EWI-2 Inhibits Cell-Cell Fusion at the Virological Presynapse

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Abstract

Cell-to-cell transfer of virus particles through the virological synapse (VS) is a highly efficient mode of HIV-1 transmission. Formation of the VS, a transient multiform adhesion structure, is mediated through an interaction between the HIV-1 envelope glycoprotein (Env) and the viral receptor CD4 on the surface of infected cell and target cell, respectively. Given that Env, unlike many other viral fusogens, can mediate the merger of membranes at neutral pH, the close encounter of infected and uninfected cells would seem prone to result in cell-cell fusion and thus the formation of syncytia. However, while it is being recognized now that small, T cell-based syncytia are indeed a defining feature of the natural history of HIV-1, the majority of VSs nevertheless resolve without fusion, thus securing continued virus spread. Gag, the main viral structural component, is partially responsible for restraining Env and preventing it from becoming fusogenic before being incorporated into particles. In addition, a few cellular factors, including tetraspanins and ezrin, have also been shown to inhibit Env's activity while this fusogen is still part of the producer cell.

Here, we identify EWI-2, a protein that was previously shown to associate with the tetraspanins CD9 and CD81 and also with ezrin, as a host factor that contributes to the inhibition of Env-mediated cell-cell fusion. Using fluorescence microscopy, flow cytometry, and TZM-bl fusion assays, we show that EWI-2, comparable to tetraspanins, while overall being downregulated upon HIV-1 infection, accumulates at the producer cell side of the VS (i.e. the presynapse), where it contributes to the fusion-preventing activities of the other viral and cellular components.

1 Introduction

HIV-1 spreads between T cells primarily through two modes of transmission: the release of cell-free virus particles followed by their uptake by (more or less distantly located) cells expressing the viral receptor/co-receptor, and cell-to-cell transmission of particles to an adjacent cell via the virological synapse (VS). Formation of the HIV-1 VS is initiated by the viral envelope glycoprotein (Env) on the surface of productively infected cells binding to its receptor, CD4, on target T cells (1), and is followed by polarization of Gag at the cell-cell contact site (1, 2). Virus particles are then released in high concentrations towards the target cell (3), facilitating efficient infection while also possibly shielding virus particles from some neutralizing antibodies ((4) and reviewed in (5)). Given that Env is fusogenic at neutral pH, it may seem likely at first that VS-mediated contacts would frequently result in cell-cell fusion, thus forming a multinucleated infected cell (syncytium). Indeed, we now know that small, T cell based syncytia arise early in HIV-1 infection (6-9). However, the majority of infected T cells observed in lymphoid tissue are mononucleated, indicating that most HIV-1 VSs ultimately result in complete cell separation and generation of a new, productively infected cell, likely due to tight regulation that acts to prevent excessive syncytium formation (reviewed in (10, 11)).

Multiple independent studies have identified viral and host functions which, together, prevent excessive HIV-1-induced cell-cell fusion at the VS. Firstly, Env is rapidly downregulated from the surface of infected cells in the absence of Gag, thus preventing premature Env-mediated membrane fusion (12, 13). Further, upon Gag multimerization at the plasma membrane, Env is trapped by Gag through Env's cytoplasmic tail and maintained in a non-fusogenic state (14). This trapping by Gag ends only after Env's incorporation into virus particles, when Gag precursor gets cleaved, i.e. upon maturation (15-18). Additionally, several host membrane proteins that accumulate at the producer cell side of the VS, including tetraspanins and ezrin, have been identified as fusion-inhibitory proteins (19-21). Tetraspanins inhibit HIV-1-induced cell-cell fusion at a post-hemifusion stage (20), while ezrin is implicated in F-actin organization and recruitment of tetraspanin CD81 to the VS (21). It remains unclear how and whether these protein functions are coordinated, though based on other cell-cell fusion regulation paradigms (discussed below), additional host proteins are likely required to mediate efficient inhibition of HIV-1-induced fusion by tetraspanins and ezrin.

EWI-F (CD9P-1/FPRP) is an immunoglobulin superfamily (IgSF) member and partner of tetraspanins CD9 and CD81 (22). EWI-F was shown to be a potent inhibitor of cell-cell fusion in myoblasts, where EWI-F knockout resulted in more efficient fusion than CD9/CD81 double knockout (23). However, EWI-F is poorly expressed in T cells (24), the primary host cell type for HIV-1 infection. Another IgSF member and tetraspanin partner protein which is expressed in T cells (22, 24), EWI-2 (IGSF8/PGRL) (25, 26), has been documented to play a role in HCV entry (27, 28) and T cell immunological synapse (IS) formation (29). That latter study by Yáñez-

Mó and colleagues also suggested that EWI-2 has a yet undetermined involvement in HIV-1 particle production (29). Furthermore, both EWI-F and EWI-2 interact with ezrin to organize the cytoskeleton in concert with tetraspanins (24). EWI-2 thus lies at the nexus of tetraspanins, ezrin, and the actin cytoskeleton (which can also inhibit cell-cell fusion; (30)) and we therefore sought to test whether EWI-2 contributes to the inhibition of HIV-1-induced cell-cell fusion.

2 Materials and Methods

2.1 Cell Lines and Cell Culture

The following cells were obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH: HeLa cells from Dr. Richard Axel (31), TZM-bl cells from Dr. John C. Kappes, Dr. Xiaoyun Wu, and Tranzyme Inc. (32-36) and CEM-SS cells from Dr. Peter L. Nara (31, 37, 38).

HEK 293T, HeLa, and TZM-bl cells were maintained in Dulbecco's Modification of Eagle's Medium (DMEM) (Corning, Corning, NY, Cat. #10-017-CV) containing 10% fetal bovine serum (FBS; Corning, Cat. #35-010-CV) and antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin; Invitrogen). CEM2n, a kind gift from R. Harris (39), and CEM-SS cells were maintained in RPMI 1640 medium (Corning, Cat. #10-104-CV) supplemented with 10% FBS and antibiotics.

Human primary blood mononuclear cells (PBMCs) were isolated as buffy coats from whole blood of healthy donors by Ficoll density centrifugation. $CD4^+$ T cells were enriched from PBMCs by negative selection using the MACS $CD4^+$ T Cell Isolation Kit (Miltenyi Biotec, Auburn, CA, Cat. #130-096-533) according to manufacturer's instructions. Primary $CD4^+$ T cells were activated in RPMI containing 10% FBS, 50 units/mL IL-2, antibiotics, and 5 µg/mL phytohemagglutinin. After 48 h of activation, cells were washed and subsequently maintained and expanded in the same medium but without phytohemagglutinin. Cells were used for infections at 4 to 7 days post isolation.

2.2 Antibodies

Mouse monoclonal antibody (mAb) to EWI-2 (8A12) was a kind gift from Dr. Eric Rubinstein (22). Mouse mAb to HIV-1 p24 (AG3.0) was obtained through the NIH AIDS Reagent Program, Division of AIDS, NIAID, NIH, from Dr. Jonathan Allan (40). Rabbit antiserum to HIV-1 p6 was a kind gift from David E. Ott. Rabbit polyclonal antibody (pAb) to HIV-1 p24 was obtained from Advanced Biotechnologies (Cat. #13-203-000). Secondary antibodies were as follows: Alexa Fluor 488-conjugated donkey pAb to mouse IgG (Invitrogen, Cat. #A21202), Alexa Fluor 488-conjugated donkey pAb to rabbit IgG (Invitrogen Cat. # A21206), Alexa Fluor 594-conjugated donkey pAb to mouse IgG (Invitrogen Cat. #R37115), Alexa Fluor 594-conjugated donkey pAb to rabbit IgG (Invitrogen Cat. # A21207), Alexa Fluor 647-conjugated donkey pAb to mouse IgG (Invitrogen Cat. #A31571), and Alexa Fluor 647-conjugated goat pAb to mouse IgG (Invitrogen Cat. #A21235). Zenon labeling of primary antibodies with either Alexa Fluor 488 or Alexa Fluor 594 was carried out using Zenon Labeling Kits according to the manufacturer's instructions (Molecular Probes, Eugene, OR, Cat. #Z25002 and #Z25007).

2.3 Plasmids and Virus Strains

pcDNA3, pCDNA3.1, and pCMV SPORT6 (Invitrogen) were vectors for EWI-2, CD81, and L6 overexpression, respectively (EWI-2 was a kind gift from Dr. Eric Rubinstein; Université Paris-Sud, Villejuif, France). Proviral plasmids pNL4-3 and pNL4-3 ΔEnv (KFS) were kind gifts from Dr. Eric Freed (National Cancer Institute, Frederick, MD, USA) (41). NL4-3-derived fluorescent protein-tagged proviral plasmids pNL-sfGI, pNL-sfGI ΔEnv, pNL-CI, and pNL-CI ΔEnv (9) were kind gifts from Dr. Benjamin Chen (Mount Sinai School of Medicine, New York, NY). Vesicular stomatitis virus glycoprotein (VSV-G) was used to pseudotype viral stocks produced in HEK 293T cells.

2.4 Virus Stocks and Infections

VSV-G-pseudotyped virus stocks of NL4-3, NL4-3 ΔEnv, NL-sfGI, NL-CI, and NL-CI ΔEnv were produced in HEK 293T cells transfected with the proviral plasmid and pVSV-G (at 17:3 ratio) using calcium phosphate precipitation. Supernatants were harvested 2 days after transfection, cleared by centrifugation at 2000 rcf for 10 min, filtered, and stored at -80 ˚C.

To infect CEM2n cells by spinoculation, two million cells were incubated with RPMI/10% FBS containing 90 µL of virus stock (resulting in ~3% of the cells being infected) or medium alone (for uninfected controls), for 20 min at 37 ˚C, followed by centrifugation at 1200 rcf for 2 h at 37 ˚C. Cell pellets were allowed to recover at 37 ˚C for 15 min, centrifuged at 300 rcf for 2 min, and resuspended in fresh RPMI/10% FBS. Cells were incubated at 37 ˚C, the medium was refreshed 2 days post infection, and the cells were used 1 day later for all subsequent experiments.

To infect primary $CD4^+$ T cells, 1 or 2 million cells were incubated in RPMI/10% FBS/IL-2 containing 200 or 400 µL of virus, respectively, and spinoculated as described above. Cells were resuspended in fresh RPMI/10% FBS/PS/IL-2 and incubated at 37 ˚C/5% CO_2. Cells were used 2 days post infection for all subsequent experiments.

To infect CEM-SS cells by shaking, one or two million cells suspended in CO_2-independent medium (Gibco) supplemented with 10% FBS were mixed with VSV-G-pseudotyped virus stocks and shaken at 220 rpm for 2 h at 37 ˚C. Cells were then washed and plated in fresh RPMI/10% FBS, and used for experiments as described. For CEM-SS infection by spinoculation, the procedure was performed as described above with some modifications; one million cells were incubated in RPMI/10% FBS containing 50 µL of virus stock or medium alone (for uninfected controls). Following spinoculation, cells were incubated at 37 ˚C for 2 days before being used for subsequent experiments (analyzing post-synapse enrichment of EWI-2 as described below in 2.5).

2.5 Imaging and quantification of EWI-2 accumulation at the VS

CEM-SS and primary $CD4^+$ T cells were infected with VSV-G-pseudotyped WT or ΔEnv virus by shaking or spinoculation, respectively, then treated as follows: For CEM-SS cells, two days post infection, uninfected CEM-SS target cells were labeled with CMAC (Invitrogen) according to manufacturer's instructions, mixed with infected cells at a 1:1 or 1:2 ratio (infected:target), seeded onto the microwell of a 35 mm glass-bottom dish (MatTek Corporation, Ashland, MA, Cat. #P35G-1.5-14-C) coated with poly-L-Lysine (Sigma), and incubated at 37˚ C for 3 to 4.5 h. Cells were then chilled on ice and surface-labeled with 1:200 mouse anti-EWI-2 mAb in RPMI/10% FBS for 45 min at 4 ˚C. Surface-labeled cells were fixed with 4% PFA in PBS at 4 ˚C for 10 min, and blocked and permeabilized overnight with 1% BSA and 0.2% Triton X-100 in PBS (block/perm buffer). All CEM-SS conditions were labeled with Alexa Fluor 647-conjugated anti-mouse secondary pAb in block/perm buffer at 1:500 dilution. Cells were subsequently stained with Alexa Fluor 594 Zenon-labeled anti-p24 AG3.0 mouse mAb, and fixed again with 4% PFA in PBS. Cells were kept in PBS for imaging.

For primary cells, uninfected cells were mixed with infected cells at a 1:1 ratio (infected:target), seeded onto 8-well glass-bottom plates (CellVis, Mountain View, CA, Cat. #C8-1.5H-N) coated with 1:10 poly-L-Lysine in double-distilled water (ddH_2O), and incubated for 2 h at 37˚ C. Cells were surface-labeled for EWI-2 and fixed as above, then blocked and permeabilized with block/perm buffer for 10 min. Cells were then labeled with a mixture of rabbit anti-p24 and anti-p6 antibodies, each at 1:1000 dilution, in PBS with 1% BSA (block) for 45 min. Subsequently, cells were labeled with Alexa Fluor-conjugated secondary pAbs as indicated. Cells were kept in PBS for imaging.

To visualize only producer cell-associated EWI-2 at the VS, 10,000 target TZM-bl cells (which have nearly-undetectable levels of EWI-2; unpublished observation) were seeded onto 8-well glass-bottom plates (CellVis) coated with 1:10 poly-L-Lysine in ddH_2O. The next day, those TZM-bl cells were labeled with CMAC at 1:250 dilution in serum-free DMEM, and then co-cultured with 150,000 CEM-SS cells (either uninfected or infected with NL-CI or NL-CI ΔEnv 2 days prior as described above) per well for 2.5 hours at 37 ˚C in RPMI/10% FBS. The cells were then surface-labeled with 1:200 mouse anti-EWI-2 mAb in RPMI/10% FBS on ice for 45 min. Cells were subsequently fixed with 4% PFA in PBS and permeabilized with block/perm for 10 min. After permeabilization, the cells were labeled using a mixture of rabbit anti-p24 and anti-p6 antibodies, each at 1:1000 dilution, in block for 45 min. Cells were subsequently labeled using Alexa Fluor-conjugated secondary pAbs (anti-mouse-Alexa Fluor 647 and anti-rabbit-Alexa Fluor 488) each at 1:500 in block for 45 minutes. Cells were kept in PBS for imaging.

To visualize only target cell-associated EWI-2 at the VS, HeLa producer cells (which have nearly-undetectable levels of EWI-2; unpublished observation) were plated (10,000 cells per well) in 8-well glass-bottom plates (CellVis) coated with 1:10 poly-L-Lysine in ddH_2O. Twenty-four hours later, cells were transfected with NL-sfGI, NL-sfGI ΔEnv, or empty vector, using Fugene6 (see section 2.8). Twenty-four hours post-transfection, 100,000-150,000 uninfected CEM-SS cells (labeled with CMAC at a 1:250 dilution in serum-free RPMI) were added to form VSs with provirus-transfected HeLa cells. After 2-2.5 h of coculture, cells were surface-labeled with 1:200 mouse anti-EWI-2 mAb in RPMI/10% FBS for 45 min at 4 ˚C. Surface-labeled cells were fixed with 4% PFA in PBS at 4 ˚C for 10 min, and then incubated with block/perm for 10 min, before labeling with a mixture of rabbit anti-p24 and anti-p6 antibodies, each at 1:1000 dilution, in block for 45 min. Subsequently, cells were labeled with secondary pAbs (anti-mouse-Alexa Fluor 647 and anti-rabbit-Alexa Fluor 594), each at 1:500 in block. Cells were kept in PBS for imaging.

Images were acquired on a DeltaVision epifluorescence microscope (GE/Applied Precision, Issaquah, WA, USA) with an Olympus IX-70 base using an Olympus 60× PlanApo 1.42 NA objective and equipped with a CoolSNAP HQ CCD camera (Photometrics). Images were imported into Fiji for analysis following deconvolution and cropping using Softworx software. The VS was identified using the Gag channel and the level of EWI-2 accumulation was determined by measuring its signal intensity at the VS. For ΔEnv controls, cell-cell contacts were identified using the differential interference contrast (DIC) channel and treated analogous to a VS. The EWI-2 associated signal intensity at non-contact sites was determined by manually outlining the surface of the cell, excluding any regions that were in contact with an adjacent cell, and calculating the mean EWI-2 intensity within the selected area. To determine the level of enrichment at the VS (or cell-cell contact for ΔEnv controls), an "unbiased" approach was applied to account for EWI-2 signal contributed by both the target and producer cell at each VS/contact. Enrichment was calculated as the EWI-2 signal intensity at the VS/contact divided by the sum of the EWI-2 signal at non-contact sites of the producer and target cell in that particular VS/contact. A "biased" approach, where only the producer cell's non-contact sites were used to normalize the VS/contact signal, yielded very similar results to the unbiased approach described above (unpublished observations).

2.6 Determining surface levels of EWI-2 by microscopy

To compare EWI-2 surface expression between infected and uninfected cells, CEM2n cells and primary $CD4^+$ T cells were infected with VSV-G-pseudotyped NL-sfGI as described above. Three days post infection, 3×10^5 infected cells were plated onto each well of 8-well glass-bottom plates coated with 1:10 poly-L-Lysine in ddH_2O. Two additional wells were used for uninfected controls. After 2 h of incubation at 37 ˚C, the medium was replaced with ice cold RPMI/10% FBS containing mouse anti-EWI-2 mAb at 1:200 dilution for surface labeling, and

incubated for 45 min at 4 ˚C. Following the primary antibody incubation, cells were washed with RPMI/10% FBS and fixed with 4% PFA in PBS for 10 min at 4 ˚C, blocked and permeabilized with PBS containing 1% BSA and 100 µg/mL digitonin for 10 min, and incubated with the indicated secondary antibody in block for 45 min at room temperature. Cells were washed with block and imaged in PBS. At least 50 fields containing infected cells were selected for each biological replicate and imaged, deconvolved and cropped using the DeltaVision microscope and Softworx software described above. After deconvolution in Softworx, Fiji was used to manually select the cell surface at the midline of each cell and the mean intensity of EWI-2-associated signal was quantified and subsequently subtracted by the mean intensity of an area that did not contain cells. Cell-cell contact sites were excluded from the quantification. The virus-associated fluorescent reporter channel was used to segregate measurements into uninfected and infected. The data shown in Figure 3B and C are pooled from 3 and 2 independent biological replicates, respectively, each consisting of 2 technical replicates, all of which were sampled randomly until a minimum of 50 infected cells were quantified.

2.7 Determining surface EWI-2 signal on infected cells by flow cytometry

CEM2n cells infected as described above were harvested after three days and incubated in cold PBS with 5 mM EDTA for 15 min (3.0 x 10^5 cells/tube). Cells were pelleted at 400 rcf for 7 min at 4 °C and resuspended in cold RMPI/10% FBS containing mouse anti-EWI-2 mAb at 1:200 dilution. After a 45 min incubation at 4 °C, cells were washed with cold RPMI/10% FBS and resuspended in ice cold PBS with 5 mM EDTA. To fix, an equal volume of PBS with 8% PFA was added and samples were incubated on ice for 10 min. Cells were washed and stained with Alexa Fluor 594-conjugated secondary antibody at 1:500 in block for 45 min at room temperature, before being washed, resuspended in PBS, and analyzed using a BD LSRII flow cytometer. Data were analyzed using FlowJo V10 (Becton, Dickinson & Company, Franklin Lakes, NJ). Samples were gated for infected and uninfected populations by GFP expression. EWI-2 wild-type (WT) and EWI-2 low gates were set based in part on controls lacking primary antibody, and in part by adjusting the gates to reflect the number of uninfected EWI-2 WT cells as measured by microscopy. The data shown are the collection of 3 independent biological replicates, each consisting of 2 technical replicates.

2.8 HIV-1-induced cell-cell fusion assay

50,000 HeLa cells were plated in each well of a 24-well plate and, the next day, transfected using FuGene6 transfection reagent at a ratio of 3:1 (Fugene6:DNA) according to manufacturer's instructions (Promega, Madison, WI, Cat. #E2691) in duplicate with 0.1 µg of either pNL-sfGI or pNL-sfGI ΔEnv along with 0.5 µg total expression vector carrying CD81 or EWI-2. L6, a tetraspanin-like protein that does not inhibit cell-cell fusion, was co-transfected instead of CD81 or EWI-2 as a positive control for maximum fusion activity, For dose response assays, 0.125,

0.25, or 0.5 µg of either EWI-2 or CD81 plasmid was ”stuffed” with L6 expression plasmid to maintain 0.5 µg of total protein expression plasmid in each condition. No cytotoxicity was observed upon transfection for any of the experimental conditions. 24 h post-transfection, producer HeLa cells were co-cultured with 10^6 TZM-bl target cells (which, upon producer-target cell fusion, express firefly luciferase under control of the HIV-1 LTR) per well for 3 h before unattached target cells were washed off and the medium was refreshed. 14-18 h later, cells were lysed for at least 30 min on ice using 1% Triton X-100, 2mM EDTA, 50 mM Tris-HCl, 200 mM NaCl, with 1% protease inhibitor cocktail (Millipore Sigma, Darmstadt, Germany, Cat. #P8340). Lysates were precleared by centrifugation at 20,000 rcf for 5 min at 4 °C and stored at -80°C until use for luciferase activity assays. Note that the timepoints used here ensure that there is not enough time for the development of any luciferase signal resulting from productive infection of target TZM-bl cells through virus transmission (unpublished observation) and that only cell-cell fusion contributes to the luciferase activity measured.

Each lysate was incubated with an equal volume of firefly luciferase reagent (Promega, Cat. #E1500) for 1 min in a 96-well white-walled plate (ThermoFisher Scientific, Waltham, MA, Cat. #7571) before collecting luminescence signal intensity on a microplate reader (BioTek Synergy 2). Background luminescence was determined using a lysis buffer blank and subtracted from all experimental samples. Luminescence intensity was used as a quantitave measurement of relative HeLa-TZM syncytium formation against the non-fusogenic (therefore incabable of forming syncytia) ΔEnv control by dividing each value by the ΔEnv value (which effectively corresponds to any leaky expression of luciferase in TZM-bl cells as no cell-cell fusion occurs at all in this condition). To then determine relative fusion activity of cells transfected with EWI-2 and CD81, those values were normalized to the L6 condition. Normalized fusion is therefore the fold difference of cell-cell fusion activity taking place when cells were co-transfected with the indicated amount of either CD81 or EWI-2 plasmid, compared to the activity taking place when cells were co-transfected with L6. The data shown are the collection of 4 independent biological replicates.

2.9 Statistical Analysis

All statistical analyses were carried out in GraphPad Prism 7 as indicated in Figure legends.

3. Results

3.1 EWI-2 accumulates at the virological presynapse in HIV-1-infected cells

Because EWI-2 is known to associate with ezrin and CD81 (22, 24), two cellular factors that we have previously found to accumulate at the producer cell side of the VS (21, 42), we first sought to determine whether this protein also localizes to the virological synapse (VS). CEM-SS cells were infected with (VSV-G-pseudotyped) NL4-3 WT, or NL4-3 ΔEnv (virus that does not express Env) and mixed with target CEM-SS cells (labeled with a cytoplasmic dye). Upon imaging with a 60× objective, the VS was identified as clusters of immunolabeled Gag present at producer-target contact sites. DIC was used to select cell-cell contacts between ΔEnv producers and uninfected target cells as Gag will not accumulate at these contacts in the absence of Env (1). To calculate enrichment at the VS/contact, we divided the EWI-2 signal intensity at the VS/contact site by the sum of the EWI-2 surface intensity at non-contact sites on the producer and target cell at each VS/contact. This unbiased approach prevents potential inflation of the enrichment value that could occur if we assumed that EWI-2 was solely contributed by either the target or producer cell. Similarly to ezrin and CD81 (21, 42), EWI-2 was observed to co-accumulate with Gag at the VS in an Env-dependent manner (Figure 1A). EWI-2 signal intensity was ~4-fold enriched at the VS in CEM-SS cells infected with NL4-3 WT, while no EWI-2 enrichment was seen at cell-cell contacts in cells expressing NL4-3 ΔEnv (Figure 1A). EWI-2 signal intensity was also enriched ~1.6-fold at the VS in infected primary $CD4^+$ T cells at Env-dependent VSs, and was again not enriched at non-VS contact sites (ΔEnv) (Figure 1B).

To determine whether EWI-2 enrichment at the VS takes place within the infected cell (rather than the apposed uninfected target cell), i.e. at the presynapse, HIV-1-infected CEM-SS cells were co-cultured with uninfected target TZM-bl cells (which have nearly-undetectable levels of EWI-2 on their surface) and imaged as described above. Significant EWI-2 enrichment (~5.3-fold) was observed at the VS as before (Figure 2A), demonstrating that the observed EWI-2 accumulation in CEM-SS-CEM-SS co-cultures takes place at least partially within the producer cell. To evaluate the relative contribution of any postsynaptic (i.e. target cell-side) accumulation of EWI-2, HIV-1-producing HeLa cells (which, like TZM-bl cells, also exhibit nearly-undetectable levels of EWI-2 on their surface) were cocultured with uninfected target CEM-SS cells. In this case, minimal EWI-2 accumulation was detected at synapses (~1.1-fold; Figure 2B), showing that EWI-2 enrichment seen at T cell-T cell VSs takes place (almost) exclusively at the presynaptic side of the VS, i.e. in the producer cell. Together, these results conclusively document that EWI-2 is recruited to the virological presynapse during HIV-1 cell-to-cell transmission.

3.2 Overall surface levels of EWI-2 are decreased upon HIV-1 infection

Despite their enrichment at the virological presynapse, the EWI-2 partner proteins CD81 and ezrin are overall downregulated in HIV-1-infected cells (21, 42). We therefore tested whether surface levels of EWI-2 are similarly decreased in lymphocytes infected with HIV-1 NL-sfGI, a strain in which superfolder GFP (sfGFP) replaces the Nef gene and Nef expression is restored using an IRES (9). We chose to utilize a GFP reporter virus rather than immunolabeling Gag after fixation because Gag-negative cells still in the early phase of infection can still exhibit host protein downregulation due to early Nef expression (unpublished observation and reviewed in (43)). We also observed that surface levels of EWI-2 in uninfected CEM-SS cells were considerably higher than in other T lymphocytes, including primary $CD4^+$ T cells, and that EWI-2 levels in the near-diploid CEM-derived cell line CEM2n (39) were most similar to primary cells (unpublished observations). We therefore here present data from CEM2n and primary cells only, though we found similar effects in other lymphocytes including CEM-SS cells (unpublished observations).

HIV-1-infected CEM2n cells adhered to glass-bottom dishes were surface-labeled with EWI-2 primary antibody on ice, and fixed before incubation with Alexa Fluor 594-conjugated secondary antibody. Uninfected and HIV-1-infected cells were imaged with a 60× objective and the resulting images were deconvolved. The mean fluorescence intensity (MFI) of EWI-2 on the surface of each cell was determined by measuring the EWI-2-associated signal intensity of manually-selected regions of the cell surface (representative image shown in Figure 3A). After measuring surface MFI, on average across three independent biological replicates, infected (GFP-expressing) cells had ~1.8-fold lower EWI-2-associated signal than uninfected (GFP-negative) cells, after subtracting background signal (Figure 3B). Similar downregulation of surface EWI-2 was also observed in HIV-1-infected primary $CD4^+$ T cells analyzed in the same way. On average, infected primary cells had ~1.6 fold lower EWI-2-associated signal than uninfected cells (Figure 3C).

We also sought to quantify EWI-2 surface expression by flow cytometry as a means of high-throughput analysis that would include EWI-2 regulation at all stages of HIV-1 infection. HIV-1 NL-sfGI-infected CEM2n cells, surface-labeled for EWI-2 and analyzed by flow cytometry, were gated for WT or low levels of EWI-2 using appropriate controls. These data showed that a much lower proportion of infected cells (identified as GFP^+) had WT levels of EWI-2 surface expression than of uninfected cells (identified as GFP^-) in the same culture (Figure 3D, left). Additionally, the mean fluorescence intensity of EWI-2-associated signal was lower within the total population of infected cells compared to that of the uninfected cells (Figure 3D, right).

Overall, both the quantitative imaging (Figures 3B and 3C) and the flow cytometric analyses (Fig. 3D) showed that EWI-2 surface levels, similar to those of CD81 and ezrin, are decreased in HIV-1-infected T lymphocytes.

3.3 EWI-2 overexpression lowers the frequency of HIV-1-induced syncytium formation

Likely through their accumulation at the producer cell side of the VS, the EWI-2 partner proteins CD81 and ezrin repress fusion of infected and uninfected cells, i.e. syncytium formation (19-21). Given that EWI-2 also accumulates at the VS (Figure 1), we sought to test whether it also contributes to the inhibition of HIV-1-induced syncytium formation. Attempts to determine whether knocking down EWI-2 expression in lymphocytes enhances HIV-1-induced syncytium formation were unsuccessful at this time due to technical difficulties. In parallel, and as we have done previously to examine the fusion-inhibitory capacity of tetraspanins (19, 20), we chose to test whether EWI-2 may act as an inhibitor of HIV-1-induced syncytium formation by overexpressing EWI-2 in HeLa cells. The results of those experiments are presented here.

NL-sfGI-producing HeLa cells overexpressing either EWI-2, CD81, or L6 (a tetraspanin-like surface protein that does not repress HIV-1-induced cell-cell fusion; (20, 44)) were co-cultured with uninfected target TZM-bl cells. As a negative control for HIV-1-induced cell-cell fusion, Env-deleted (ΔEnv) NL-sfGI-expressing HeLa cells were also co-cultured with target TZM-bl cells. HIV-1-induced HeLa-TZM-bl syncytia express firefly luciferase under control of the HIV-1 LTR (19). After 3 h of co-culture (and another 14-18 h to allow for reporter expression), cells were lysed, the lysates were incubated with luciferase substrate, and luminescence was measured using a microplate reader. Overexpression of increasing amounts of EWI-2 (0.125, 0.25, or 0.5 µg of plasmid) in NL-sfGI-producing cells resulted in robust and dose-dependent decrease of cell-cell fusion (at 0.25 and 0.5 µg of input plasmid), though repression was not as extensive as that observed upon CD81 overexpression (Figure 4).

4 Discussion

Transient alignment of infected (producer) and uninfected (target) cells allows for very efficient transmission of virus particles. However, because of the presence of viral Env and viral receptors at the surface of producer and target cell, respectively, rather than separating after particle transfer, these cells could also easily fuse with each other, thus forming a syncytium. Various viral and cellular factors have been identified which, together, reduce the likelihood of such syncytium formation, and this study now allows us to add EWI-2 to the list of host factors that repress HIV-1 Env-mediated cell-cell fusion.

Our investigations were prompted partially by two recent reports. In one of those studies, Rubinstein and colleagues documented a role for EWI-F, a close relative of EWI-2, in myoblast fusion regulation (23). EWI-F was shown to act as fusion repressor in cooperation with the tetraspanins CD9 and CD81. With the other study, Yáñez-Mó and colleagues (29) showed the presence of EWI-2 at sites of contact between uninfected T cells and T cells stably expressing HIV-1 Env. In separate experiments, HIV-1-infected EWI-2 knockdown cells were also shown

to have somewhat increased virus production and the authors mentioned (as data not shown) that this was accompanied by augmented syncytium formation, indicating that EWI-2 could be involved in the regulation of HIV-1-induced membrane fusion. Importantly, however, the study did not address the question of whether the reported increase in syncytium formation was (potentially) caused by the action of EWI-2 in producer or target cells, nor did it provide a dissection of where EWI-2 accumulates (producer and/or target cells), though the authors speculated that EWI-2, together with α-actinin, might be active in target cells, there possibly contributing to α-actinin's actin bundling activity, thus ultimately inhibiting virus entry/fusion. They also explicitly stated, however, that even if their speculation about where α-actinin acts during virus replication should eventually turn out to be confirmed (with subsequent studies), they cannot exclude an involvement of the partner protein EWI-2 in "subsequent steps of the viral life cycle".

Our study now reveals that EWI-2 indeed acts during the late phase of the HIV-1 replication cycle: It accumulates on the producer cell side of the VS (Figures 1-2), while, to our surprise, no enrichment can be seen at the postsynapse. This does not reflect what is known about tetraspanins, which is that they have a fusion-inhibitory role at both sides of the HIV-1 VS (and thus likely localize at both sides of the contact site) (19, 45). However, paralleling what we observed for tetraspanins (19), we found that fusion with uninfected target cells was inhibited by EWI-2 in a dose-dependent manner (Figure 4). Furthermore, the background of these expression experiments effectively corresponds to an EWI-2-null condition, because HeLa cells exhibit nearly-undetectable levels of EWI-2. In turn, the low EWI-2 plasmid condition is a "knockdown" of the high EWI-2 plasmid condition, and indeed a specific one where no off-target effects are seen (as often happens when RNA interference is used). Thus, while we could not test the effect of EWI-2 knockdown or knockout on cell-cell fusion *per se*, we can confidently predict what would happen based on these data.

While, at first sight, CD81 appears to be a more potent inhibitor of cell-cell fusion than EWI-2, at this point we cannot determine whether this is a consequence of other factors, e.g. the levels of EWI-2 or CD81 achieved upon transfection, or whether the (near) absence of endogenous EWI-2 in HeLa producer cells means that overexpressed EWI-2 is quickly downregulated (unlike CD81, which is highly expressed at baseline, thus likely saturating the ability of the virus to downregulate it already before overexpression). Further investigations are required to appropriately compare the contribution of each fusion inhibitor. Additionally, in the tetraspanin case, we also show that tetraspanin knockdown enhanced cell-cell fusion while tetraspanin overexpression reduced cell-cell fusion (19), and because EWI-2 is associated with tetraspanins, we expect that it will also behave similarly when knockdown is achieved in future work.

Interestingly, and paralleling our findings about tetraspanin expression in HIV-1-infected cells (42, 46), while EWI-2 accumulates at the virological presynapse, overall this protein is

downregulated in infected cells (Figure 3). Importantly, and again similar to our tetraspanin data which were confirmed by an independent study (47), in two recent proteomics screens (48, 49) EWI-2 was found to be one of the host proteins whose expression is diminished in HIV-1-infected T cells. The combination of these two features, i.e. enrichment during one step of the viral replication cycle (assembly and transmission at the VS) and overall downregulation specifically in infected cells, strongly suggests that a particular host factor plays an important role in virus replication, and given the fusion-preventing functions documented in this study, we certainly think this holds true for EWI-2.

Finally, EWI-2's contribution to repressing the formation of HIV-1-induced syncytia may be relevant mostly because this allows for continued spread of the virus since the number of infected cells duplicates each time when producer and target cells separate after virus transmission. Additionally, we and others have recently shown that lowering Env's fusion activity and thus its syncytium forming ability also allows HIV-1 to overcome a restriction factor (APOBEC3G; (50)), and even antiviral drugs (Van Duyne and Freed, pers. communication). Importantly, the action of this protein along with others could also help the virus, or, rather, HIV-1-infected cells, to evade the host immune response. For instance, simply because syncytia are larger and thus more "visible" than individual infected cells, they may be more prone to be attacked by innate immune cells. In addition, unpublished studies in our lab suggest that these entities have a different surface profile of immune regulatory proteins compared to mononucleated infected cells, similar to how e.g. measles virus-induced syncytia show an upregulation of ligands of activating natural killer cell receptors (51). Thus, fusion-repressing host factors such as EWI-2, whose syncytium-inhibiting function we identify in this report, besides supporting efficient virus spread, probably also help HIV-1 escape attacks by the human immune system.

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6 Author Contributions Statement

EW, MS, and MT conceived and designed the experiments. EW, MS, and PM performed the experiments and analyzed the results. EW and MS prepared the figures. EW, MS, and MT wrote and edited the manuscript.

7 Conflict of Interest Statement

The authors declare no competing commercial or financial interests.

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Figure Legends

Figure 1. EWI-2 co-accumulates with Gag at the HIV-1 VS in T cells

(A) CEM-SS cells infected with HIV-1 NL4-3 WT or ΔEnv were co cultured with CMAC-labeled CEM-SS target cells (blue) for 5 h, and subsequently stained for surface EWI-2 (red) and Gag (green). The EWI-2-associated fluorescence intensity at cell-cell contacts either enriched with Gag (WT) or not Gag-enriched but identified by DIC (ΔEnv) was measured. This value was then divided by the sum of the EWI-2-associated fluorescence intensity on non-contact sites on the producer and target cell in each VS/contact to yield EWI-2 enrichment (i.e. the values shown here). The data quantified are from one biological replicate consisting of two technical replicates. Similar trends were observed in a second dataset; not shown. (B) Primary $CD4^+$ T cells infected with NL-sfGI WT or NL-CI ΔEnv were co-cultured with uninfected target primary cells for 2 h and stained for EWI-2 (red) and Gag (green), followed by secondary pAbs (Alexa Fluor 647-conjugated for EWI-2, and either Alexa Fluor 594 or Alexa Fluor 488-conjugated for Gag in the case of WT and ΔEnv, respectively). Because different secondary antibodies were used for Gag in either condition, the scaling shown for that channel is not the same across the two conditions, and was based on corresponding no primary and uninfected controls done alongside each dataset. Enrichment of EWI-2 at Env-dependent (WT) or Env-independent (ΔEnv) infected-uninfected cell contacts was quantified as described in (A). The data quantified are pooled from two independent biological replicates consisting of two technical replicates. Scale bars = 10 µm. In both data plots, each data point represents one cell-cell contact site (as opposed to one cell). The dotted horizontal line indicates a theoretical fold enrichment value of 1, which indicates no enrichment. Error bars = standard deviation of the mean (SD). *p*-values are the result of two-tailed non-parametric Mann-Whitney *U* tests.

Figure 2. EWI-2 accumulation takes place on the producer cell side of the VS

(A) To evaluate presynaptic accumulation of EWI-2, CEM-SS cells infected with HIV-1 NL-CI WT or ΔEnv were co cultured with CMAC (blue) labeled TZM-bl target cells (which have nearly-undetectable EWI-2 surface levels compared to CEM-SS cells) for 2.5 h, and subsequently stained for surface EWI-2 (red) and Gag (green). EWI-2 enrichment was quantified as described in Figure 1. Quantification is the result of pooled VS/contacts from two independent biological replicates. (B) To evaluate postsynaptic accumulation of EWI-2, HeLa cells (which, like TZM-bl cells, also have nearly-undetectable EWI-2 surface levels) were transfected with HIV-1 NL-sfGI or NL-sfGI ΔEnv and cocultured with uninfected CEM-SS target cells (blue) for 2-2.5 h. Cells were stained for surface EWI-2 (red) and Gag (green). Note that Gag expression in the ΔEnv condition was not detected above the somewhat high background binding of the primary antibodies, especially since Gag expression in this virus is already expected to be considerably reduced (52). As a result, in the merged image for ΔEnv, the GFP reporter is shown

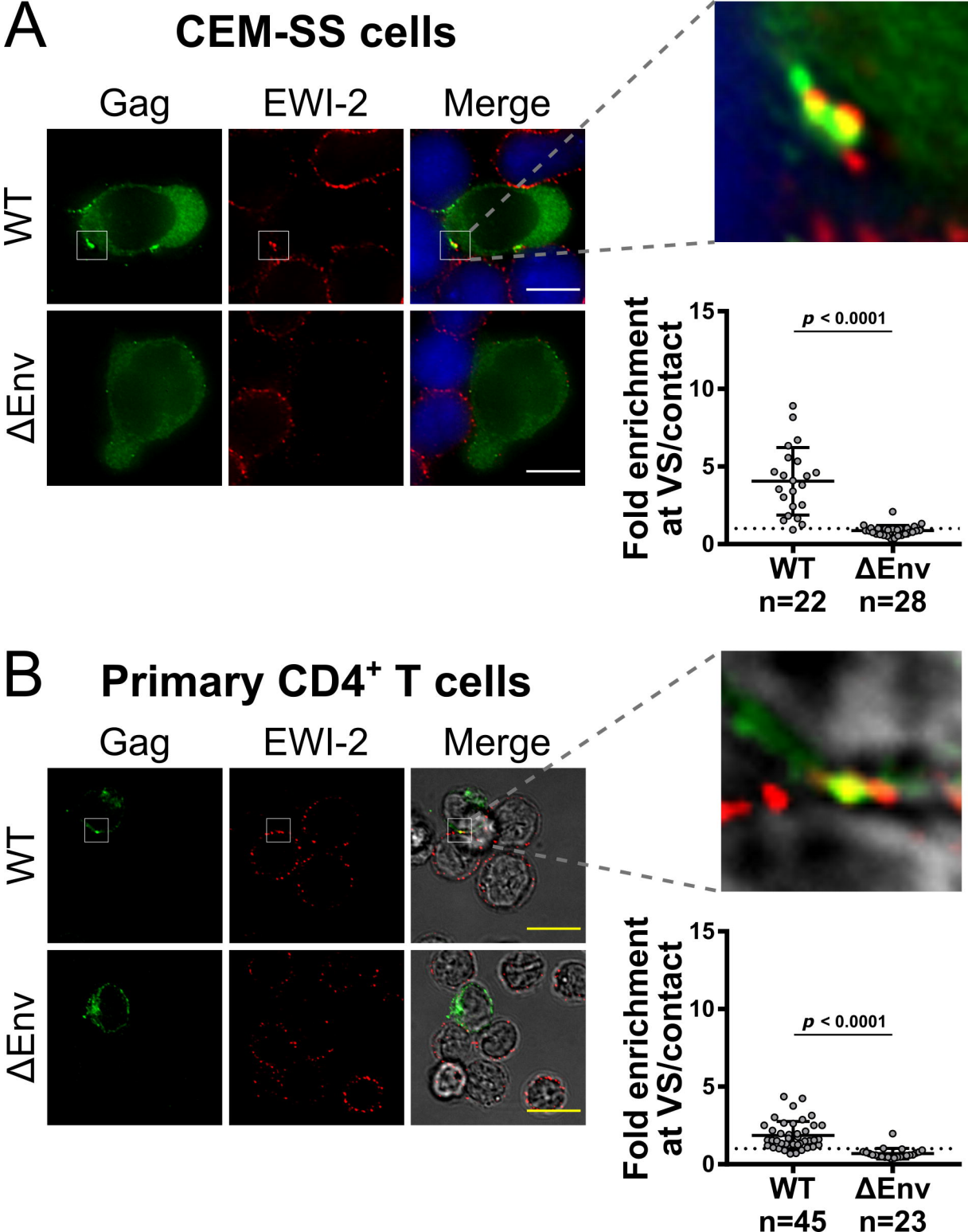
in cyan to mark the producer HeLa cell's location. EWI-2 enrichment was calculated as described in Figure 1. Quantification is the result of pooled VSs/contacts from two independent biological replicates. Scale bars = 10 µm. In both data plots, each dot represents the EWI-2 enrichment value of one VS/contact. The dotted horizontal line indicates a theoretical fold enrichment of 1, which indicates no enrichment. Error bars = standard deviation of the mean (SD). *p*-values are the result of two-tailed non-parametric Mann-Whitney *U* tests.

Figure 3. EWI-2 is downregulated from the surface of infected cells

(A) CEM2n cells were infected with NL-sfGI and surface-labeled for EWI-2, fixed, stained with DAPI (shown in blue) and Alexa Fluor 594-conjugated secondary antibody, and imaged. GFP signal (green) was used to identify infected cells, and EWI-2-associated signal is shown pseudocolored in red. Representative cells are shown. Scale bars = 10 µm. (B) Cells were prepared as in (A) and EWI-2 levels at the plasma membrane in infected and uninfected cells were measured by manually selecting the plasma membrane at the midline of each cell and quantifying the mean EWI-2-associated fluorescence intensity. Data shown are pooled from three biological replicates, each consisting of two technical replicates. Only non-contact sites were quantified. (C) Primary CD4+ T cells were infected, plated, and surface-labeled for EWI-2 as described in (A). EWI-2 levels at the plasma membrane in infected and uninfected cells were measured as in (B). Data shown are pooled from two biological replicates, each consisting of two technical replicates. Only cells which were not in contact with other cells were quantified. Error bars = SD. *p*-values are the result of a two-tailed non-parametric Mann-Whitney *U* test. (D) CEM2n cells were infected with NL-sfGI and surface-labeled for EWI-2, fixed, and stained with Alexa Fluor 647-conjugated secondary antibody, and analyzed by flow cytometry. Left; Points represent the percentage of uninfected and infected cells that fell into the "EWI-2 WT" gate from 3 independent biological replicates, averaged across 2 technical replicates within each. Right; EWI-2 surface expression was measured by mean fluorescence intensity (MFI) of EWI-2-associated signal. In both panels, lines connect paired data points, i.e. infected cells and uninfected cells (within an infected tube) from the same biological replicate.

Figure 4. EWI-2 overexpression represses infected-uninfected cell fusion.

HeLa-TZM-bl fusion assays were performed using producer HeLa cells that were co-transfected with either pNL-sfGI ΔEnv (ΔEnv) or pNL-sfGI (WT) in combination with either EWI-2, CD81, or L6 overexpression plasmids. Luminescence readings (across 4 independent biological replicates, each with 2 technical replicates) were divided by the ΔEnv condition to obtain the fold increase in fusion, and then normalized to the WT co-transfected with L6 condition (thus making L6 have the value 1.0, shown as a dashed line). Values from within each biological replicate are linked by a grey line.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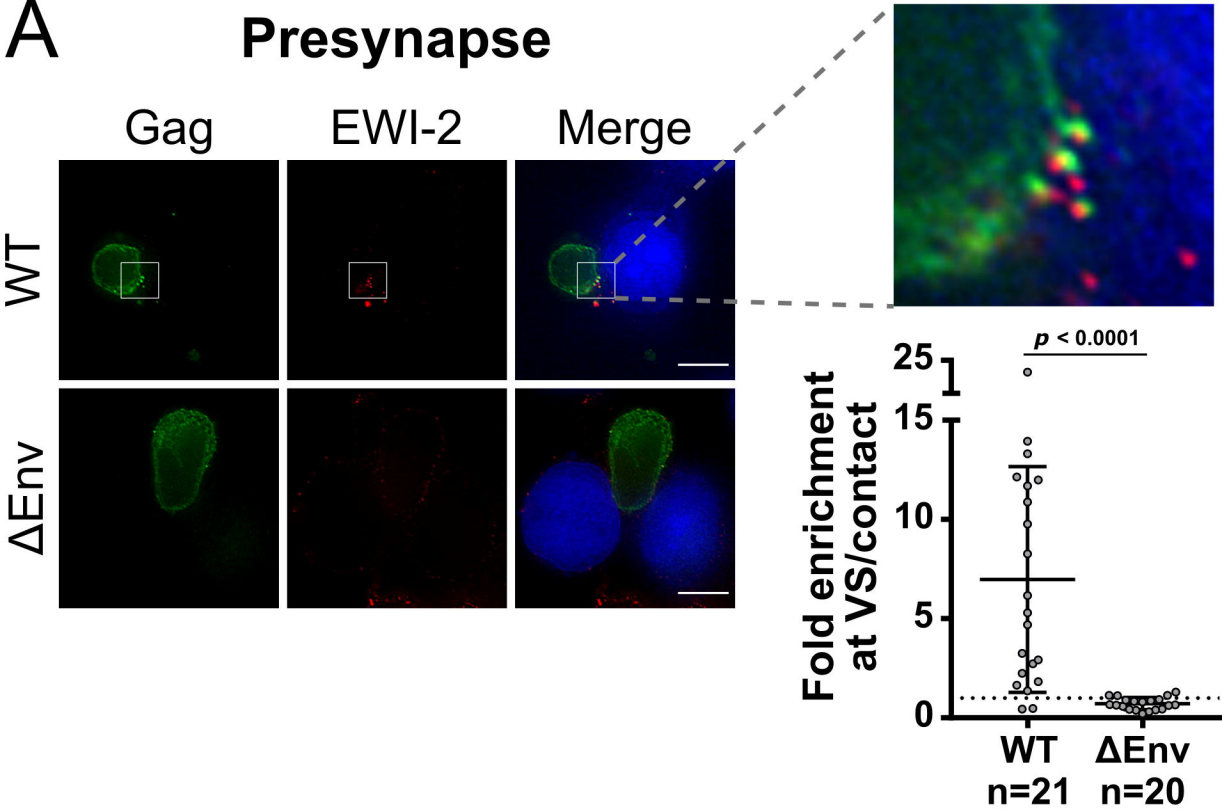
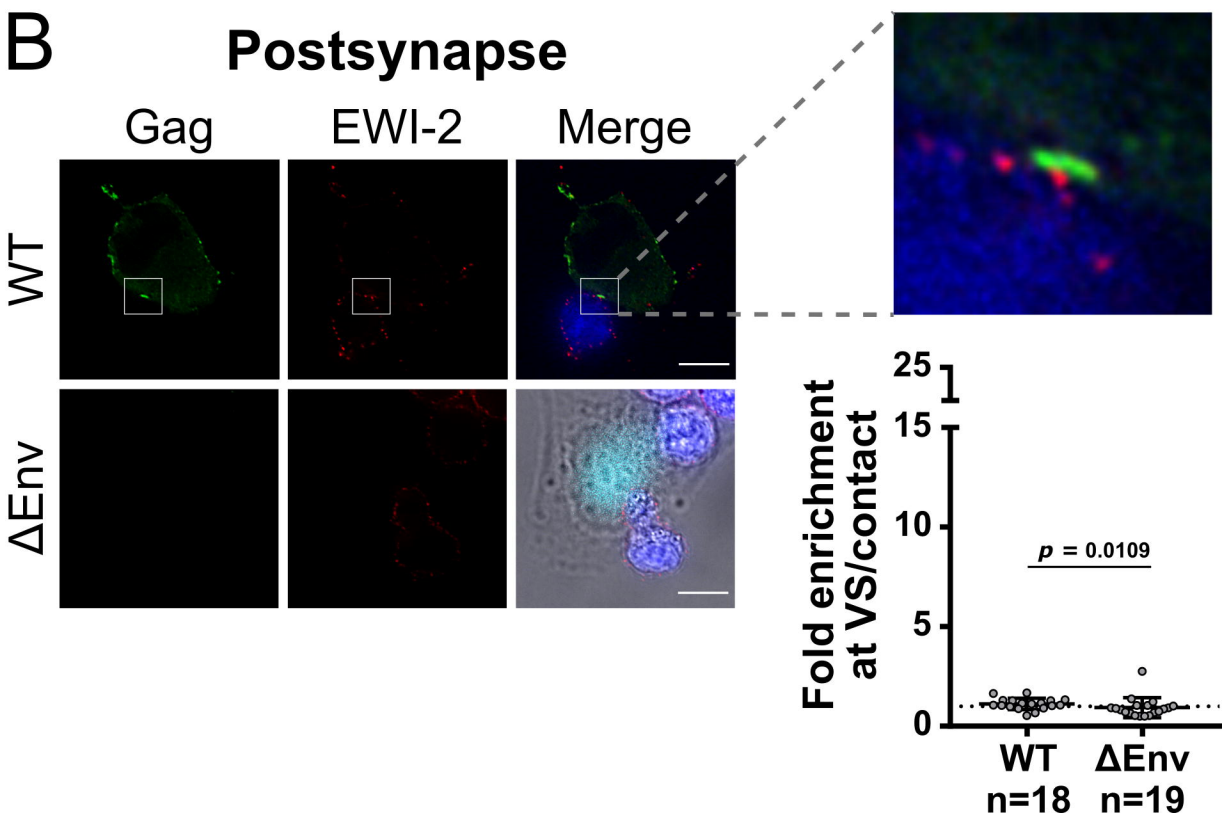
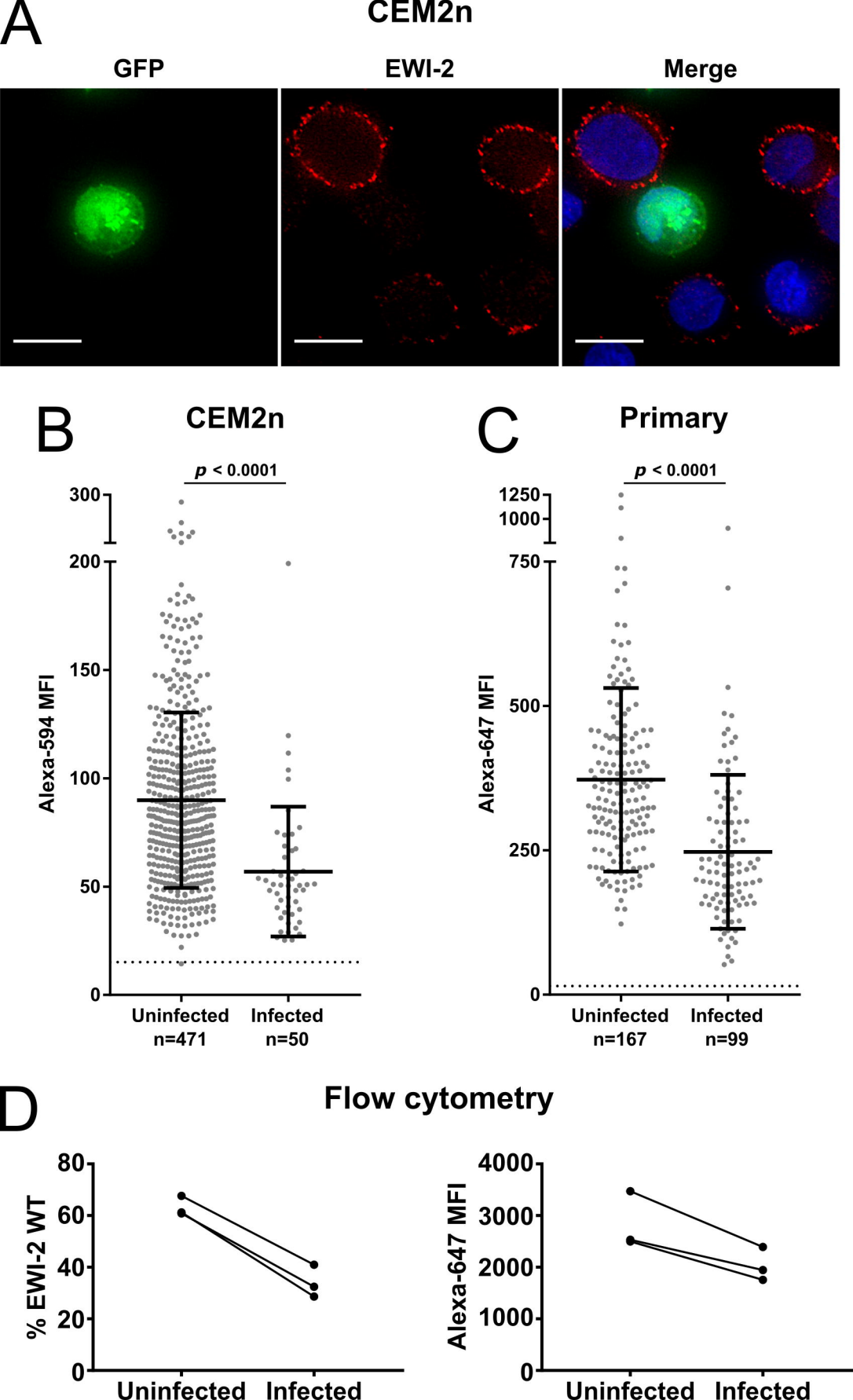
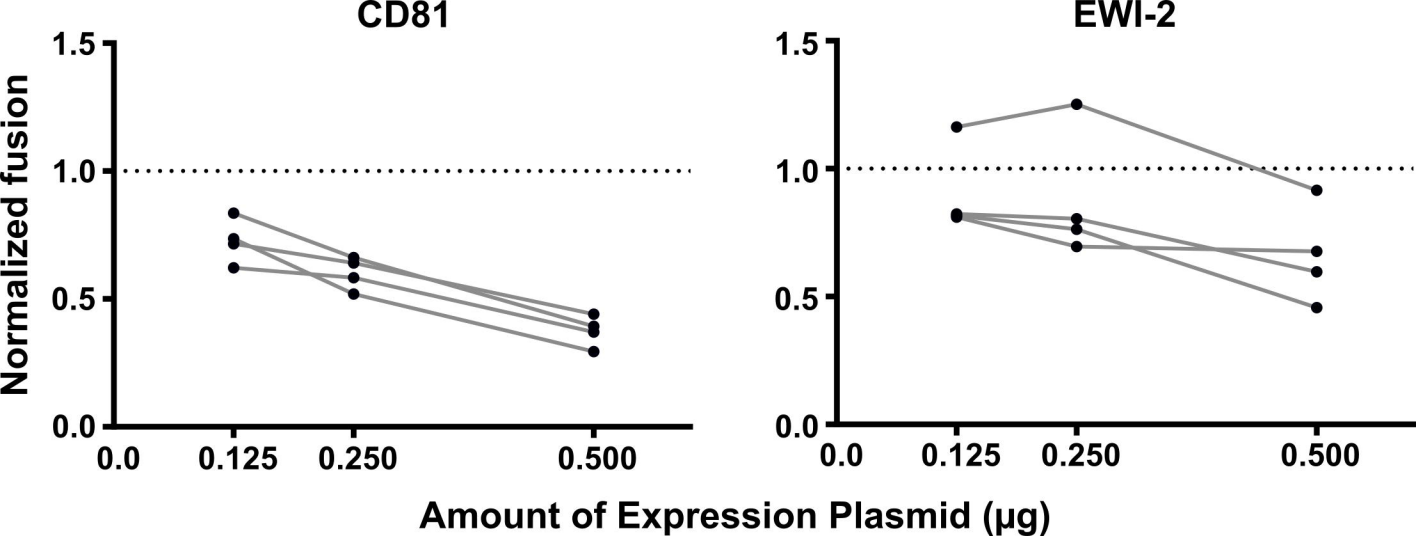
Gag
EWI-2
Merge
WT
ΔEnv
Fold enrichment at VS/contact
p < 0.0001
15
10
5
0
WT n=22
ΔEnv n=28
B
Primary CD4+ T cells
Gag
EWI-2
Merge
WT
ΔEnv
Fold enrichment at VS/contact
p < 0.0001
15
10
5
0
WT n=45
ΔEnv n=23


A
Presynapse
Gag
EWI-2
Merge
WT
ΔEnv
p < 0.0001
Fold enrichment at VS/contact
25
15
10
5
0
WT n=21
ΔEnv n=20


B
Postsynapse
Gag
EWI-2
Merge
WT
ΔEnv
p = 0.0109
Fold enrichment at VS/contact
25
15
10
5
0
WT n=18
ΔEnv n=19


A
CEM2n
GFP
EWI-2
Merge
B
CEM2n
p < 0.0001
Alexa-594 MFI
300
200
150
100
50
0
Uninfected
n=471
Infected
n=50
C
Primary
p < 0.0001
Alexa-647 MFI
1250
1000
750
500
250
0
Uninfected
n=167
Infected
n=99
D
Flow cytometry
% EWI-2 WT
80
60
40
20
0
Uninfected
Infected
Alexa-647 MFI
4000
3000
2000
1000
0
Uninfected
Infected


CD81
EWI-2
Normalized fusion
1.5
1.0
0.5
0.0
0.0
0.125
0.250
0.500
Amount of Expression Plasmid (µg)