Supplementary Materials and Methods

Cell Culture

All cell cultures were maintained at 37°C in 5% CO_2 humidified atmosphere. Virus producer cells (293T and BOSC cells (1)) were maintained in D10 medium [DMEM medium (Gibco) supplemented with 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM glutamine (Pen-Strep-Glutamine, Invitrogen), 10 mM HEPES (Invitrogen), 10% fetal bovine serum (Sigma, Invitrogen), and 0.022% plasmocin (Invivogen)]. All other cells were maintained in R10 medium [RPMI-1640 medium (Gibco) supplemented as D10]. Primary T cells were cultured in R10-50 [R10 plus 50IU/mL interleukin-2 (IL-2, Fisher 202IL010)]. CEM cell lines expressing recombinant, HA-tagged MHC-I molecules (2, 3) were maintained in R10 supplemented with 1mg/mL geneticin (Gibco).

Viral constructs and infections

(i) HIV constructs: NL4-3-ΔGPE-GFP (ΔGPE) wild type and HXBePLAP wild type and *nef* mutants have been described previously (4) (5). 454-Gag-GFP was constructed from a molecular clone isolated from a donor who was treated with combination ART and had undetectable plasma viral levels (6). Briefly, we utilized 454-LTR-GFP, created as previously described (6), in which *gfp* was inserted by gene synthesis in frame at position 809, which corresponded to position 19 in the *gag* open reading frame and created a Gag-GFP fusion protein when expressed. To reconstruct the remainder of the genome, we used PCR to generate donor derived sequence from position 4761 in *pol* through the XhoI site at 9255 in *nef* using the re-constructed near full length 454 genome as a template. The PCR product, which contained 11-15 base pair overlaps with 454-LTR-GFP, was inserted using the GeneArt Seamless Cloning Enzyme Mix (Thermo Fisher A14606). Nef mutations were introduced into ΔGPE and 454-Gag-GFP by filling in a unique Xho I site using klenow and re-ligating.

(ii) HIV infections: Infectious supernatants were prepared by co-transfection of 293T cells using polyethylenimine (PEI) as previously described (7) with each viral construct, the HIV packaging plasmid pCMV-HIV, and pHCMV-G at a mass ratio of 1:1:1. 293T cells were maintained and transfected in D10 medium. Infections were performed by spinoculation at 1,050*xg* for 2 hours at room temperature at a density of $1.0x10^6$ cells/mL. Primary cells were spinoculated in undiluted infectious supernatants supplemented with 4μg/mL hexadimethrine bromide (polybrene, Sigma-Aldrich, H9268). Cell lines were spinoculated with infectious supernatants diluted in D10 to achieve the desired MOI (approximately 50% infection) in the absence of polybrene. Following spinoculation, infectious supernatants were replaced with the appropriate culture medium for the infected cell type.

(iii) MSCV: Murine stem cell virus internal ribosome entry site GFP (pMIG) constructs containing various *nef* alleles were generated as previously described (8). Retroviral supernatants were prepared using BOSC cells transfected with the pMIG constructs (5.5μg), the retrovirus packaging vector pCL-Eco (4.0μg)(9) and pHCMV-G (0.5μg) using PEI as for HIV. Viral supernatants were collected 48 hours post-transfection, clarified by centrifugation, stored at -80°C, and transductions were performed as described for HIV constructs.

(iv) Adenoviral vectors: Nef-expressing and control adenoviral vectors were obtained from the University of Michigan Gene Vector Core (vector clone: Ad-Ef1a.dIE3 #6, Nef clone: Ad-EF1 Nef.dIE3 #2) as previously described (10). CEM-A2 cells were transduced in serum-free R10 medium for 6 hours at a concentration of $1.0x10^6$ cells/mL, then R10 with 20% FBS was added to achieve a density of $5.0x10^5$ cells/mL in R10.

Flow cytometry surface staining

All flow cytometry stains were performed on ice in FACS buffer (2% fetal bovine serum, 1% human AB serum (Fisher, BP2525), 2 mM HEPES, 0.025% sodium azide (Sigma) in PBS). Briefly, cells were resuspended in primary antibody diluted in FACS buffer for 20 min., washed once in FACS buffer, resuspended in secondary antibody diluted in FACS buffer for 15 min., washed once in FACS buffer, and fixed in 2% paraformaldehyde. Primary antibodies against the following proteins were used: HLA-A2 (BB7.2 from HB-82 hybridoma as previously described (11), 0.5μg/mL), Bw4 (Bw4-PE (Miltenyi Biotec, 130-103-847, 1:50), Bw6 (Bw6-APC (Miltenyi Biotec, 130-099-845, 1:50)), pan MHC (w6/32 (Fisher, MA1-70111, 1:1000), PLAP (PLAP-647 (Santa Cruz Biotechnology, clone 8B6, sc47691, 1:1000), CD4 (BD Bioscience, 555344, 1:1000), and HA (HA.11, clone 16B12, Covance, MMS-101R 1:100).

Secondary antibody for BB7.2 was goat anti-mouse IgG2b-AF647 or -AF546 (Invitrogen, 1:2000), secondary antibody for w6/32 was goat anti-mouse IgG2a-PeCy7 (Abcam, 1:1000), secondary antibody for CD4 was goat anti-mouse IgG1-PE (Invitrogen, 1:1000), secondary antibody for HA.11 was goat anti-mouse IgG1-AF647 (Invitrogen, 1:1000).

2μg/mL 7-aminoactinomycin D (7-AAD; Calbiochem) or 4ng/mL DAPI (4′,6-diamidino-2-phenylindole; Thermo Scientific) viability dyes were included with secondary antibodies in staining protocols. In all experiments, cells were gated sequentially by forward scatter vs. side scatter for cells, doublet exclusion (forward scatter area vs. height) for singlets, and exclusion of viability dye for viable cells. Flow cytometry data were collected with a BioRad Ze5 cytometer, a MoFlo Astrios cytometer (Beckman Coulter), or a BD FACScan cytometer with Cytek 6-color upgrade, and all flow cytometry data were analyzed with FlowJo software.

Primary screen and counter-screen

CEM-A2 cells were transduced with an adenoviral vector expressing Nef derived from NL4-3 driven from the Ef1α promoter at the minimal multiplicity of infection that demonstrated downmodulation of HLA-A2 in at least 90% of cells as assessed by flow cytometry, which was determined for each viral prep. Cells were incubated for 48 hours, then counted and re-suspended in R10 with only 0.2% FBS at a density of $2.0x10^6$ cells/mL. Experimental plates were prepared separately using robotic equipment provided by the University of Michigan Life Sciences Institute Center for Chemical Genomics by delivering 4µL of PBS into each well on a 384 well plate followed by Natural Product Extracts (NPEs). CEM T cells transduced with adenoviral vector were then dispensed into experimental plates (4µL/well) and incubated overnight at 37°C and 5% CO_2. 2µL of a 5x antibody solution of 7-AAD and BB7.2-AF488 in 5x FACS buffer (10% FBS, 5% Human A/B serum, 5% HEPES and 0.025% sodium azide in PBS) was added directly to the cells and culture medium per well and incubated at 4°C for 30 minutes. Each sample was then diluted and fixed with 20µL of 1.5% paraformaldehyde (Sigma-Aldrich) in 1x FACS buffer (2% FBS, 1% Human A/B serum, 1% HEPES ad 0.025% sodium azide in PBS). After fixation, plates were read on an Accuri C6 flow cytometer (BD Biosciences, San Jose, CA) within one week. The counter screen was performed exactly as the primary screen but with parental CEM-SS T cells that do not express HA-HLA-A2.

Relative activity among the averaged control samples was set to 100% for control-vector-transduced cells, and 0% for Nef-transduced cells. Activity was defined as an increase in HLA-A2 cell surface expression greater than three standard deviations above the negative control samples for each plate, within the entire assay, or greater than 25% activity. Wells in which fewer than 25 live cell events were recorded were discarded, interpreted as cytotoxic, or considered worthy of re-testing due to potential sampling error. Only compounds which tested as hits in a minimum of 2 of 3 replicate wells were counted as confirmed hits. Hits from the primary screen

were then subjected to the counter screen. Any compound that registered as a hit in the counter screen using the same criteria was eliminated as a false positive.

Initial screening was performed on the Spectrum library of FDA-approved compounds, Chembridge and Maybridge collections of drug-like compounds, and the ChemDiv 100,000 library of small molecules. We subsequently screened the natural product extract library housed in the Natural Products Discovery Core (NPDC) at the University of Michigan. This library consists of over 40,000 natural product extracts and over 8,000 microorganisms from a variety of locations worldwide. Hits that were deemed to be confirmed positives in the counter screen were then subjected to secondary screening using NL4-3-ΔGPE-GFP-transduced CEM-A2 T cells. Activity was defined as percent inhibition of MHC-I fold downmodulation.

Secondary screen

CEM-A2 cells were infected with ΔGPE and maintained in R10 for 48 hours post-infection. Cells were counted, re-suspended and plated in a 96-well flat-bottom plate at $1x10^5$ cells/100μL in R10 with only 0.1% FBS, supplemented with hits from the primary screen. After 24 hours, downregulation of HLA-A2 was assessed by BB7.2 staining of viable cells, comparing the MFI of HLA-A2 in GFP^+ infected cells to that in uninfected GFP^- cells to obtain the fold inhibition of Nef.

Flow cytometric viability assay

Primary, PHA-activated $CD4^+$ T cells were treated as in the MTT assay prior to harvest after 72 hours of exposure to plecomacrolides. Cells were pelleted and incubated on ice in FACS buffer with 2μg/mL 7-AAD for 15 minutes, washed once in FACS buffer, and fixed in 2% paraformaldehyde. The frequency of cells excluding the 7-AAD vital dye was assessed using a BD FACScan cytometer with Cytek 6-color upgrade and analyzed with FlowJo software.

MTT assay

$CD4^+$ T cells were plated at a density of $1x10^5$ cells in 200μL R10-50 in flat-bottom 96-well plates 4 days post-stimulation with PHA. Cells were exposed to titrations of plecomacrolides or solvent controls for 72 hours in culture, at which point viability was assessed relative to solvent by MTT assay in experimental duplicates. Equal volumes of cell culture medium containing $CD4^+$ T cells were pelleted in 96-well round-bottom plates and incubated in 4.5mg/mL MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide, Fisher, M6494) in R10 with no Phenol Red at 37°C until the purple formazan signal was clearly visible. The absorbance of cell pellets resuspended in DMSO was measured at 595nm on a Molecular Devices Emax precision microplate reader and compared a standard curve of known viable cell numbers to ensure the experimental samples fell within the linear range of the assay.

DAPI cell cycle analysis

DAPI cell cycle analysis was performed as previously described (12), and data were collected on a BioRad Ze5 cytometer and analyzed with FlowJo software.

LysoTracker fluorescence microscopy

$CD4^+$ T cells were treated at a density of $1x10^6$ cells/mL in R-10/50 for 24 hours, at which point 500,000 $CD4^+$ T cells were incubated in 500 μL of PBS containing 100 nM LysoTracker Red DND-99 and 5 μg/mL Hoechst 33342 (Invitrogen, H3570) on a Poly-L-lysine (Sigma Aldrich) coated chambered slide (ThermoFisher, 154534) for 1 hour at 37°C. Slides were then fixed in PBS + 2% PFA for 20 minutes at room temperature and washed once in PBS. ProLong Gold Antifade Mountant (Invitrogen, P36930) was applied before adding coverslips. Images were acquired on a

Leica SP5 confocal microscope using identical instrument settings for each sample, and maximum projections were created using ImageJ.

Western blotting

Sorted PLAP$^+$ CD4$^+$ T cells isolated as previously described (13) were pelleted and lysed in Blue Loading Buffer (Cell Signaling Technology, 56036S) with DTT according to manufacturer's protocol. Lysates were sonicated with a Misonix Sonicator (QSonica) at 100 amps for four minutes, boiled at 95°C prior to loading onto Criterion Tris-HCl gels (Bio-Rad Laboratories, Hercules CA), and separated by gel electrophoresis. Gels were transferred onto PVDF transfer membrane (MilliporeSigma, IPVH00010) for 90 minutes at 350 mA. Membranes were blocked in 5% milk (LabScientific Inc., Highlands, NJ) in TBS-T (0.05% Tween 20, 0.15M NaCl, 0.01M Tris pH 8.0) for 1 hour. Antibodies against the following proteins were used for western blotting: clathrin adaptor protein AP-1 γ (Fisher, 610386, 1:100); Nef (2949, AIDS Research and Reference Reagent Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, NIH, Ron Swanstrom, 1:500); MHC-I heavy chain (HC.10, prepared as described (14)); CD4 (Abcam, 133616, 1:1000); HA (HA.11, Covance), glyceraldehyde-3-phosphate dehydrogenase (Abnova, clone 32C, H00002597) and AP-1 μ1 (RY/1, Dr. Linton Traub, University of Pittsburgh). The secondary antibody for GAPDH and HA.11 was Rat anti-Mouse IgG1-horesradish peroxidase (Invitrogen, 18401582). The secondary antibody used for Nef 2949, CD4, and RY/1 was Goat anti-Rabbit IgG-HRP (Invitrogen, 656120). The secondary antibody used for AP-1 γ was Goat anti-Mouse IgG1-HRP (Zymed Laboratories Inc.). The secondary for HC.10 was Rat anti-Mouse IgG2a-HRP (Invitrogen, 046220).

Western blotting results were quantified using Photoshop by determining the mean pixel density in a box of equal size over each band from a single, unedited film displaying a single gel.

Background pixel density was subtracted. No quantification comparisons were made from bands on different films or gels at any point.

In vitro investigations of AP-1:Nef:MHC-I complexes

Recombinant protein expression and purification

The His6- and GST-tagged AP-1 core, mouse AP1μ1 (157-423) (referred as μ1-CTD), human Arf1 (17-181)-Q71L, human MHC-I (338-365)-NL4-3 Nef, HIV-1 NL4-3 Nef constructs and protein purification were previously described (15, 16). For the GST pull down assay, codon-optimized human MHC-I (338-365) was subcloned into pGST parallel2 vector using BamHI/XhoI sites, and fused to an N-terminal GST tag and a TEV cleavage site (17). PCR encoding HIV-1 Nef or SIVsmm Nef fused with GFP was subcloned into LIC 2BT vector (Macrolab) and expressed as a TEV-cleavable N-terminal His6 tag and C-terminal uncleavable GFP tag.

His-NL4-3 Nef-GFP or His-SIVsmm Nef-GFP constructs were expressed in BL21 (DE3) star cells (Life technologies, Grand Island, NY), induced with 0.3mM IPTG at 25°C overnight. The purification was carried out using Ni-NTA resin. The eluate was subjected to a HiLoad 16/60 Superdex 75 column in the buffer of 20mM Tris pH 8, 300mM NaCl, 0.1mM TCEP.

His-MBP tagged μ1-CTD was expressed in BL21 (DE3) star cells and induced with 0.3mM IPTG at 20°C overnight. The clarified lysate was purified by Ni-NTA resin. The protein was eluted with 0.1 M imidazole in 50mM Tris pH 8, 300mM NaCl, followed by TEV cleavage at 4°C overnight. The sample was then diluted 2 times by SP buffer A (30mM Tris pH 8), and then loaded onto a HiTrap SP HP 5mL column (GE healthcare). The SP column elution was performed with a 10 CV linear gradient from 0-1 M NaCl in SP buffer A. The sample fractions were pooled together and subjected to a 16/60 Superdex 75 column in 20mM Tris pH 8, 300mM NaCl, 0.1mM TCEP.

GST tagged MHC-I tail was expressed in BL21 (DE3) star cells by induction at 20ºC overnight. The purification was carried out using glutathione-Sepharose 4B resin, the elution was then subjected to a HiLoad 16/60 Superdex 75 column in 20mM Tris pH 8, 150mM NaCl, 0.1mM TCEP.

AP-1:Arf1: MHC-I-Nef complex assembly

Recombinant AP-1 core was mixed with Arf1-GTP and MHC-I-Nef at a molar ratio of 1:4:6, then incubated at 4ºC overnight. The mixture was then subjected to a Superose6 10/100GL column in 20mM Tris pH 8.0, 150mM NaCl, 5mM $MgCl_2$, 0.3mM TCEP. The early eluted peak, corresponding to AP-1 trimer assembly, was pooled together and concentrated to 25μM. Each AP-1 trimer complex consists of three AP-1 core, three MHC-I-Nef, and six Arf1-GTP molecules.

Differential Scanning Fluorimetry (DSF) assay

DSF assays were performed using a Stratagene Mx3000P RT-PCR machine (Stratagene, La Jolla, CA) to monitor protein unfolding by the florescence increasing of SYPRO Orange (Invitrogen, Carlsbad, CA). SYPRO Orange (5000x concentration in DMSO) was first diluted to 1000x using DMSO, then diluted to 100x using the assay buffer. The final volume of the reaction was 20 μl. Protein samples with CMA (6 or 12μM) or with the DMSO control were first incubated at 4 ºC for one hour, then mixed with SYPRO Orange dye in a 96-well polypropylene plate (Agilent Technologies, Santa Clara, CA). DMSO concentration in each well was fixed at 5% (v/v). Final concentrations of the proteins were 6μM in the assay buffer (20mM HEPES pH 7.5, 200mM NaCl, 1mM TCEP), and the final dye concentration was 8x. The fluorescence intensity was measured using the SYBR green filter over the temperature range of 25 to 90ºC in 1 degree/min increments. After subtracting fluorescence from the DMSO control reaction without protein, the average fluorescence intensities were plotted as a function of temperature. Measurements were repeated three times and the data were processed using Origin software (OriginLab, Northampton, MA).

The fluorescence intensity (before post-peak region) was fitted to Boltzmann equation to obtain melting temperature (Tm).

GST pull down assay

35 μg of recombinant GST-MHC-I tail proteins were incubated with His-MBP tagged μ1-CTD and HIV-1 NL4-3 Nef-GFP or SIVsmm Nef-GFP proteins (10 μM each), with or without CMA (40 μM) at 4°C overnight in 20 mM Tris pH 8, 150 mM NaCl, 0.1 mM TCEP. DMSO concentration in each tube was fixed at 2.5%. 30 μl glutathione-Sepharose 4B resin was then added into the mixture, which was rocked at 4°C for 2 hours. The beads were washed 4 times, mixed with 60 μl of 2x lithium dodecylsulfate (LDS)/ βME buffer and heated at 90°C for 3 min. 28 μl of each sample and 1 μg inputs were subjected to SDS/PAGE gel and stained with Coomassie blue.

CTL clones

CTL clones were isolated by limiting dilution from HIV-1 infected individuals. Clonality of the line was established by demonstration of unique T cell receptor usage. The CTL clones were maintained in culture with periodic re-stimulation as previously described (74, 75) except for the following changes; CTL clones were stimulated with anti-CD3 clone 12F6 (NIH AIDS Reagent Program) and cultured with IL-2 (NIH AIDS Reagent Program, Hoffman-La Roche, 136). Peripheral blood mononuclear feeder cells were isolated from leukopaks (New York Blood Center) and X-irradiated with 30 cGy in R10 medium. Irradiations were performed using a Kimtron IC 225 (Kimtron Medical) at a dose rate of approximately 2 Gy/min in the University of Michigan Comprehensive Cancer Center Experimental Irradiation Core (Ann Arbor, MI). CTL clones 115B15 and 161JXA14 both recognize HIV gag amino acids 77–85; SLYNTVATL presented by MHC-I HLA-A2 (76).

CTL killing of peptide-pulsed JY cells

The HLA-A2 expressing B-cell line JY was maintained in R10 medium. Non-peptide pulsed cells were labeled with 1μM CellTrace Violet (Invitrogen, C34557) according to the manufacturer's protocol. Cells for gag SL9 peptide loading were labelled with 0.5μM CellTracker Green CMFDA (Invitrogen, C7025) in R10 medium for 15 min at 37°C and quenched with 5 volumes warm R10 medium. Peptide loading was performed in 10μg/mL gag SL9 peptide in R10 medium for 1 h. Unbound peptide was removed with 3 washes of cold R10 medium. Violet (no peptide) and green (peptide-pulsed) cells were combined 1:1 and 20,000 cells per well were added to CTLs at 5:1 and 10:1 effector:target ratios in duplicate. CMA assays included solvent (DMSO, Sigma) or 0.5nM CMA (Cayman Chemical). Target cells were gated by light scatter and viability dye exclusion, then plotted by violet and green fluorescence to distinguish peptide loaded and peptide negative cells. Specific killing was calculated by dividing the percent of viable target cells that were green in the sample by the percent observed in the control wells lacking CTLs and subtracting from 1.

Natural Product extraction and purification

***Streptomyces* sp. (39098-H2N) Fermentation and Extraction**

Streptomyces sp. (39098-H2N) was cultured on R2YE agar (18) for 3-5 days until sporulation occurred. A 1 x 1 cm^2 lawn was inoculated into 2 L of ISP2 (0.4% yeast extract, 1% malt extract, 0.4% dextrose, 3% NaCl), and incubated at 28 °C with agitation (175 RPM) for 48-72 h. Using this growth, nutrient poor media for marine bacteria (0.025% yeast extract, 0.064% malt extract, 0.025% dextrose, 3% NaCl) was then inoculated with 3% (v/v) seed culture. Growths were performed in 50 L batches (300 L total) in baffled 2.8 L Fernbach flasks containing 1 L of media per flask, or 10-15 L bioreactors with agitation (175 RPM) and filtered air bubbled through solution. All growths were performed at 28 °C for 10 days. Completed growths were passed through coffee filters to remove the majority of bacteria and cell debris. MeOH-activated Amberlite XAD16N

absorbent resin (20% w/v) was added to the clarified broth and stirred at room temperature for 24 h. The resin was filtered from the solution, and washed with copious amounts of H_2O (~80 L). Material was eluted by washing with methanol (3 × 3 L), followed by acetonitrile (3 × 3 L). Organic extracts were dried via rotary evaporation. Dry material was dissolved in minimal HPLC grade methanol and filtered to remove any insoluble material. This solution was subsequentially dried yielding 1.573 g of crude extract from 300 L of fermentation.

***Streptomyces* sp. (39098-H2N) fractionation and dereplication**

The sample was dissolved in a minimal volume of methanol, loaded onto C18 resin, and dried *in vacuo*. Flash chromatography was performed using an Isolera One (Biotage®) utilizing a pre-packed SiliCycle® reversed-phase C18 column (40 g). Material was eluted with a flow rate of 50 mL/min collecting 120 mL fractions. Material was eluted using a three-solvent gradient system, consisting of H_2O (solvent A), methanol (solvent B) and acetonitrile (solvent C). The column was first washed with 10% methanol in H_2O for 1 CV, followed by a linear increasing gradient from 10% to 95% methanol in H_2O over 12 CV. An isocratic gradient of 95% methanol in H_2O was then applied for 5 CV, followed finally by an additional isocratic gradient of 95% acetonitrile in H_2O for 5CV. Fractions were dried into pre-weighed vials using a V10-touch evaporator (Biotage®) coupled with a Gilson GX-271 liquid handler. Samples were analyzed in the secondary screen for Nef activity**.** Active fractions (F6-F9) were combined (92.5 mg) for further HPLC purification.

Purification of active metabolites was accomplished utilizing preparative HPLC (Shimadzu LC-20AT), using a reversed-phase Phenomenex Luna 5 µm Phenyl-Hexyl 100 Å column (250 × 10.00 mm) run at a flow rate of 4 mL/min. Compound elution was monitored using a Shimadzu diode array detector (SPD-M20A). The sample was brought up in 1 mL of HPLC grade MeOH (~100 $mgmL^{-1}$), and injected in 100 µL aliquots (using a 200 µL loop). Elution was accomplished using H_2O + 0.1% formic acid (solvent A) and acetonitrile (solvent B). The gradient involved an initial isocratic step of 15% acetonitrile for 2 min. This was followed by a linear increasing gradient

from 15% to 95% acetonitrile over 38 min (40 min total). The column was washed with a isocratic gradient of 95% acetonitrile for an additional 10 min (50 min total), finally followed by an isocratic equilibration step of 15% acetonitrile for 10 min (60 min total). From this nine peaks with similar UV-profiles were collected and immediately dried using the V10-touch evaporator. All fractions were identified as active in the Nef inhibition assay. Samples were analyzed by HRESIMS and screened against the Antibase 2017 database (19), identifying a series of ions related to the bafilomycin family of natural products. Ions of interest include: F3 *m/z* 667.4085 $[M - H + FA]^-$ (bafilomycin A1); F4 *m/z* 764.4253 [M – H + FA] (bafilomycin C1); F5 *m/z* 814.4410 $[M - H]^-$ (bafilomycin B1); F6 *m/z* 719.4054 $[M - H]^-$ (bafilomycin C1); F7 *m/z* 719.4054 $[M - H]^-$ (bafilomycin C1); F8 *m/z* 814.4404 $[M - H]^-$ (bafilomycin B1); F9 *m/z* 828.4561 $[M - H]^-$ (bafilomycin B2). Preliminary NMR data coupled with MS-dereplication, strongly suggested the active metabolites to be the bafilomycin family of natural products. Due to low yields from *Streptomyces* sp. (39098-H2N) and access to a higher producing strain within the Sherman laboratory, efforts towards isolation of bafilomycin analogs shifted to *Streptomyces lohii* Δ*bafY*.

Streptomyces lohii Δ*bafY* fermentation and extraction

Streptomyces lohii Δ*bafY* was cultured on R2YE agar for 3-5 days until sporulation occurred. A 1 x 1 cm^2 lawn was inoculated into 1 L of 2× YT media (1.6% tryptone, 1% yeast extract, 0.5% NaCl), and incubated at 28 °C with agitation (175 RPM) for 48-72 h. Bafilomycin production media (20) was then inoculated with 3% (v/v) seed culture. Growths were performed on 10 L scale in baffled 2.8 L Fernbach flasks containing 1 L of bafilomycin production media per flask. Fermentations were conducted at 28 °C with agitation (175 RPM) for 7 days. Upon completion of the growth, cells were pelleted via centrifugation at 5500 RPM (4°C) for 45 min. The supernatant (containing both aqueous and oil layers) was removed from the cell pellet and stored for workup, as described below. The cell pellets were combined and extracted into acetone (10 L) for 24 h. Cell debris was removed via filtration through coffee filters, and reextracted with acetone (5 L).

Cell debris was removed again via filtration through coffee filters. The acetone layers were combined and dried via rotary evaporation until only water remained. Residual oil that separated as the acetone was dried off, separated from the final aqueous layer using a separatory funnel, and combined with the oil layer from the supernatant produced via centrifugation as described above. This aqueous solution was then extracted 3X with equal volumes of ethyl acetate, followed by a fourth extraction of an equal volume of dichloromethane. The organic layers were combined, dried over anhydrous Na_2SO_4, filtered, and dried via rotary evaporators yielding a viscous, yellow-brown oil.

The supernatant, containing the mixture of oil and aqueous broth generated from centrifugation, was separated using a separatory funnel. The aqueous broth was discarded, and the combined oil layers were extracted 3X with equal volumes of methanol. The methanol extracts were combined and dried immediately via rotary evaporation to minimize any degradation. This yielded a viscous, yellow-brown oil that was combined with the cell extract sample described above, yielding ~6 g of material.

***Streptomyces lohii* Δ*bafY*: Bafilomycin A1 and C1 purification**

The extract was diluted with an equal volume of acetone and loaded onto minimal silica. The loaded silica was dried via rotary evaporation and separated into 4 equal aliquots. Aliquots were loaded into separate dry load vessels and run individually. Flash chromatography was performed using an Isolera One (Biotage®) utilizing pre-packed SiliCycle® normal phase silica columns (40 g). Material was eluted with a flow rate of 50 mL/min collecting 27 mL fractions. Purification was accomplished using a four-solvent gradient system, consisting of hexanes (solvent A), ethyl acetate (solvent B), dichloromethane (solvent C), and methanol (solvent D). Initially, the column was washed with 1 CV of 5% ethyl acetate in hexanes, followed by a linear increasing gradient of 5% to 60% ethyl acetate in hexanes over 13 CV. This was followed by an isocratic gradient of 60% ethyl acetate in hexanes over 6 CV. Bafilomycin A1 eluted at

approximately 13-15 CV as a broad peak absorbing at λ = 235 and 285 nm. To purify bafilomycin C1, the gradient was switched to dichloromethane and methanol. Material was eluted starting with an isocratic gradient of 10% methanol in dichloromethane for 5 CV, followed by a final wash of 20% methanol in dichloromethane for 5 CV. Bafilomycin C1 eluted at approximately 27-28 CV within the 20% methanol in dichloromethane step as a broad peak absorbing at λ = 235 and 285 nm. Fractions were checked by TLC for purity and combined yielding bafilomycin A1 and C1 as impure yellow oils.

Final purification of bafilomycin A1 was accomplished utilizing preparative HPLC (Shimadzu LC-20AT), using a reversed-phase Phenomenex Luna 5 µm C18(2) 100 Å column (250 × 10.00 mm) run at a flow rate of 4 mL/min. Elution was monitored at 235 and 285 nm using a Shimadzu diode array detector (SPD-M20A). The sample was dissolved in HPLC grade acetone to a final concentration of 100 $mgmL^{-1}$. Purification was accomplished by injecting 100-180 µL aliquots of this solution (using a 200 µL loop). Material was eluted using H_2O (solvent A) and acetonitrile (solvent B). The gradient used an initial isocratic step of 60% acetonitrile for 2 min. This was followed by a linear increasing gradient from 60% to 95% acetonitrile over 38 min (40 min total). The column was washed with a isocratic gradient of 95% acetonitrile for an additional 20 min (60 min total), finally followed by an isocratic equilibration step of 60% acetonitrile in water for 10 min (70 min total). Bafilomycin A_1 eluted at 28.1 min. All fractions containing the purified bafilomycin A1 were combined, and dried immediately using a V10-touch evaporator (Biotage®). Material was lyophilized for 24 hrs yielding 48.7 mg of bafilomycin A1 as a white powder; HRESIMS *m/z* 667.4085 $[M - H + FA]^-$ (expected *m/z* 667.4063). Mass spec, elution time and NMR all matched commercial standard.

Final purification of bafilomycin C1 was accomplished utilizing preparative HPLC (Shimadzu LC-20AT), using a reversed-phase Phenomenex Luna 5 µm C18(2) 100 Å column (250 × 10.00 mm) run at a flow rate of 4 mL/min. Elution was monitored at 235 and 285 nm using a Shimadzu diode array detector (SPD-M20A). The sample was dissolved in HPLC grade

methanol to a final concentration of 100 mgmL^{-1}. Purification was accomplished by injecting 250-450 µL aliquots of this solution (using a 500 µL loop). Material was eluted using H_2O + 0.1% (v/v) formic acid (solvent A) and acetonitrile (solvent B) following the same gradient described for bafilomycin A1. Due to instability of the bafilomycins in acidic conditions, material was collected into test tubes containing 5 mL of phosphate buffered saline (pH 7.5). Bafilomycin C1 eluted at 25.1 min. Fractions were combined and the acetonitrile was removed via rotary evaporation. The buffered aqueous layer was then extracted 3X with equal volumes of ethyl acetate. The organic layers were combined and dried *in vacuo*, then lyophilized for 24 hrs, yielding 6.7 mg of bafilomycin C1 as a white powder; HRESIMS *m/z* 719.4053 $[M - H]^-$ (expected *m/z* 719.4012). Mass spec, elution time and NMR all matched commercial standards.

Global Natural Products Social Molecular Networking (GNPS)

The strains *Streptomyces sp. (34893-N3I), Streptomyces sp. (54875-N1N), Streptomyces sp. (5736-A1I),* and *Streptomyces sp. (39098-H2N)* were cultivated in liquid medium and subsequently extracted to be analyzed with HPLC coupled with HRMS and automated fragmentation. The resulting MS^2 data were factored with media blank and analyzed using GNPS (21) to generate respective molecular networks consisting of multiple nodes, with precursor mass tolerance of 2.0 Da and fragment ion mass tolerance of 0.5 Da. The obtained data was then visualized and interpreted using Cytoscape 3.6.1. High-resolution mass spectrometry provided a $[M+ Na]^+$ ion peak at 645.3988 m/z, from which the molecular formula of $C_{35}H_{58}O_9$, containing seven degrees of unsaturation, was deduced. H^1 and C^{13}-NMR comparison along with co-elution LCMS experiment using Baf A1 standard confirmed the identity as bafilomcyin A1.

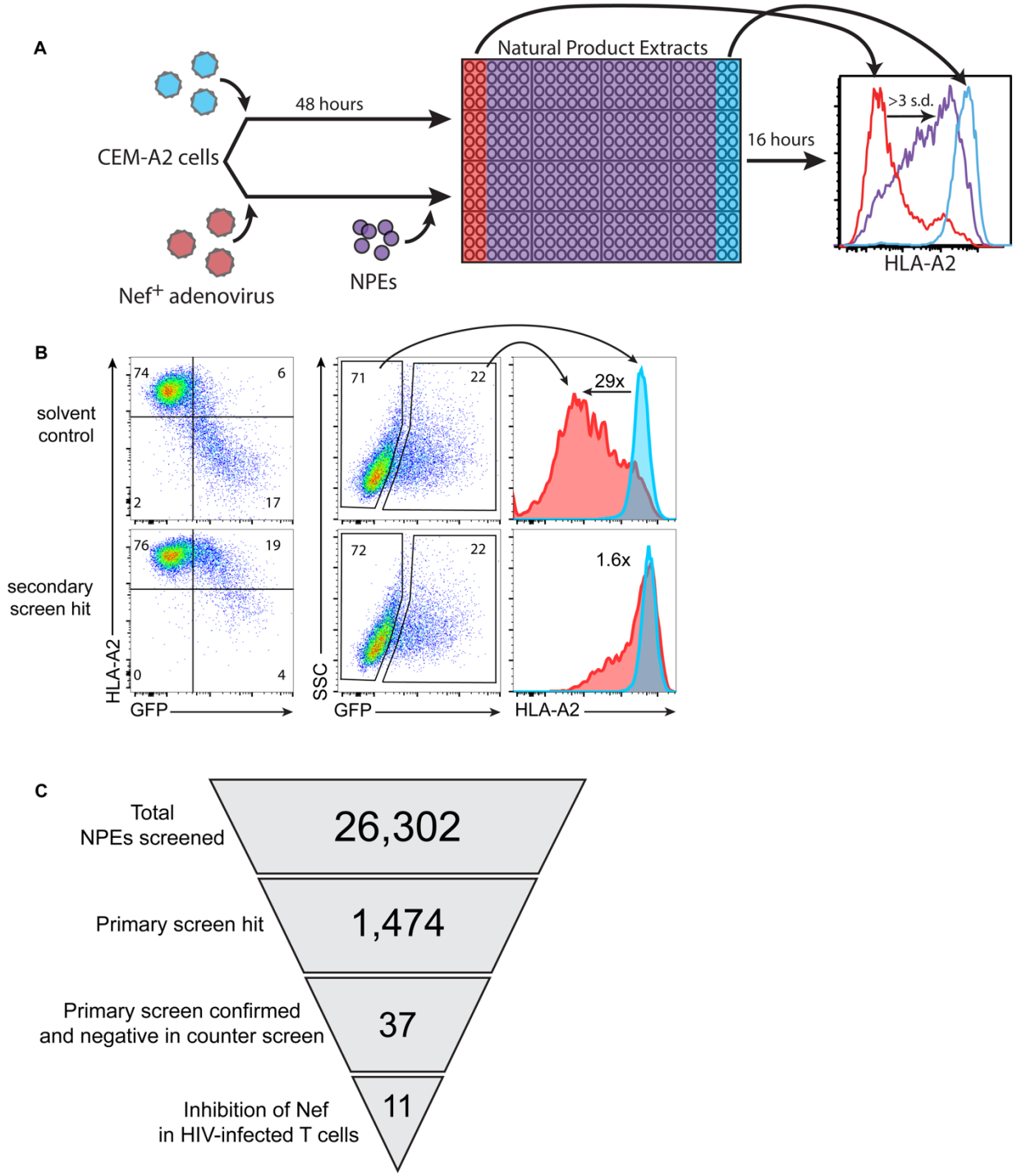

Fig. S1: Screening of natural product extracts for inhibitors of HIV Nef. (A) Schematic representation of primary screen assay. Blue histogram represents CEM-A2 cells transduced with Nef-negative adenoviral vector and treated with solvent control, red histogram represents cells

transduced with Nef-expressing adenoviral vector and treated with solvent control, purple histogram represents cells transduced with Nef-expressing adenoviral vector and treated with a representative positive hit in the primary screen. (B) Representative flow cytometry plots from secondary screen assay showing the solvent control and a representative positive hit in the secondary screen. Blue histograms are from uninfected, GFP^- cells, red histograms are from infected, GFP^+ cells. Numbers in histograms indicate the fold decrease in HLA-A2 MFI in infected cells relative to uninfected cells. (C) Summary of NPE screening results, yielding the identification of 11 microbial strains producing Nef inhibitory compounds for further purification.

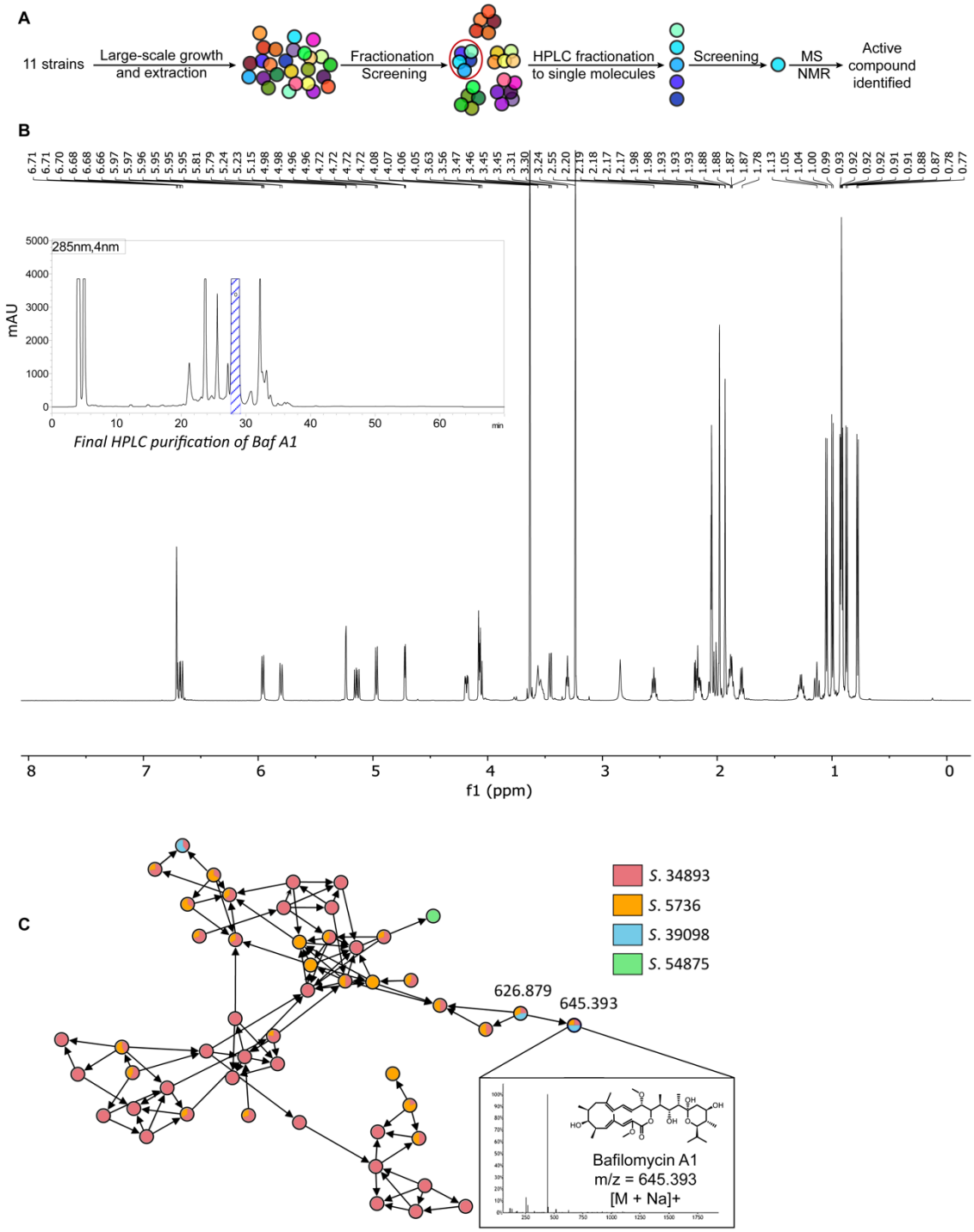

Fig. S2: Plecomacrolides identified as Nef inhibitors in multiple NPE strains. (A) Schematic representation of the isolation of a single active compound from a natural product extract by sequential fractionation and re-screening. (B) Fractionation from one strain yields a single HPLC peak that

possesses activity, and NMR identifies Bafilomycin A1. (C) MS/MS molecular networking reveals Baf A1 in 3 of 4 lead candidate extracts.

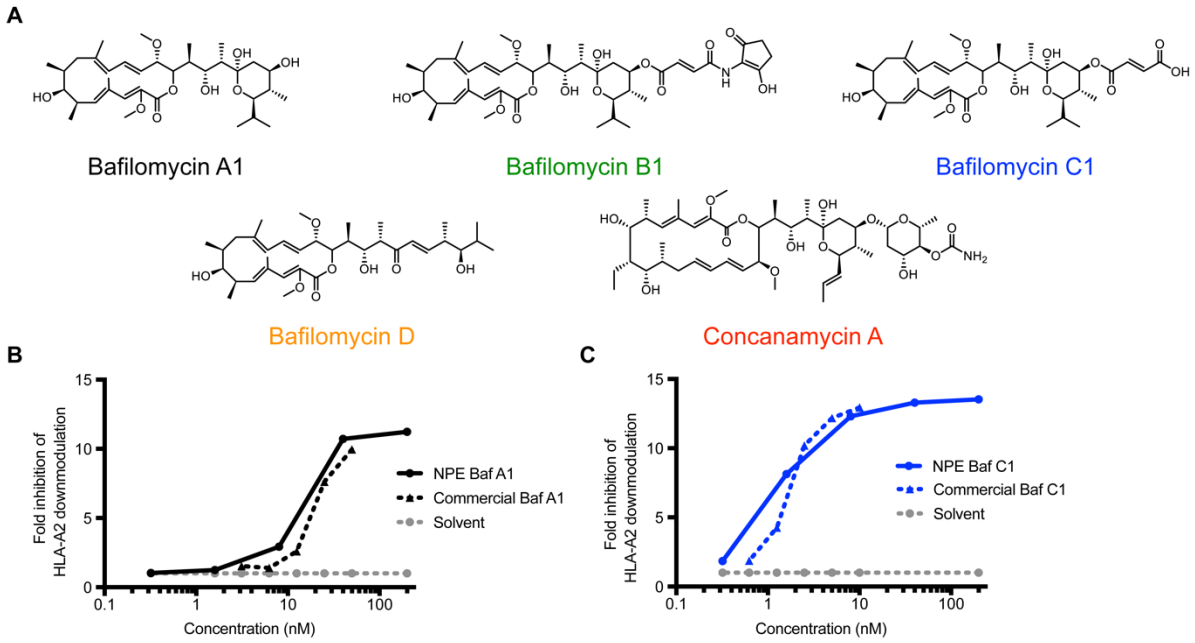

Fig. S3: Natural product extract-derived plecomacrolides mirror commercially available compounds. (A) Chemical structures of plecomacrolides. (B-C) Summary graphs of flow cytometric data from the secondary screen assay (as in Fig. S1B) using the indicated compounds. NPE Bafs were isolated as in Fig. S2 (n=1).

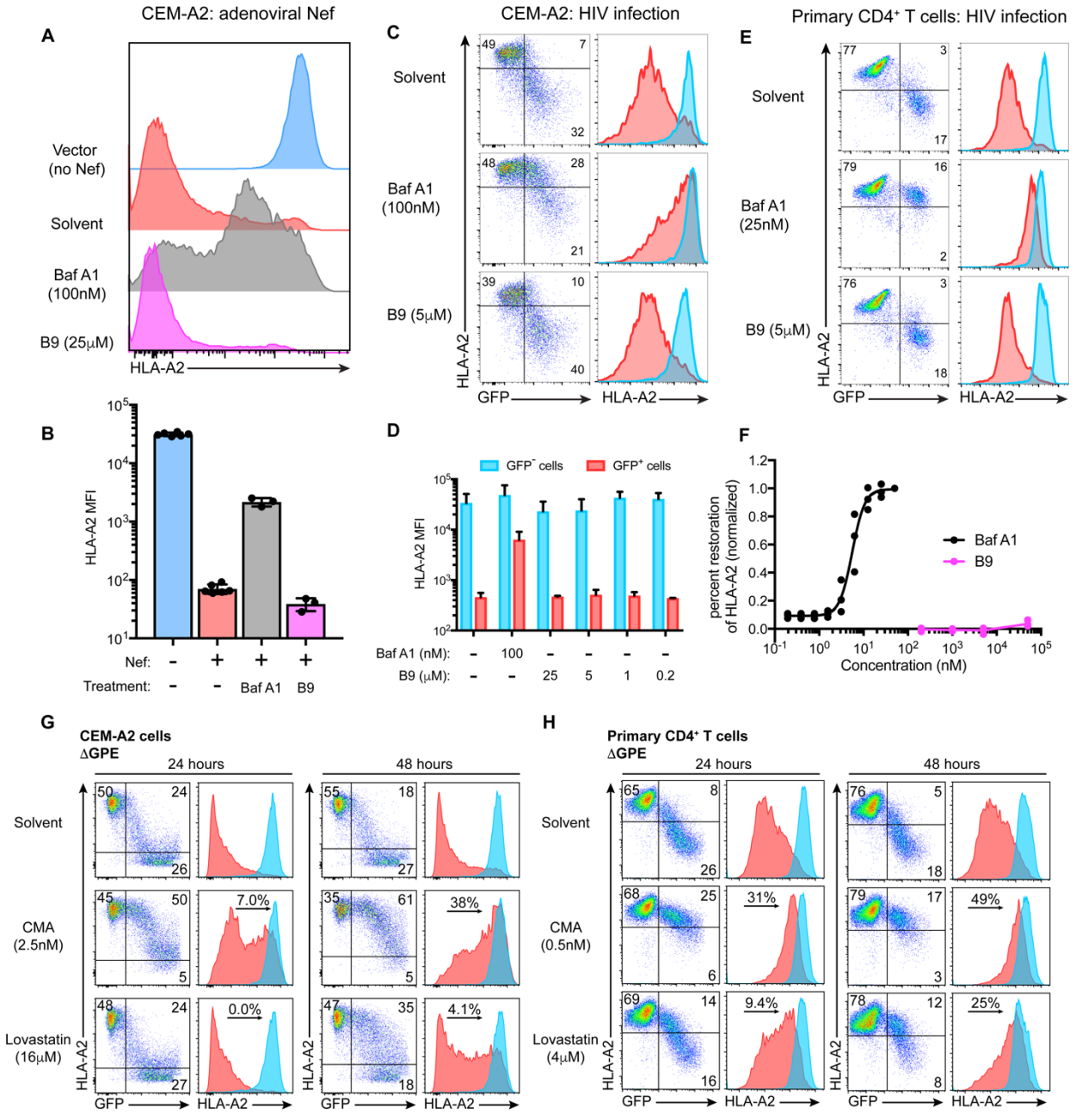

Fig. S4: B9 fails to restore MHC-I in cell line screens and HIV-infected primary cells, while lovastatin restores less effectively than CMA. (A) Representative flow cytometry histograms and (B) summary graph (n=6 for controls, n=3 for experimental samples) from CEM-A2 cells treated with B9 and Baf A1 in the primary screen as in Fig. S1A. (C) Representative flow cytometry plots and (D) summary graph (n=3) from CEM-A2 cells infected with ΔGPE and treated with B9

and Baf A1 in the secondary screen as in Fig. S1B. Blue histograms are from GFP^- cells, red histograms are from infected GFP^+ cells. (E) Representative flow cytometry plots from primary activated $CD4^+$ T cells infected with ΔGPE and analyzed as in the secondary screen in Fig. S1B. Blue histograms are from GFP^- cells, red histograms are from infected GFP^+ cells. (F) Summary graph of assays performed as in E using the indicated compound. Percent restoration of HLA-A2 is calculated as described in Materials and Methods and normalized to percent restoration achieved at 50nM Baf A1 (n=3). (G-H) Representative flow cytometry plots from ΔGPE-infected (G) CEM-A2 cells and (H) primary $CD4^+$ T cells, treated side-by-side with the indicated compound for the indicated time. Numbers in histogram panels indicate percent restoration of HLA-A2 (n=3). Error bars indicate standard deviation, MFI = median fluorescence intensity. Solvent control is DMSO.

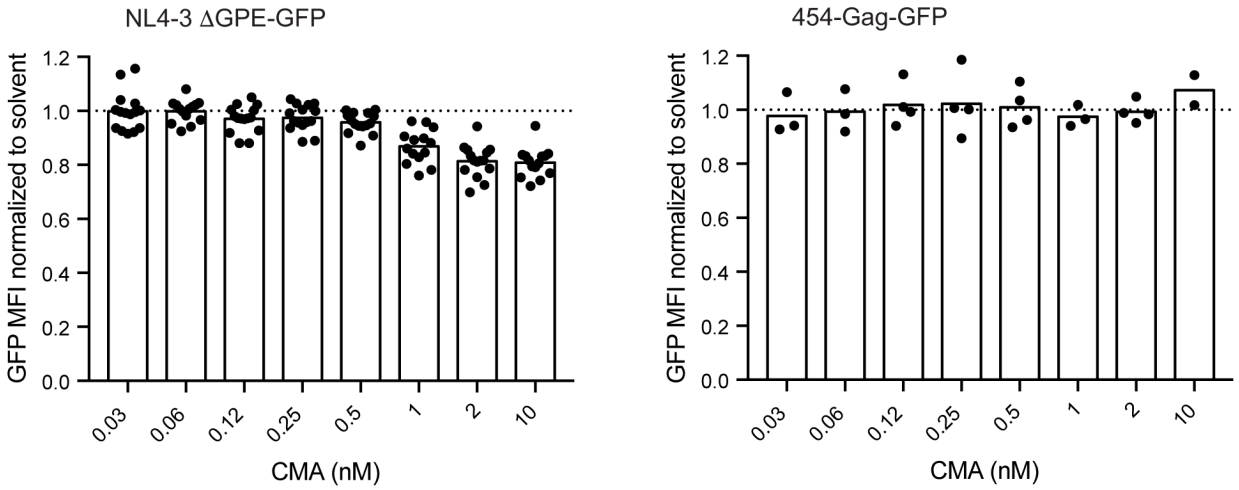

Fig. S5: CMA does not counteract Nef by reducing HIV gene expression in primary CD4$^+$ T cells. Summary graph of flow cytometric data analyzing the mean fluorescence intensity (MFI) of GFP in the GFP$^+$ cells gate of CD4$^+$ T cells infected with the indicated GFP-expressing viral constructs and treated with CMA as in Figure 1. Dots represent biological replicates with independent donors.

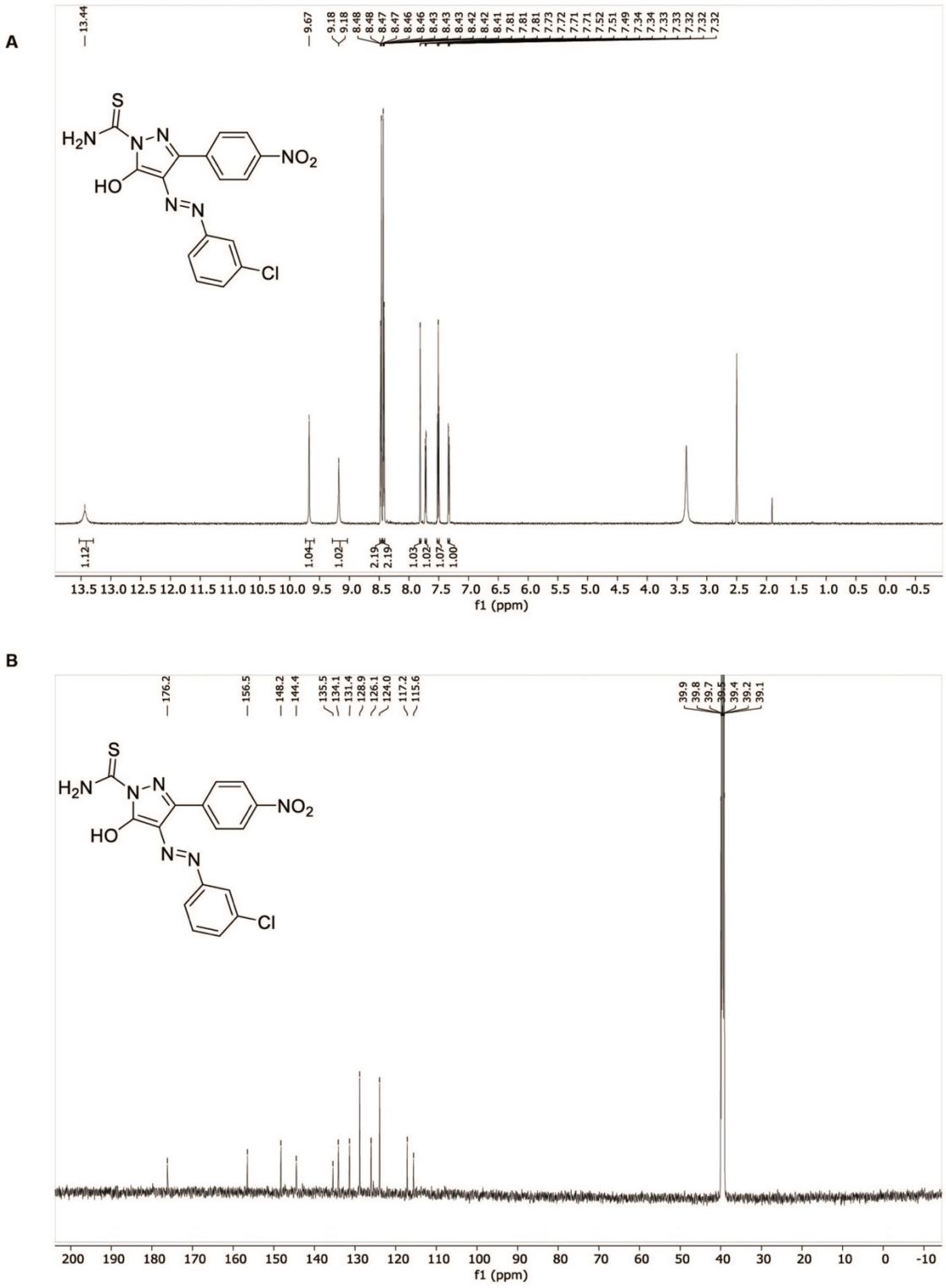

Fig. S6: Confirmation of B9 structure.

(A) 1H-NMR of B9 in DMSO-d_6 (600 MHz). (B) 13C-NMR of B9 in DMSO-d_6 (125 MHz).

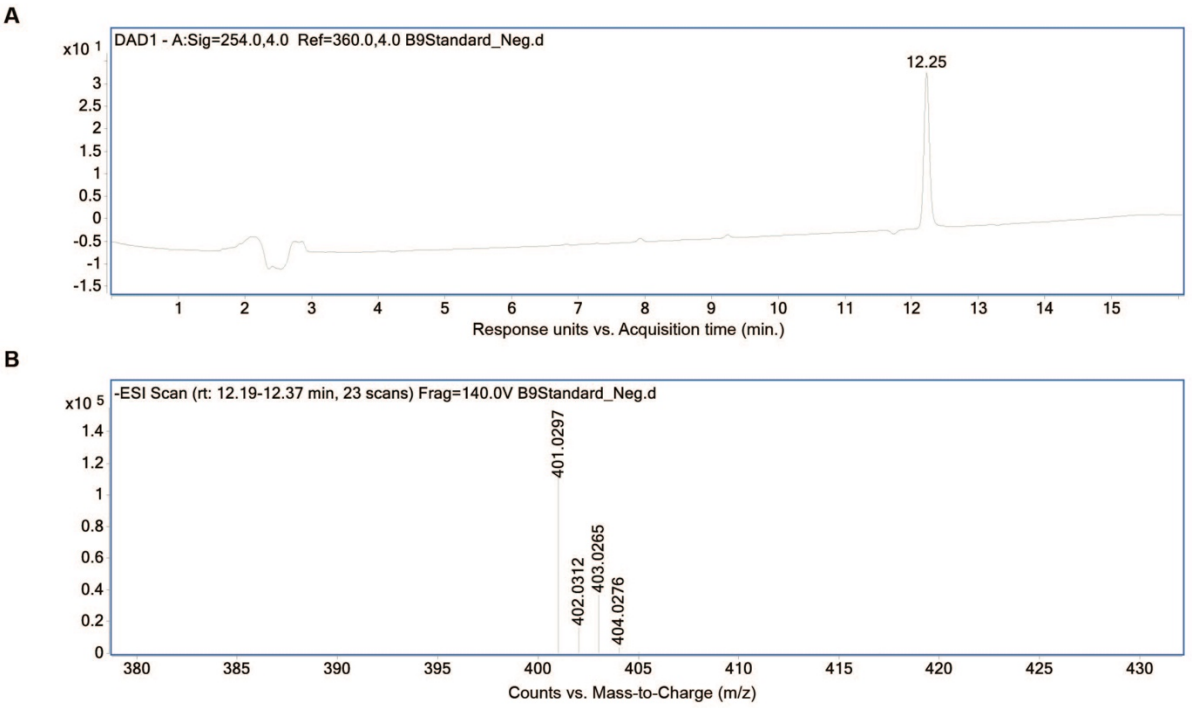

Fig. S7: Confirmation of B9 structure, continued.

LCMS trace of standard B9 illustrating (A) diode array detector (DAD) trace (Rt = 12.25 min) and (B) mass spectrum at 12.15 – 12.41 min. All confirm that the structure of commercially-acquired B9 matches the published structure.

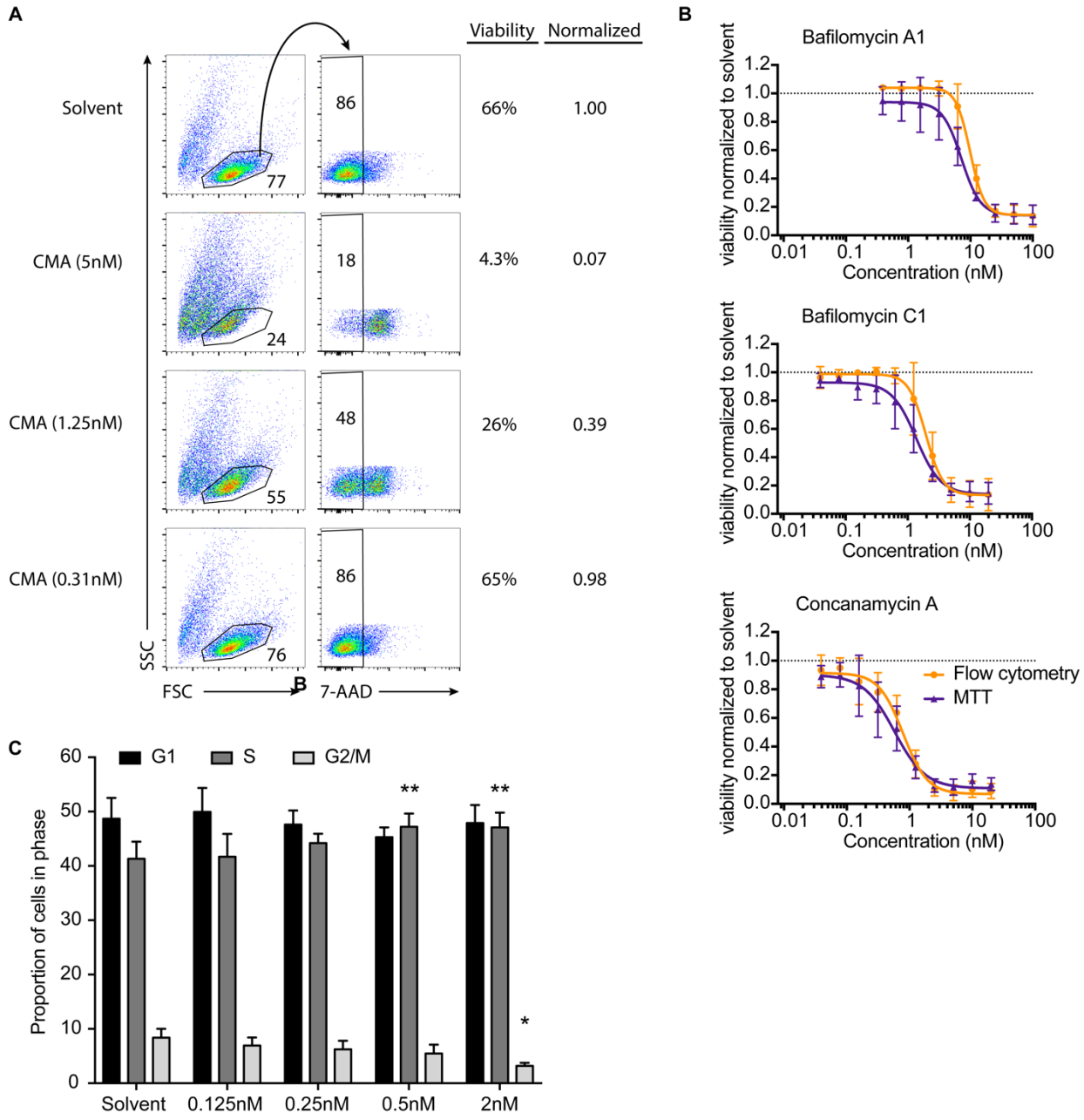

Fig. S8: Plecomacrolide toxicity is consistent between assays and cell cycle arrest requires higher concentrations than Nef inhibition. (A) Gating strategy and representative flow cytometric plots for data in Fig. 1E. (B) Comparison of MTT and flow cytometric viability assays, showing comparable results in primary $CD4^+$ T cells incubated with the indicated

compounds for 72 hours. Summary graph of data from flow cytometric (circles, orange, as in A) and MTT assay (triangles, purple) assessing the viability of $CD4^+$ T cells treated with titrations of the indicated plecomacrolides for 72 hours (n=3 for Baf A1 and C1, n=6 for CMA). Data are normalized to viability of cells treated with matched DMSO solvent control. (C) Summary graph of DAPI flow cytometric cell cycle data from primary $CD4^+$ T cells treated with CMA or matched DMSO solvent control for 24 hours as indicated (n=5). All error bars represent standard deviation. (** = $p < 0.01$, * = $p < 0.05$, Dunnett's multiple comparisons test, compared to solvent)

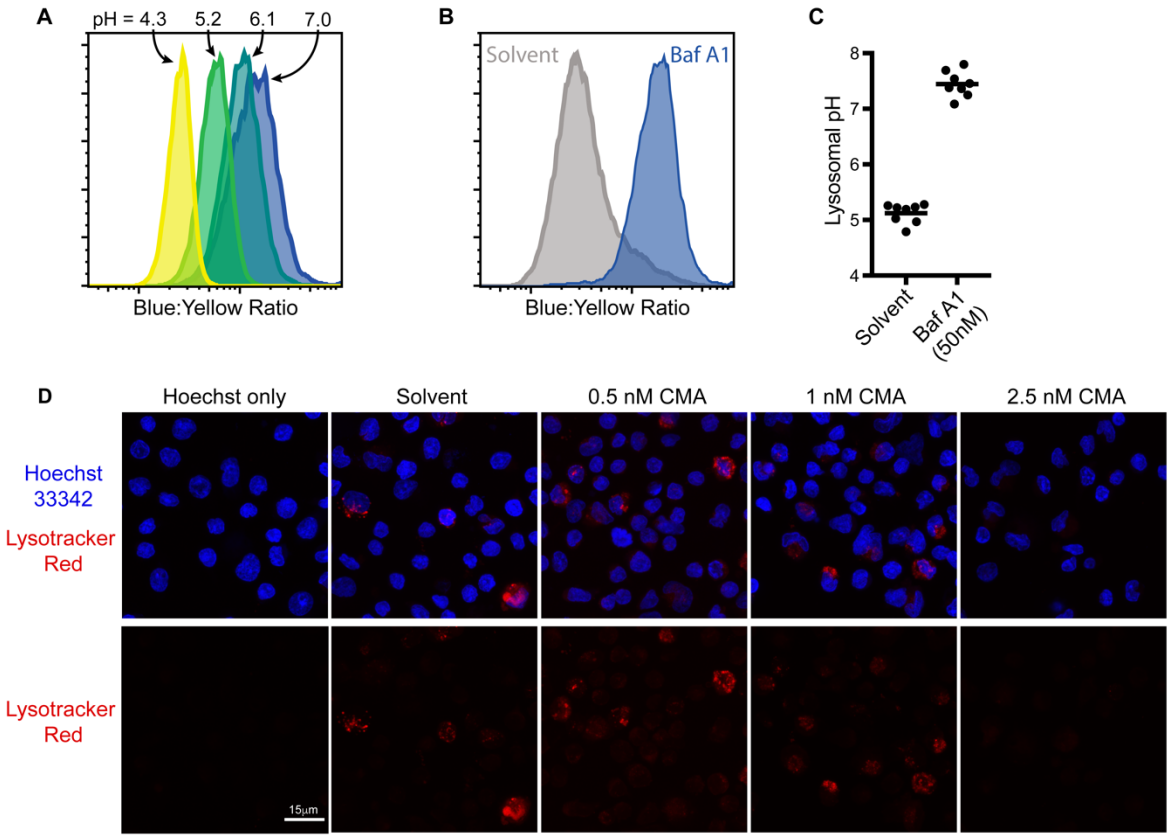

Fig. S9: Supplement to lysosome and trafficking studies in Fig. 2. (A) Flow cytometric data derived from primary monocyte-derived macrophages (MDM) incubated with Lysosensor Yellow/Blue dextran, 10,000 MW for 24 hours and analyzed in buffers of known pH as indicated. The ratio of blue:yellow fluorescence was calculated for each cell, and the median ratio for each sample was used to generate a standard curve to calculate lysosomal pH. (B-C) Representative flow cytometric plot (B) and summary graph (C) describing the lysosomal pH of primary monocyte-derived macrophages (MDM) treated with 50nM Baf A1 for 1 hour after incubation with Lysosensor Yellow/Blue dextran, 10,000 MW as in A (n=8). Lysosomal pH was calculated using a standard curve generated for each donor as in A. (D) Representative confocal microscopy max projections from multiple z-stack images of primary $CD4^+$ T cells treated for 24 hours with CMA as indicated and incubated with Lysotracker Red and Hoechst 3342 for 1 hour. All images were captured with identical microscope settings (representative from 3 independent experiments).

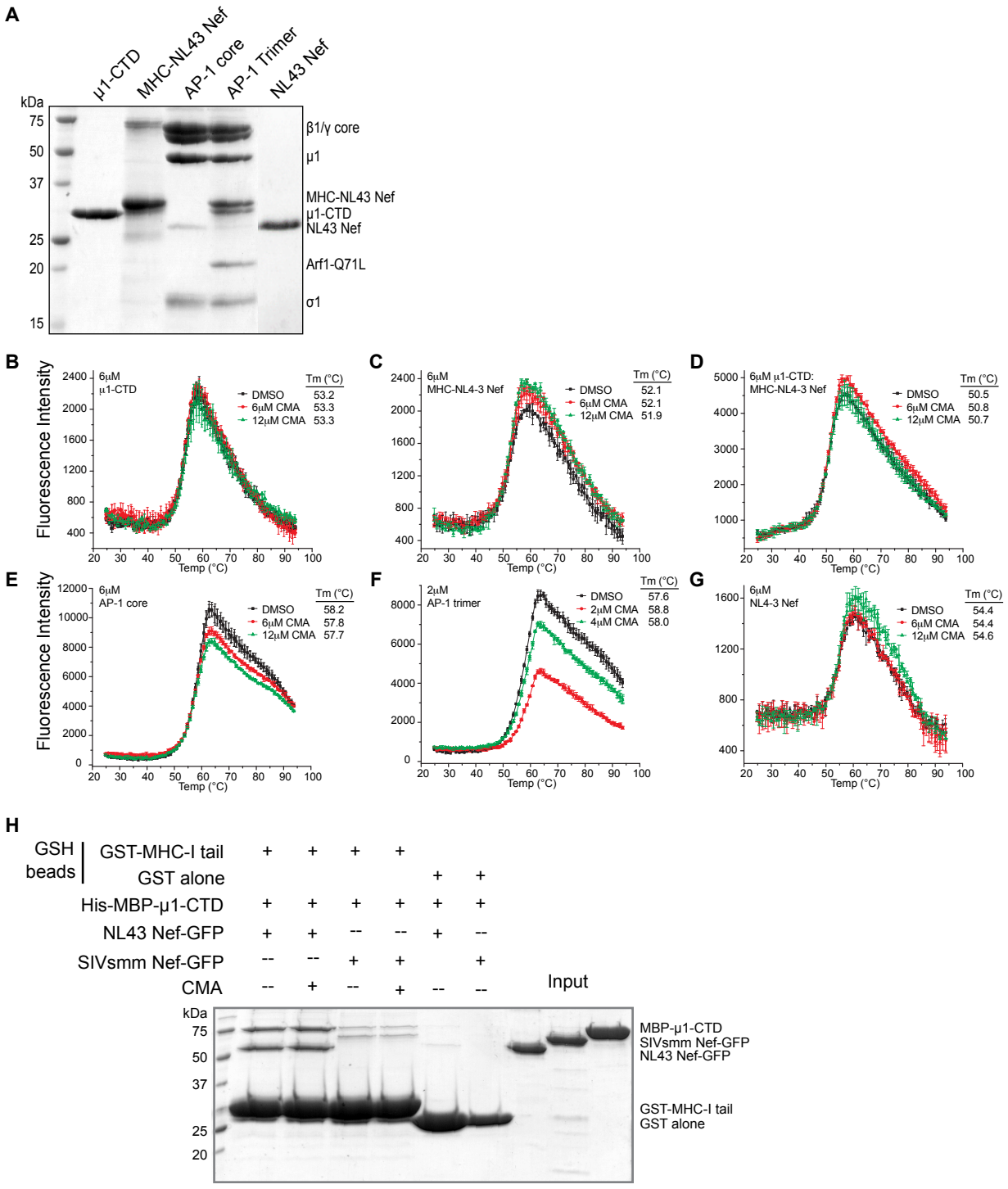

Fig. S10: CMA does not directly alter the interaction between the MHC-I tail, AP-1, and Nef.

(A) SDS PAGE gel of protein samples used in DSF assay in Fig. 3D-I, which was visualized by Coomassie blue staining. (B-G) CMA does not affect the AP-1:MHC-I:Nef interaction in vitro. Differential scanning fluorimetry (DSF) plots of protein thermal stability with or without CMA

treatment. Reaction mixtures contained SYPRO orange and 2-6 μM proteins in the presence or absence of 2-12 μM CMA. SYPRO orange fluorescence intensity was plotted as a function of temperature for (B) μ1-CTD domain, (C) MHC-I tail fused with HIV-1 NL4-3 Nef (MHC-NL43 Nef), (D) μ1-CTD: MHC-NL43 Nef, (E) AP-1 core, (F) AP-1 trimer containing AP-1 core: Arf1-GTP: MHC-NL43 Nef, and (G) NL43 Nef alone. DMSO concentration in each reaction was fixed at 5%. Measured fluorescence intensity (before post-peak region) was fitted to Boltzmann equation to obtain melting temperature (Tm). The error bars represent the corresponding standard deviation among three replicates. (H) The effect of CMA in μ1-CTD:MHC-I tail:Nef interaction analyzed by GST pull down assay. Glutathione sepharose beads were used to immobilize GST tagged MHC-I tail and subsequently pull down MBP tagged μ1-CTD and Nef-GFP in the presence or absence of 40μM CMA. The pull-down results were visualized by SDS-PAGE and Coomassie blue staining.

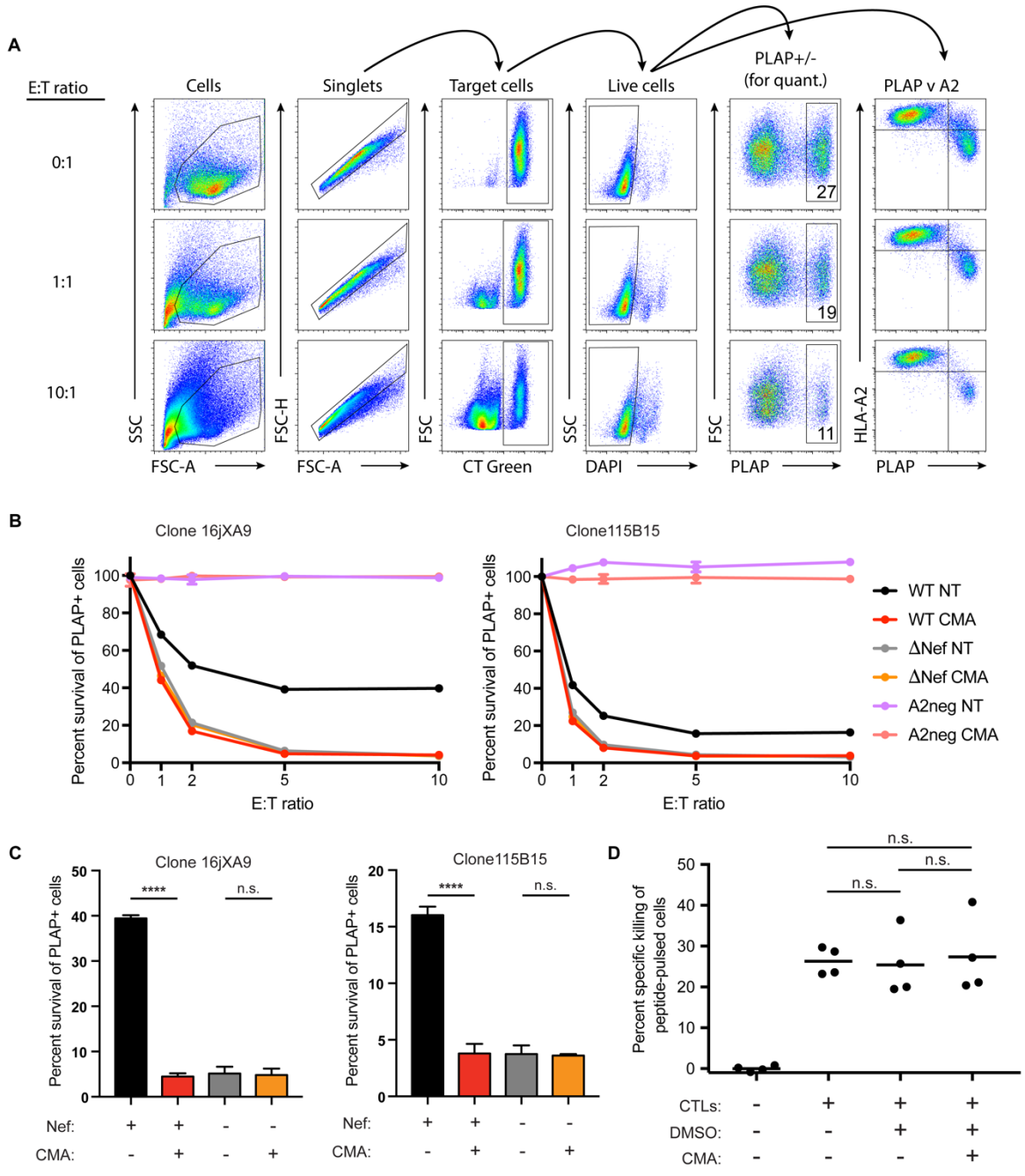

Fig. S11: Supplement to CTL killing assay as in Fig. 4. (A) Gating strategy for results in Fig. 4. Representative flow cytometric plots from CD4$^+$ T cells infected with HXBePLAP (Fig. 1A) and co-cultured with increasing ratios of HIV-specific CTLs. CD4$^+$ T cell target cells are stained with CellTracker Green dye (CT Green) 24 hours before co-culture to distinguish the CD4$^+$ T cell

targets from CTL effectors. DAPI is used as a viability dye, and PLAP marks infected cells. Data for summary graphs in B are generated from PLAP vs. FSC plots as indicated. (B) Summary graphs for assays described in Fig. 4A-B, where elimination of $PLAP^+$ cells is only observed for $HLA\text{-}A2^+$ target cells. WT = HXBePLAP, ΔNef = HXBePLAP with Nef deletion, A2neg = target cells derived from a donor lacking the HLA-A2 allele of MHC-I infected with HXBePLAP with Nef deleted, NT = no treatment (matched DMSO solvent control), CMA = 0.5nM CMA. (C) Summary graphs of flow cytometric data as in Fig. 4 in which the 5:1 and 10:1 E:T ratios were pooled into a single condition where CTL killing was saturated, yielding four individual replicates within each experiment (unpaired t test, **** = $p<0.0001$, n.s. = not significant). (D) Summary graphs of flow cytometric data from co-culture experiments of CTL clones with Gag SL9 peptide-pulsed JY cells, reporting specific elimination of peptide-pulsed JY target cells relative to unpulsed target cells, which were differentially dyed. Co-cultures were performed in the presence or absence of 0.5nM CMA and the solvent DMSO. Viable cells were identified by light scatter and viability dye exclusion, and specific killing was calculated by dividing the frequency of viable cells that were peptide-pulsed observed in the sample by the frequency observed in the control and subtracting from 1. Individual points represent experimental duplicates from each of two independent biological replicates (unpaired t tests, n.s. = not significant).

A

HLA genotypes for donor in Fig. 5

Locus	Allele	Reactivity
HLA-A	A*02:01:01:01	BB7.2
HLA-A	A*03:01:01:01	none
HLA-B	B*51:01:01:01	anti-Bw4
HLA-B	B*07:02:01	anti-Bw6
HLA-C	C*15:02:01:01	none
HLA-C	C*07:02:01:03	anti-Bw6

*note: HLA-C is reportedly expressed at 6-fold lower levels than HLA-B, so most anti-Bw6 signal is expected to come from B*07:02:01 for this donor

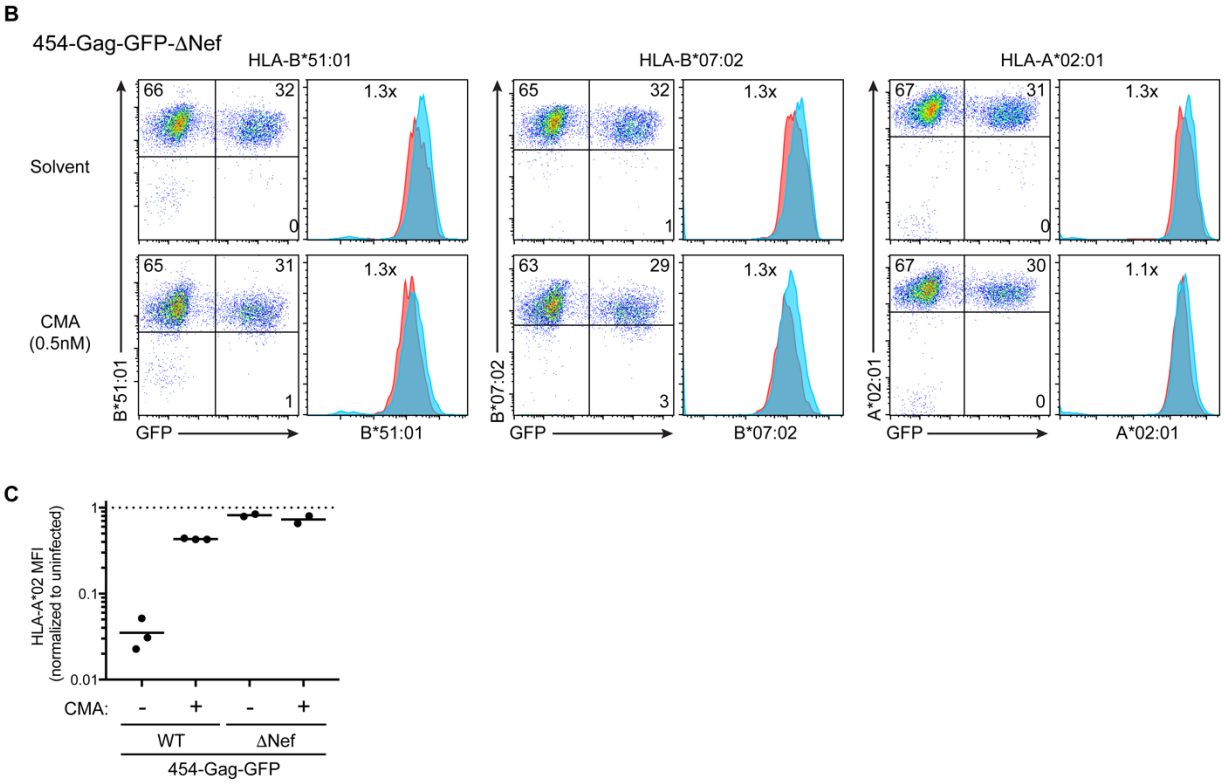

Fig. S12: Supplemental information for primary cell experiments with HLA-B and 454-Gag-GFP. (A) HLA genotypes for the donor used in Fig. 5. Only A*02:01:01:01 reacts with BB7.2. Only B*51:01:01:01 reacts with Bw4. Both B*07:02:01 and C*07:02:01:03 react with Bw6, although B*07:02:01 is expected to contribute the majority of the staining, since HLA-B is expressed at 6-fold higher levels than HLA-C (22). (B) Representative flow cytometry plots (n=3 independent replicates from a single donor) from $CD4^+$ T cells infected with Nef-deleted 454-Gag-GFP for 48 hours, treated with 0.5nM CMA for 24 hours, and stained with monoclonal antibodies to Bw4 (B*51:01) and Bw6 (B*07:02), and monoclonal antibody BB7.2 (HLA-A*02:01). Blue histograms

are from GFP^- cells, red histograms are from infected GFP^+ cells. (C) Summary graph of data from B and Fig. 5D plotting the HLA-A*02 MFI normalized to that in uninfected cells treated with solvent control. Data are from independent experiments with 3 donors for 454-Gag-GFP, 2 donors for 454-Gag-GFP-ΔNef.

Supplementary references

1. Pear WS, Nolan GP, Scott ML, & Baltimore D (1993) Production of high-titer helper-free retroviruses by transient transfection. *Proceedings of the National Academy of Sciences* 90(18):8392-8396.
2. Roeth JF, Williams M, Kasper MR, Filzen TM, & Collins KL (2004) HIV-1 Nef disrupts MHC-I trafficking by recruiting AP-1 to the MHC-I cytoplasmic tail. *J Cell Biol* 167(5):903-913.
3. Thammavongsa V*, et al.* (2009) Assembly and intracellular trafficking of HLA-B* 3501 and HLA-B* 3503. *Immunogenetics* 61(11-12):703-716.
4. McNamara LA, Ganesh JA, & Collins KL (2012) Latent HIV-1 infection occurs in multiple subsets of hematopoietic progenitor cells and is reversed by NF-kappaB activation. *J Virol* 86(17):9337-9350.
5. Collins KL, Chen BK, Kalams SA, Walker BD, & Baltimore D (1998) HIV-1 Nef protein protects infected primary cells against killing by cytotoxic T lymphocytes. *Nature* 391(6665):397-401.
6. Zaikos TD*, et al.* (2018) Hematopoietic stem and progenitor cells are a distinct HIV reservoir that contributes to persistent viremia in suppressed patients. *Cell reports* 25(13):3759-3773. e3759.
7. Kuroda H, Kutner RH, Bazan NG, & Reiser J (2009) Simplified lentivirus vector production in protein-free media using polyethylenimine-mediated transfection. *Journal of virological methods* 157(2):113-121.
8. Leonard JA, Filzen T, Carter CC, Schaefer M, & Collins KL (2011) HIV-1 Nef disrupts intracellular trafficking of major histocompatibility complex class I, CD4, CD8, and CD28 by distinct pathways that share common elements. *J Virol* 85(14):6867-6881.
9. Naviaux RK, Costanzi E, Haas M, & Verma IM (1996) The pCL vector system: rapid production of helper-free, high-titer, recombinant retroviruses. *Journal of virology* 70(8):5701-5705.
10. Kasper MR & Collins KL (2003) Nef-mediated disruption of HLA-A2 transport to the cell surface in T cells. *Journal of virology* 77(5):3041-3049.
11. Kasper MR & Collins KL (2003) Nef-mediated disruption of HLA-A2 transport to the cell surface in T cells. *J Virol* 77(5):3041-3049.

12. Painter MM, Zaikos TD, & Collins KL (2017) Quiescence promotes latent HIV infection and resistance to reactivation from latency with histone deacetylase inhibitors. *Journal of virology* 91(24):e01080-01017.
13. Zaikos TD, Painter MM, Kettinger NTS, Terry VH, & Collins KL (2018) Class 1-selective histone deacetylase (HDAC) inhibitors enhance HIV latency reversal while preserving the activity of HDAC isoforms necessary for maximal HIV gene expression. *Journal of virology* 92(6):e02110-02117.
14. Kasper MR*, et al.* (2005) HIV-1 Nef disrupts antigen presentation early in the secretory pathway. *Journal of Biological Chemistry* 280(13):12840-12848.
15. Shen QT, Ren X, Zhang R, Lee IH, & Hurley JH (2015) HIV-1 Nef hijacks clathrin coats by stabilizing AP-1:Arf1 polygons. *Science* 350(6259):aac5137.
16. Morris KL*, et al.* (2018) HIV-1 Nefs Are Cargo-Sensitive AP-1 Trimerization Switches in Tetherin Downregulation. *Cell* 174(3):659-671 e614.
17. Sheffield P, Garrard S, & Derewenda Z (1999) Overcoming expression and purification problems of RhoGDI using a family of "parallel" expression vectors. *Protein Expr Purif* 15(1):34-39.
18. Shepherd MD, Kharel MK, Bosserman MA, & Rohr J (2010) Laboratory maintenance of Streptomyces species. *Current protocols in microbiology* 18(1):10E. 11.11-10E. 11.18.
19. Laatsch H (2011) *AntiBase 2011: the natural compound identifier* (Wiley-Vch).
20. Li Z*, et al.* (2017) Complete elucidation of the late steps of bafilomycin biosynthesis in Streptomyces lohii. *Journal of Biological Chemistry* 292(17):7095-7104.
21. Wang M*, et al.* (2016) Sharing and community curation of mass spectrometry data with Global Natural Products Social Molecular Networking. *Nature biotechnology* 34(8):828.
22. Apps R*, et al.* (2015) Relative expression levels of the HLA class-I proteins in normal and HIV-infected cells. *The Journal of Immunology* 194(8):3594-3600.