from unstimulated ACH-2 cells and A3.01 cells were subjected to the nested *Alu*-Gag qPCR to serve as positive and negative controls of HIV-1 integration, respectively. The data are representative of three independent experiments. B) A fraction of latently infected primary resting CD4<sup>+</sup> T cells contains replication-competent provirus. In each FACS plot, the percentage of p24-positive primary CD4<sup>+</sup> T cells or ACH-2 cells in response to RMD stimulation in the boxed region is shown. Anti-CD3/anti-CD28 beads and PMA (10 ng/ml) plus ionomycin (1  $\mu$ g/ml) were used as positive stimulants to stimulate primary CD4<sup>+</sup> T cells and ACH-2 cells, respectively. The experiments were performed in the presence of SQV (1.25  $\mu$ M) to prevent spreading infection as previously described (48). Uninfected resting primary CD4<sup>+</sup> T cells and A3.01 cells were treated as the same as the latently infected cells. They were used as controls for background staining with the anti-Gag mAb (KC57). Staining with an isotype-matched control mAb was also used to obtain staining background. C) Pooled data show the percentage of p24-positive cells as determined by intracellular p24 staining from latently-infected primary cells from 3 donors (n=3) or from ACH-2 cells in response to RMD

stimulation for 3 days. D) Anti-Env pAbs triggered ADCML of provirus-activated primary CD4<sup>+</sup> T cells (open bars). Neither heat-inactivated complement (gray bars) nor medium alone or irrelevant rabbit IgG pAbs (black bars) triggered cytolysis of these cells. The results are represented by mean  $\pm$  SD of ADCML of three blood donors. Open bars, cells treated with BRIC229 (20  $\mu$ g/ml) + anti-HIV-1 pAbs + complement; Gray bars (complement controls), cells treated with BRIC229 (20  $\mu$ g/ml) + anti-HIV-1 pAbs + heat-inactivated complement; Black bars, cells treated with BRIC229 (20  $\mu$ g/ml) + anti-HIV-1 pAbs + medium. Statistical significance (\*\* $p < 0.01$  versus no complement or heat-inactivated complement) is indicated by asterisks, while ns indicates no statistical difference.

Table 1

Profiles of HIV-1-infected patients on ART or ART-naïve

Patient ID	Age (Years)	Sex (M/F)	Race	Ethnicity	CD4 T cells (Count/ $\mu$ l)	Plasma HIV-1 RNA (Copies/ml)	ART	Anti-HIV-1 EnvAb Titer
<b>ART-Naïve</b>								
G022	24	M	W	NH	386	5,600	Naïve	409,600
G023	31	F	B	NH	315	13,000	Naïve	409,600
G025	54	F	B	NH	754	26,877	Naïve	819,200
G026	18	F	B	NH	365	5,700	Naïve	204,800
G031	34	F	B	NH	913	47,800	Naïve	25,600
G041	42	M	W	NH	763	10,837	Naïve	819,200
<b>Suppressive ART</b>								
R01	56	M	B	NH	648	<50	Suppressive	51,200
R02	53	F	B	NH	680	<50	Suppressive	128,000
R03	52	M	W	NH	855	<50	Suppressive	12,800
R04	31	M	B	NH	456	<50	Suppressive	204,800
R05	23	M	B	NH	300	<50	Suppressive	12,800
R06	26	M	B	NH	764	<50	Suppressive	51,200
R07	47	M	B	NH	482	<50	Suppressive	12,800
R08	33	M	B	NH	566	<50	Suppressive	12,800
R09	60	M	W	H	417	<50	Suppressive	51,200

**Note**

M, Male; F, Female; W, white; B, Black; NH, non-Hispanic; H, Hispanic