**Cell-to-cell transmission of HIV-1 is required to trigger pyroptotic death of lymphoid-derived CD4 T cells**

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**SUPPLEMENTAL DATA**

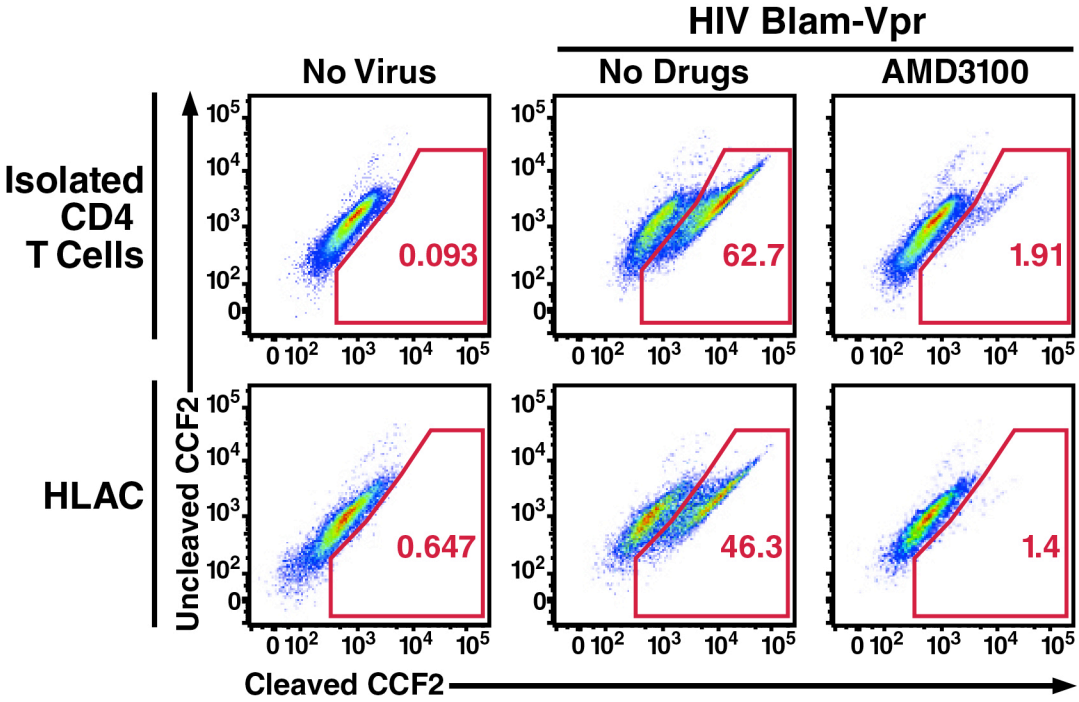


**Figure S1**. **Free HIV-1 particles fuse into target lymphoid CD4 T cells at high levels.** To assess the capacity of free HIV-1 particles to fuse into target cells, we used an HIV virion-based fusion assay that measures β-lactamase (BlaM) enzymatic activity delivered to target cells upon the fusion of virions containing BlaM fused to the Vpr protein (BlaM-Vpr)(Cavrois et al., 2002). HLAC or CD4 T cells isolated from HLAC were infected with 100 ng of X4-tropic HIV-1 containing Blam-Vpr. Target cells were then loaded with the Blam substrate CCF2, which fluoresces in the green channel. Upon HIV-1 BlaM-Vpr fusion into a CCF2 loaded target cell, BlaM cleaves CCF2, changing its fluorescence from green to blue and allowing for measurement of the amount of viral fusion into target cells. Of note, HIV-1 fused to 62.7% of isolated CD4 T cells and 46.3% of CD4 T cells in HLAC, which was blocked upon the addition of the CXCR4 antagonist AMD3100.

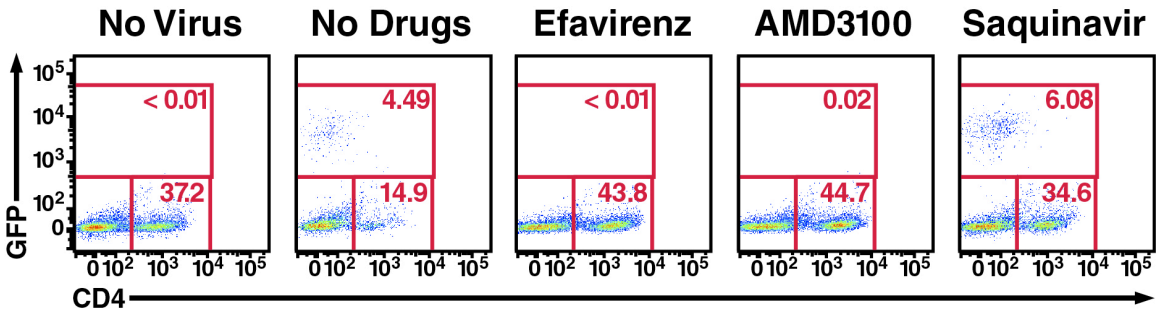


**Figure S2. Treatment with saquinavir, a viral protease inhibitor, does not inhibit productive infection but prevents CD4 T-cell death by newly released HIV-1 virions.** To discriminate between CD4 T-cell death mediated by free virions and direct cell-to-cell transmission across virological synapses, we treated the cells with saquinavir, an HIV protease inhibitor that acts late in the viral life cycle and leads to the generation immature virions. These immature virions are unable to fuse to target cells either when transmitted by the cell-free route or via cell-to-cell interaction. Importantly, saquinavir does not impair syncytia formation mediated by membrane expression of HIV Env nor the function of ICAM-1 and LFA-1 that form the virological synapse (Wyma et al., 2004). HLACs were spinoculated with multiple-round viruses containing a GFP reporter (NLENG1) alone or in the presence of, efavirenz, AMD3100, or saquinavir. Drugs were added 1 hour before spinoculation with the virus. After 4 days, spinoculation of HLACs with free HIV-1 particles produced productive infection and extensive depletion of target CD4 T cells. As expected, productive infection and loss of CD4 T cells were prevented by the NNRTI efavirenz, which allosterically inhibits HIV-1 reverse transcriptase, and by AMD3100, which prevents viral entry by blocking gp120 engagement with CXCR4. Interestingly, spinoculation of HLACs in the presence saquinavir produced similar levels of productive infection but the loss of target CD4 T cells was prevented. Because saquinavir acts during a late stage of the viral life cycle it does not affect production infection of permissive target cells. However, the immature particles generated in the presence of saquinavir were not able to spread into and kill target CD4 T cells. Thus, the introduction of free virions by spinoculation is not sufficient to induce cell death. These findings also indicate that viriological synapse formation alone is insufficient. Instead, infectious virus must be transmitted across these synapses. Of note, the fact that saquinavir does not interfere with syncytia formation indicates that the cell death we observe does not involve this process. Data represent viable CD4 T cells 4 days after spinoculation. FACS plots are representative of three independent experiments performed with tonsils from different donors.

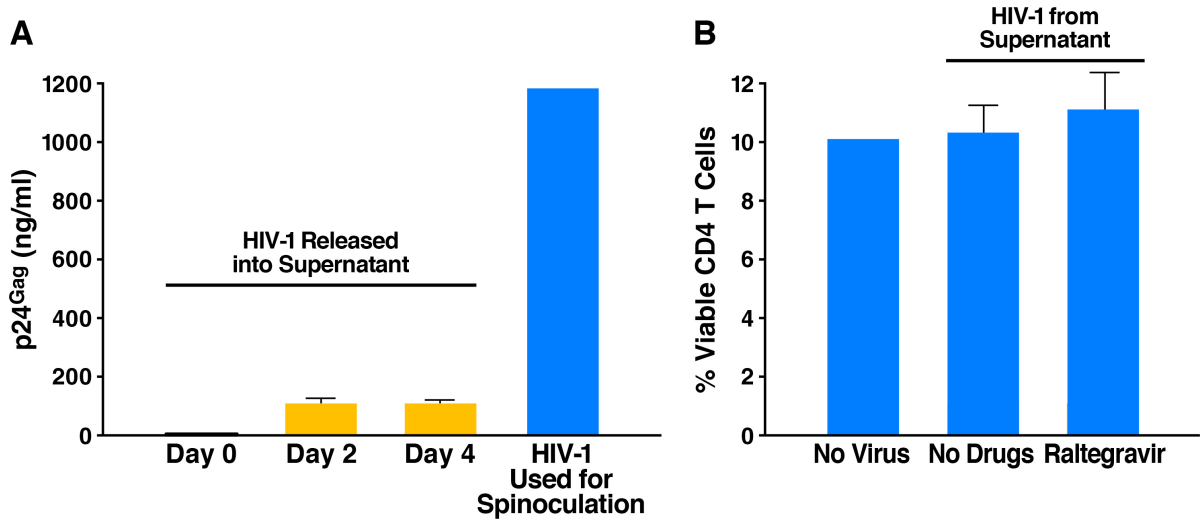


**Figure S3. Supernatants from HIV-infected HLACs do not induce cell death of target CD4 T cells.** The amount of HIV-1 used for spinoculation of 1 million tonsillar cells is 100ng $p24^{gag}$, which is roughly equivalent to 500 x $10^6$ free HIV-1 particles. Considering that 1 million tonsil cells equates to approximately 250,000 target CD4 T cells, spinoculation experiments achieve a high MOI of 200 (in a very low culture volume of only 100µl). Nevertheless, spinoculation of free virions into these cells, despite the high MOI, fails to trigger pyroptosis in the target CD4 T cells.

(**A**) We quantified and compared the amount of free HIV-1 particles released in infected HLACs versus the amount of input free HIV-1 particles used for spinoculation. To this end, 1 million tonsillar cells were spinoculated with 100ng of free HIV-1 particles. Cells were incubated for four hours at 37°C to allow viral fusion into target cells, and then extensively washed to remove excess input free viral particles. The amount of de-novo HIV-1 particles released into supernatants was determined by $p24^{gag}$ ELISA assay after 0, 2 or 4 days of culturing. After four days in culture, when virtually all target CD4 T cells were depleted, the amount of de-novo free HIV-1 particles released into the supernatant was 8-fold lower than the amount of free viral particles used for spinoculation. Thus, the amount of free virus released from HLAC cultures does not exceed the amount used for spinoculation, in fact it is far less. Data represent the average amounts of $p24^{gag}$ released in a single well of a 96-well plate containing 1 million HIV-infected tonsillar cells.

(**B**) Next, we assessed whether supernatants containing free virus released from infected HLACs was sufficient to promote CD4 T-cell killing of fresh HLACs. After 4 days, CD4 T cells were not depleted in cultures spinoculated with these supernatants, either in the absence of antiviral drugs or in the presence of raltegravir. These findings indicate that neither free HIV-1 particles released into supernatant of infected cultures nor the large quantities of free virus introduced during spinoculation are able to induce pyroptotic death of CD4 T cells. Since supernatants containing virus were used, these findings also argue against release of a soluble factor(s) from productively infected cells that drives the death of target CD4 T cells. Of note, these findings are in agreement with our previous transwell culture experiments arguing in favor of a close cell-to-cell interaction for the induction of pyroptosis (Doitsh et al., 2010). The levels of viable CD4 T cells were determined using flow cytometry, and were normalized to uninfected samples.

**A**

| Sample | Live Cells CD8 | Live Cells CD4 | CD4/CD8 Survival Ratio | Normalized % CD4 T Cell Survival |
|---|---|---|---|---|
| No Virus | 440 | 5275 | 11.99 | 100.0 |
| Multiple-Round | 567 | 430 | 0.76 | 6.3 |
| Single-Round | 435 | 4078 | 9.37 | 78.2 |
| Integrase-deficient | 410 | 4159 | 10.14 | 84.6 |

**B**

| Sample | Patient 3317 | 3318 | 3319 | AVG | STD | SQRT | STD Error of Mean | P Val |
|---|---|---|---|---|---|---|---|---|
| No Virus | 99.9 | 100.0 | 100.0 | 100.0 | 0.03 | 1.73 | 0.02 | |
| Multiple-Round | 12.7 | 6.3 | 4.5 | 7.8 | 4.29 | 1.73 | 2.48 | |
| Single-Round | 77.7 | 78.2 | 73.1 | 76.3 | 2.80 | 1.73 | 1.62 | 0.00002 |
| Integrase-def. | 82.2 | 84.6 | 72.4 | 79.7 | 6.47 | 1.73 | 3.73 | 0.00008 |

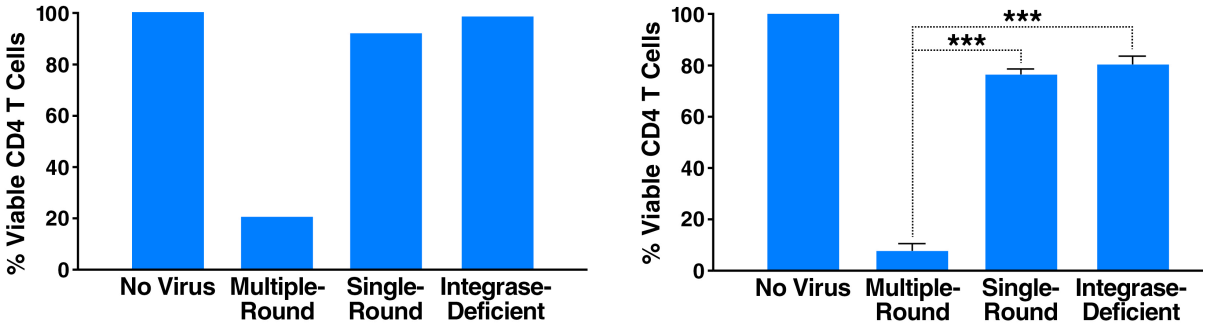


**Figure S4. Data collection and calculation of CD4 T-cell death in HIV-infected HLACs.** **(A)** The viability of lymphocytes in HLACs may decrease non-specifically due to culture conditions. To carefully control for non-specific cell death we use two different approaches. First, for nonspecific cell death that might occur in individual wells, we normalized the number of live CD4 T cells to the number of live CD8 T cells in each well. This method is similar to the manner that rRNA is used to normalize for cell number in qPCR analyses, or β-actin is used to normalize specific protein levels in Western Blot analyses. As a second control, we normalize the number of viable target CD4 T cells spinoculated with HIV-1 (or co-cultured with infected cells), to the number of viable target CD4 T cells spinoculated with no virus (or co-cultured with uninfected cells). The adjusted CD4/CD8 survival ratio in sample with no virus is defined as 100%. **(B)** To calculate the amount of variation in each set of data, standard error of the mean (SEM) is calculated from independent experiments with HLACs from a minimum of three independent donors. The P-values calculated for these samples are $p<0.001$, ***. All experiments involving HIV-mediated CD4 T-cell death described in our current and previous manuscripts are analyzed in this manner.

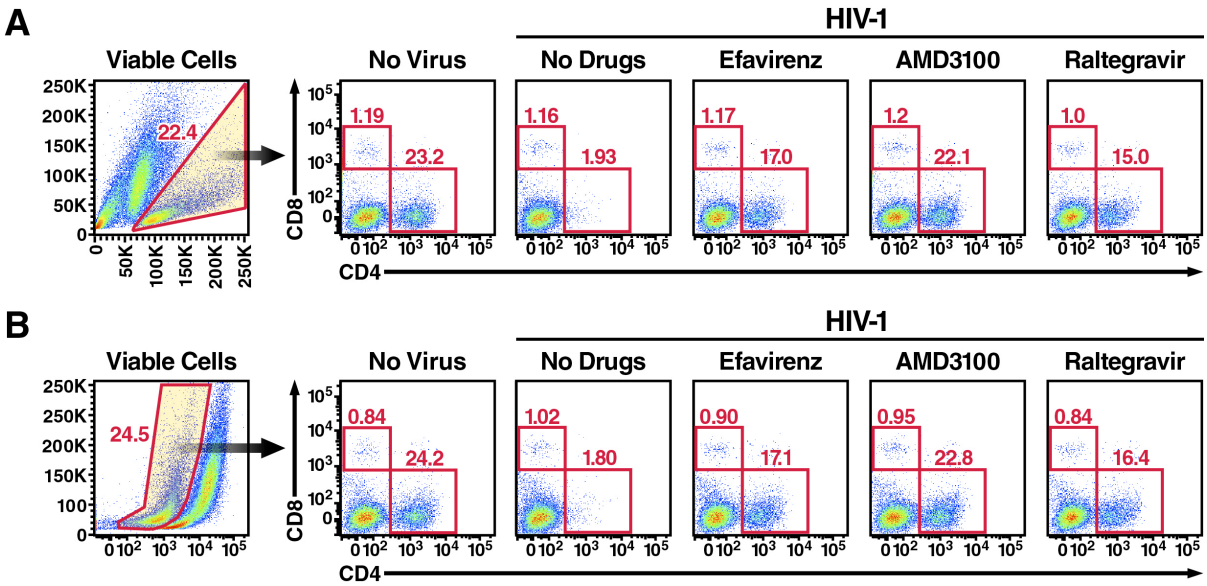


**Figure S5. Assessing the levels of viable CD4 T cells in HIV-infected HLACs using flow cytometry.** The experiment shown in Figure 1B of the main manuscript was repeated using a specific cell viability marker. Tonsillar HLACs were spinoculated with free HIV-1 particles alone, or in the presence of the indicated drugs. After 4 days, cells from the same samples were analyzed for levels of CD4 T-cell death using two independent methods: (A) Viable lymphocytes were determined using gating on forward scatter versus side scatter as indicated. The same live gate was used to assess the percentage of CD4 T cells versus the percentage of CD8 T cells in each sample as indicated. (B) Viable cells were detected using Zombie Red, an amine reactive fluorescent dye that is excluded from live cells, but readily stains cells with compromised membranes. The dye produces a distinct red fluorescence (excited by the yellow/green laser at 561 nm), making it suitable for multi-color detection. Cells negatively stained for Zombie Red (i.e. live cells) were gated to further assess the percentage of CD4 T cells versus CD8 T cells in each sample as indicated. The same tonsil cell preparation was used in panels A and B. These cultures were spinoculated with HIV in the presence or absence of the indicated drugs. The results show a close concordance between the two experimental approaches.

## SUPPLEMENTAL EXPERIMENTAL PROCEDURES

### Preparation of HIV-1 Virions

Replication-competent HIV-1 virions were produced using the X4-tropic reporter NL4-3 clones of HIV-1. For replication-competent reporter viruses we use the NLENG1-IRES (NLENG1I), and a DsRedExpress reporter NLRX-IRES (NLRXI). To produce HIV-1 particles, 80 μg total plasmids were transfected into T175 flask with 293T cells using calcium phosphate as previously described (Doitsh et al., 2014). For single-round reporter particles cell were transfected with NLENG1-ES-IRES + gp160 at a 9:1 ratio (HIV-1 reporter clone harboring a Stop codon in the Env gene, pseudotyped with HIV-1 gp160 envelope) or with NLRX-ES-IRES + gp160 at a 9:1 ratio. To produce integrase-deficient HIV-1 particles cells were transfected with NLENG1I-D116N or with NLRXI-D116N DNA clones. For NL4-3 Blam-vpr, 293T cells were transfected with NL4-3, Blam-vpr and pAdvantage at a ratio of 3:1:0.1. All virus stocks were quantitated by measuring $p24^{gag}$ levels by Elisa (Perkin Elmer, Cat # NEK050B001KT).

### Processing of HLAC, Isolation of CD4 T Cells and Cell Culture

Human tonsil tissue from routine tonsillectomies were obtained from Cooperative Human Tissue Network, processed into HLAC and cultured as described(Doitsh et al., 2010). CD4, CD8 T cells and B cells were isolated from HLAC by positive selection (Miltenyi, Cat # 130045101), or negative selection (Stem Cell Technologies).

### Infection of HLAC and Co-Culture with CFSE-Labeled Target Cells, Killing Assay with 293T Overlaid HLACs

All infections of HLAC or isolated CD4 T cells from HLAC were performed by spinoculation as described (Doitsh et al., 2010). In brief, cells were plated in a 96 V-bottom plate at $1x10^6$ cells/well/100 μl. Drugs were then added as indicated and allowed to incubate with the cells at 37°C for 15 minutes. Cells were then cooled on ice for 15 minutes before they were mixed with 100 ng of $p24^{gag}$ (infections with single-round virus required three-fold more viral particles to achieve the same amount of productive infection as multiple-round virus). The virus-cell mixture was incubated on ice for 30 minutes to allow the virus to attach

to cells, and then spun for 1 hour at 1200g at 4°C and subsequently shifted to 37°C to allow synchronized viral fusion of the attached virions. In co-culture experiments, uninfected HLAC were stained with 1 μM CFSE (Molecular Probes, Cat # C1157) and plated at $1x10^6$ cells/well/200 μl. At 2 days post-spinoculation, infected or uninfected HLAC were co-cultured with CFSE-labeled target cells in a 96 V-bottom plate in 200 μl of tonsil medium. The indicated drugs were added to the CFSE-labeled cells and incubated at 37°C for 15 minutes before co-culture with infected HLAC, unless otherwise noted. Uninfected, infected, and CFSE-labeled cultures were manipulated as described for individual experiments. To assess the killing of single-round or non-infectious HIV-1 clones, we modified the infection system by overlaying HLAC cells on a monolayer of 293T cells transfected with 50 ng pNLENG1, pNLENG1 ΔENV + gp160 (at a ratio of 9:1) or pNLENG1 D116N DNA in a 24-well plate. After 12 hours, 293T cells were overlayed with $4x10^6$ HLACs in tonsil media (i.e. RPMI 1640 supplemented with 15% heat-inactivated fetal bovine serum, 100 μg/ml gentamicin, 200 μg/ml ampicillin, 1 mM sodium pyruvate, 1% nonessential amino acids (Mediatech), 2 mM L-glutamine, and 1% fungizone), in the presence of the indicated drugs. Virus-producing 293T cells directly interact with targets present in the overlaying HLACs. After 24–72 hours, HLAC suspensions were collected from wells and analyzed by flow cytometry. Unless otherwise stated, drugs were used at the following concentrations: 250 nM AMD3100; 100 nM Efavirenz, 5 μM Raltegravir, and 15 μM Saquinavir. In some experiments, AMD3100 was added 4 hours post-spinoculation.

**FACS Analysis and Gating Strategy**

For analysis of cell death by flow cytometry, cells were stained with CD4-PE (BD Biosciences) and CD8-APC (BD Biosciences) at a concentration of 1:200 in FACS buffer (PBS supplemented with 2 mM EDTA and 2% fetal bovine serum) and fixed with a final concentration of 2% paraformaldehyde. Data was collected on a FACS Calibur (BD Biosciences) and analyzed with Flowjo software (Treestar). Cell death was analyzed in spinoculation cultures by gating live cells with forward and side scatter, followed by gating and counting subsets of CD4 and CD8 T cells. Survival of CD4 T cells was calculated by dividing the number of CD4 T cells by the number CD8 T cells in each condition. All samples were normalized to the number of uninfected cultures. For a detailed description to

our method of measuring and calculating the levels of CD4 T-cell death in HIV-infected HLACs see Figure S4.

Cell death was determined in co-culture systems by gating on live cells with forward and side scatter, followed by gating and counting of CD4 and CD8 T cells. The survival percentage of CD4 T cells was calculated by dividing the number of CD4 T cells by that of CD8 T cells. All conditions were normalized to uninfected conditions. Viable cells were also detected using Zombie Red (BioLegend, Cat # 423105), an amine reactive fluorescent dye that is non-permeant to live cells, but permeant to the cells with compromised membranes. The results showed a close concordance to the experimental approach using gating on forward scatter versus side scatter (Figure S5).

**Virion-Based Fusion Assay**

The virion-based fusion assay was performed as described (Cavrois et al., 2004; Doitsh et al., 2010). Briefly, HLAC or isolated CD4 T cells were incubated with 100 ng of $p24^{gag}$ BlaM-Vpr containing virions at 37°C for 2 hours and washed in $CO_2$-independent medium (Gibco BRL). Cells were then loaded with CCF2/AM dye as described by the manufacturer (Invitrogen) and incubated for 16 hours at room temperature with 2.5 mM probenecid (Sigma Pharmaceuticals). Cells were then washed, fixed with 2% paraformaldehyde, and analyzed by flow cytometry with LSR2 (Becton Dickinson). Data were collected with DiVa software and analyzed with FlowJo software (Treestar).

**Plate Surface Experiments**

HLACs were processed and infected, and target cells were stained with CFSE, as described above, with the exception that $5x10^5$ cells/well were spinoculated with 40 ng $p24^{gag}$ NL4-3 and CFSE target cells were plated at $5x10^5$ cells/well. At 2 days post-spinoculation, uninfected or infected HLAC were co-cultured with CFSE target cells in either a 96 V-bottom, 96 U-bottom, 96 flat-bottom, 24-well, or 12-well plate. Co-cultures in 96 V-bottom, 96 U-bottom, and 96 flat-bottom plates were plated in 200 μl of tonsil medium and co-cultures in 24- or 12-well plates were plated in 1 ml of tonsil medium. Cell death was analyzed 1, 2, 3, and 4 days post co-culture, as described above.

**Virological Synapse Blocking Antibody Experiments**

HLACs were processed, infected, and co-cultured as described above. Antibodies against ICAM-1 (Calbiochem, Cat # CP53) and LFA-1 (eBioscience, Cat # BMS102), as well as an isotype mouse IgG1 negative control (clone 1E2.2 EMD Millipore) and Efavirenz, were added to CFES target cells 4 hours before co-culture. At 2.5 days post co-culture, cells were stained and analyzed by flow cytometry as described above.

**CaspaLux1 $E_1D_2$ Staining for Active Caspase 1**

To determine intracellular activation of caspases-1, we used cell permeable fluorogenic caspase 1-specific substrates CaspaLux1 $E_1D_2$ (Cat# CPL1R1E-5, OncoImmunin). Upon cleavage of these substrates by active capase-1, the signal of their conjugated fluorophore increases significantly over the background fluorescence of the uncleaved substrate (Komoriya et al., 2000; Packard and Komoriya, 2008). CaspaLux1 $E_1D_2$ was incubated according to the manufacturer's instructions with live unfixed cells for 15-20 min at 37°C, cells were then immediately placed on ice for surface staining with anti CD4-APC antibodies. Caspase 1 activity was detected using flow cytometry by the FITC channel. To avoid interference with the FITC channel, HLACs were spinoculated with HIV clones containing the reporter DsRedExpress gene (NLRX-IRES). Similar to the NLENG1I clone, the DsRedExpress gene in NLRX-IRES is inserted immediately downstream of the envelope gene in the position occupied by the early gene nef, which is expressed from an IRES element (Gelderblom et al., 2008). For Single round viruses, the NLRX-ES-IRES clone lacking envelope was pseudotyped with gp160. For integrase-deficient viruses, NLRX-IRES-D116N clones were used. When applicable, saquinavir was added to the cells before spinoculation, and AMD3100 was added 4 hours post spinoculation.

**LDH Assay**

HLAC cultures were spinoculated as described above with NLENG1, NLENG1 ΔENV + gp160, or NLENG1 D116N. When applicable, AMD3100 was added 4 hours post spinoculation. At 3–4 days post spinoculation, culture supernatant was collected and assessed for release of cytoplasmic lactate dehydrogenase (LDH), as described (Decker and Lohmann-

Matthes, 1988). Cells in the same cultures were also collected and analyzed for productive infection (GFP+ cells) and death as described above.

**Immunoblotting**

Cells were washed in PBS and immediately lysed in cell extraction buffer (Life Technologies) with the addition of a protease inhibitor cocktail (Roche). Lysates were subjected to SDS-PAGE protein analysis using rabbit anti human CD54/ICAM-1 antibody (Cell Signaling Technology, Cat# 4915S, 1:1000), mouse anti Human Integrin alpha L/CD11a antibody (R&D systems, Cat# MAB3595, 1:1000), and mouse anti-β-Actin antibody (Sigma, Cat# A5316**,** 1:10,000**).** For SDS-PAGE immunoblotting analysis, Bio-Rad Criterion pre-cast Tris-HCL gels were used. Gels were wet transferred onto PVDF membranes (Bio-rad) at 4°C and then blocked in 5% non-fat milk for 1 hour at room temperature. Primary antibodies were incubated overnight at 4°C and secondary antibodies for 1 hour at room temperature.

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