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Supplemental Information

HIV-Captured DCs Regulate T Cell Migration and Cell-Cell Contact Dynamics to Enhance Viral Spread

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KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Human CD3	Biolegend	clone: HIT3a
Human CD4	Biolegend	clone: RPA-T4
Human CD11c	Biolegend	clone: Bu15
Human CD14	Biolegend	clone: 63D3
Human CD80	Biolegend	clone: 2D10
Human CD81	Biolegend	clone 5A6
Human CD83	Biolegend	clone: HB15E
Human CD86	Biolegend	clone: IT2.2
Human CD169	Biolegend	clone: 7-239
Human CD209	Biolegend	clone: 9E9A8
Human CCR7	Biolegend	clone: G043H7
Human CD11+CD18 (high affinity conformation)	Abcam	clone: 24
PSGL-1	Biolegend	clone KPL-1
goat anti-mouse IgG antibody conjugated with AF568	Thermo Scientific	Cat #A11004
Neutralizing Siglec-1 antibody	R&D	Cat #AF5197-SP
Human LFA-1	BD Biosciences	clone L130
Human ICAM-1	BD Biosciences	clone LB-2
Neutralizing CCR7 antibody	R&D	MAB197R-SP
Mouse LYVE-1	R&D	Clone: 223322
anti-human CD3ε/CD28 antibody coated dynabeads	Life Technologies	Cat #11131D
LPS from Salmonella Minnesota	Invivo Gen	Cat # tlrl-smlps
Fetal calf serum	VWR Seradigm	Cat #1500-500
Nunclon Sphera flasks	Thermo Scientific	Cat #174951
Bovine collagen	PureCol	Cat #5005-100ML
Dynasore hydrate	Sigma	Cat #D7693
Texas Red conjugated dextran (70kDa)	Thermo Scientific	Cat #D-1830
Texas Red conjugated phalloidin	Thermo Scientific	Cat #T7471
Collagenase D	Roche	Cat #11088866001
Bacterial and Virus Strains		
HIV-iGag/dTomato (BaL envelope)	This paper	N/A
HIV-iGag/dTomato Δenv (BaL envelope)	This paper	N/A
Biological Samples		
Chemicals, Peptides, and Recombinant Proteins		
human CCL19	Biolegend	Cat #582102

human CCL21	Biolegend	Cat #582202
human S1P	Sigma	Cat #73914
Human IL-4	Biolegend	Cat #574008
Human IL-2	Peprotech	Cat #200-02
Human GM-CSF	Biolegend	Cat #578606
FTY720-phosphate	Caymen	Cat #10008639
Raltegravir	NIH ARRRP	Cat# 11680
Critical Commercial Assays		
GenElute HP Plasmid Maxiprep Kit	Sigma	NA0310-1KT
Human CD14+ monocyte isolation kit	Stemcell Technologies	Cat #17858
Human CD4+ T cell isolation kit	Stemcell Technologies	Cat #17952
Deposited Data		
Experimental Models: Cell Lines		
HEK 293T cells	ATCC	ATCC CRL-11268
MAGI CCR5 cells	NIH ARRRP	Cat #3580
Experimental Models: Organisms/Strains		
Oligonucleotides		
Recombinant DNA		
Software and Algorithms		
Imaris 8.2	Bitplane	
ImageJ	NIH/freeware	
Prism 6	GraphPad	
FlowJo	Treestar	
Matlab	Mathworks	
Ingenuity pathway analysis (IPA)	Ingenuity Systems	
InnateDB	www.innatedb.com	
Other		

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Thomas Murooka (thomas.murooka@umanitoba.ca).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cells

Human $CD14^+$ monocytes and $CD4^+$ T cells (both from Stem cell Technologies) were isolated from PBMCs of healthy donors (NetCAD, Canadian Blood Services). All cells derived from donors are anonymized and contain no information regarding the gender, race or health status. Studies using human blood products were approved by the University of Manitoba Biomedical Research Ethics Board (B2015:030).

MAGI.CCR5 cells were obtained from the NIH ARRRP (Cat #3522) and grown in DMEM supplemented with 10% fetal calf serum (VWR Seradigm), 2 mM GlutaMAX (Gibco), 1mM sodium pyruvate (Corning) and 10mM HEPES (Sigma-Aldrich) under 37°C/5% CO_2 conditions. The parental cell line of MAGI is a HeLa cell clone, which is a female cell line. This cell line has not been authenticated.

Viral Constructs and Preparation of Viral Stocks

The R5-tropic HIV Gag-iGFP/dTomato (HIV-iGFP for short) reporter encodes for two fluorescent proteins: (1) a fusion protein between GFP and the structural polypeptide Gag (Gag-iGFP) to visualize GFP-containing HIV particles (Muller et al., 2004), and (2) a *nef-IRES-dTomato* gene cassette that express soluble dTomato (Usmani et al., 2019) after productive infection. The *env*-deleted plasmid was constructed by digesting the HIV Gag-iGFP plasmid

with PsiI to generate a frameshift mutation in the *env* gene (HIV-iGFPΔ*env)*, as described previously (Murooka et al., 2012). All plasmids were confirmed by Sanger sequencing.
All HIV-1 stocks were generated by transient transfection of HEK 293T cells using calcium phosphate. To generate HIV-iGag virus, cells were co-transfected with the pGagPol plasmid (NIH ARRRP). Viral supernatants were collected 48 hours after transfection and layered over a 20% sucrose solution before ultra-centrifugation at 35,000 rpm for 90 min using an SW70Ti rotor (Beckman Coulter). Virus pellets were resuspended in PBS, aliquoted and stored at -80ºC until use. Each HIV stock was titrated using MAGI.CCR5 cells and expressed as infectious blue focus units (bfu) per ml.

METHOD DETAILS

Cell culture

Human $CD14^+$ monocytes and $CD4^+$ T cells (Stem cell Technologies) were isolated from PBMCs of healthy donors (NetCAD, Canadian Blood Services). Cell purity for both populations were routinely >95%. Monocytes were differentiated into immature dendritic cells by seeding into Nunclon™ Sphera™ flasks (Thermo Scientific) with 50ng/mL each of human granulocyte-macrophage colony-stimulating factor (hGM-CSF; Biolegend) and interleukin 4 (IL-4; Biolegend) in complete RPMI1640 media supplemented with 10% FBS (VWR Seradigm), 2 mM GlutaMAX (Gibco), 1mM sodium pyruvate (Corning Cat #25-000-CI) and 10mM HEPES (Sigma-Aldrich), for five days. On day 5, immature monocyte-derived dendritic cells (MDDCs) were stimulated with 100ng/mL purified lipopolysaccharide (LPS) from *Salmonella minnesota* (Invivo Gen) for 24-48 hours. Naïve CD4+ T cells were activated with anti-human CD3ε/CD28 antibody coated dynabeads (1:1 bead:cell ratio, Life Technologies) in complete RPMI 1640

media. After two days, beads were magnetically removed and T cells were cultured for another 4-6 days in complete medium containing 50 IU/mL human rIL-2 (Peprotech), keeping cell densities at 2 x 10^5 cells/mL. Day 7 expanded T cells were used for all experiments. Studies using human blood products were approved by the University of Manitoba Biomedical Research Ethics Board (B2015:030).

Flow cytometry and antibodies

Phenotypic characterization of MDDCs and CD4+ T cells were performed using the BD FACSCanto-II and analyzed with FlowJo software (Tree Star). Human Fc receptors (FcR) blockade was performed using the TruStain FcX™ (Biolegend) solution. MDDC and T cells were phenotyped with a panel of directly conjugated anti-human mAb (Biolegends) as indicated in the Key Resources Table. HIV-captured DCs and HIV infected T cells were fixed with 2.5% paraformaldehyde prior to flow cytometry analysis.

Virus capture and retention assays

3-6 million DCs were pulsed with HIV-iGFP (1,550 – 3,300 bfu) and their capture efficiency measured by flow cytometry. Because HIV-iGFPΔ*env* titers were not attainable using MAGI.CCR5 indicator cell lines, we pulsed DCs with increasing amounts of either parental or HIV-iGFPΔ*env* stocks to achieve equivalent numbers of HIV-captured DCs by flow cytometry (data not shown). To evaluate the role of Siglec-1 on HIV capture efficiency, DCs were pre-treated with anti-Siglec-1 neutralizing antibody (2ug; R&D) for 15 mins at room temperature prior to virus incubation and imaging studies.

Live-cell imaging in 3D collagen chambers

Collagen type I was used to recapitulate the three-dimensional (3D) fibrillar networks found in the lymph node T cell zone (Wolf et al., 2009) and were assembled as previously described (Sixt and Lammermann, 2011). Bovine collagen (PureCol) was used to achieve a final concentration of 1.7mg/mL in each chamber. To characterize the HIV capture dynamics by DCs, 1-2 million DCs were labeled with Celltracker Blue (CMAC; 30μM) or Celltracker Red (CMTPX; 7.5μM), washed and embedded into collagen along with HIV particles. Chambers were allowed to solidify for 45 minutes at 37°C / 5% CO_2 and placed onto a custom-made heating platform attached to a temperature controller apparatus (Werner Instruments). A thermocouple device was used to continuously monitor and maintain the chamber temperature at 37°C. A multiphoton microscope with two Ti:sapphire lasers (Coherent) was tuned to between 780 and 920 nm for optimized excitation of the fluorescent probes used. For four-dimensional recordings of cell migration, stacks of 13 (or 26) optical sections (512 x 512 pixels) with 4 μm z-spacing were acquired every 15 (or 30) seconds to provide imaging volumes of 48 (or 96) μm in depth. Emitted light was detected through 460/50 nm, 525/70 nm and 595/50 nm dichroic filters with non-descanned detectors. All images were acquired using the 20X 1.0 N.A. Olympus objective lens (XLUMPLFLN; 2.0mm WD).

To perform live imaging studies of antibody-labeled DCs, DCs were pulsed with HIV particles for 4 hours, washed extensively with warm complete media to remove free virus, and then incubated with primary antibodies against Siglec-1, PSGL-1 and CD81. Secondary goat anti-mouse IgG antibody conjugated with Alexa Fluor 568 or Alexa Fluor 594 (Thermo Fisher Scientific) were added at 1:100 dilution for an additional 15 minutes, then cells were washed and embedded into collagen chambers for imaging studies. In some experiments, cells were

pretreated with either isotype or blocking antibodies for LFA-1 (BD Biosciences), CD4 (Biolegend) or ICAM-1 (BD Biosciences) prior to embedding into collagen chambers. To study the role of actin, CMAC-labeled cells were pre-treated with Dynasore (100μM) for 20 mins at room temperature and embedded into collagen chambers with HIV particles. As Dynasore is a reversible drug, for some experiments, Dynasore were washed out after 2 hours of incubation. These washed cells were then embedded with HIV-1 particles in the collagen chamber. In some studies, HIV-captured DCs were pulsed with Texas Red-conjugated dextran (70kDa; Thermo Fisher Scientific) for 15 mins at 37°C prior to embedding into collagen chambers. A 1:4 or 1:8 ratio of DCs and CD4 T cells, respectively, was used for visualizing cell-cell contact dynamics in collagen chambers.

Immunofluorescent staining in collagen chambers

To preserve the migratory DC morphology in collagen, chambers were fixed with 4% paraformaldehyde/5% sucrose overnight at 37°C / 5% CO_2, as described previously (Lopez et al., 2019). Glycine buffer (0.15M) was used to quench residual PFA. Next, chambers were permeabilized using a 0.5% triton-X solution for 48 hours at 4°C. Collagen gels were blocked using 1% bovine albumin serum (BSA) and labeled with Texas Red™-conjugated phalloidin (ThermoFisher), as per manufacturer's protocol.

Transmission electron microscopy

Transmission electron microscopy was performed to reveal the ultrastructure of the HIV-containing compartments in migratory DCs in collagen. DC-embedded chambers were fixed, quenched with glycine buffer and incubated in 0.05% Tween in PBS. Gels were fixed with 1%

osmium tetroxide for 2 hours, then dehydrated using the following solutions: 30% ethanol, 50% ethanol, 70% ethanol, 90% ethanol, 100% ethanol, 100% methanol, then 100% propylene oxide. Gels were placed in embedding solution (mixture of EMbed 812, DDSA, NMA and DMP-30) for 24 hours prior to ultra-sectioning with the Reichert ultrathin microtome at a thickness of 70-90nm. The EM grids were then stained with uranyl acetate for 30 minutes and lead citrate for 10 minutes**.** Electron micrographs were taken with a Philips CM10 transmission electron microscope.

Transwell® assay

To investigate the chemotactic migration of HIV-bearing MDDCs towards lymph node chemokines, 0.5-1 million DCs were placed into the top insert at a final volume of 100µl of a Transwell plate (5µm pore size; Corning). 600µl of media containing human CCL19 (2ug; Biolegend) and CCL21 (2µg; Biolegend) or S_1P (10nM) was added to the bottom chamber. Transwell plates were incubated for 3 hours at 37°C/5% CO_2, after which migrated DCs in the bottom well were collected and counted using counting beads by flow cytometry (Biolegend). For receptor blockade studies, DCs were pre-treated with anti-CCR7 antibody (2µg; R&D) or FTY720-P (10 µM) prior to analysis in transwell assays.

***Ex vivo* skin crawl-in assay**

A crawl-in assay using explanted mouse ears was used to assess chemokine-dependent DC migration in the dermal layer in real time, as previously described, with some modifications (Weber and Sixt, 2013). Briefly, the ears of female/male 6-8-week-old Balb/c mice were excised, and ear sheets composed of the epidermis and dermis was obtained using forceps. After FcR

blockade, the lymphatic vessels were labeled with Alexa Fluor-594 conjugated mouse anti-LYVE-1 antibody (R&D) for 30 minutes at room temperature. Ear sheets were overlaid with 0.25 million DCs with or without HIV for 20 minutes at 37°C to allow them to crawl into the dermal layer. After washing away unbound DCs, skin sheets were mounted onto a glass slide with complete media and prepared for live-cell microscopy. In some studies, DCs were pre-treated with anti-CCR7 and/or FTY720 prior to overlay onto dermal tissue layers.

Image analysis

Time lapse micrograph images were transformed using Imaris 8.3 (Bitplane) to generate maximum intensity projections (MIPs) and exported as Quicktime movies. Automated 3D tracking of DC centroids was performed for all motility analyses, and subsequent cell track parameters (arrest coefficient, mean displacement) were analyzed using a custom script in Matlab (Mathworks). To characterize the spatial distribution of HIV-containing compartments in motile DCs, line profile analyses were performed to determine fluorescent intensity profiles on the z-axis, where 0 represents the uropodia and 1 represents the leading edge. Cell surface area and cell perimeter measurements were performed using ImageJ using the wand (tracing) tool after color thresholding. Circularity measurements were also performed using ImageJ, where a measure of 0 indicated a straight line and 1 indicated a perfect circle, as previously described (Usmani et al., 2019). Contact duration and cell migration speeds during contacts were analyzed using ImageJ.

HIV infection in collagen matrix

0.5 million DCs and 2-4 million CD4+ T cells were embedded into collagen gel with HIV particles and placed into a 48-well plate. Solidified gels were incubated for 4 hours at 37°C, then cells were recovered after digestion with collagenase D (Roche), washed and subsequently placed in complete media containing 50IU/mL hrIL-2 in a 24 well plate. T cells in the culture supernatant were gently removed, whereas DCs remained attached to the plastic culture vessel (which removed >95% of DCs). Recovered T cells were incubated for another 48 hours and productive infection was assessed by flow cytometry. Cell-free infection in collagen matrix was performed in parallel but in the absence of DCs. In some experiments, cells were pretreated with either isotype or neutralizing antibodies prior and during DC co-cultures in collagen. Raltegravir (20μM) was used to prevent productive infection and served as a negative control.

Phenotypic analysis of high affinity LFA-1

One million DCs were pulsed with HIV (WT and Δ*env*) for 3 hours and washed with RPMI medium to remove unbound virus. DCs were co-cultured with 4 million resting autologous CD4+ T cells in collagen gel for 3.5 hours. T cells were recovered using collagenase D and were immediately stained for high affinity LFA-1 expression (Human CD11+CD18, clone 24) for flow cytometry analysis.

Kinome analysis

Design, construction and application of the human peptide arrays were based on a previously reported protocol (Kindrachuk et al., 2014). DCs were pulsed with either wildtype or HIV-iGFP*ΔEnv*, washed extensively and co-cultured with autologous T cells for 15 minutes at 37°C. Cells were placed on ice and T cells were separated using a negative selection magnetic column

to remove $CD11c^+$ DCs (purity >98%). T cells were pelleted, cell lysates prepared and incubated with human kinome arrays (JPT Technologies). Briefly, cell pellets were lysed with 100uL buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mM NaF, 1 g/ml leupeptin, 1 g/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride) and incubated on ice for 10 min. Cell lysates were clarified by centrifugation at 14,000 rpm. Cell lysates were transferred to fresh microcentrifuge tubes, and the total protein concentrations were measured using the Pierce BCA Protein Assay Kit. Activation mix (50% glycerol, 50 μM ATP, 60 mM MgCl2, 0.05% Brij 35, 0.25 mg/mL bovine serum albumin) was added to the equivalent amounts of total protein (100 μg) for each sample, and total sample volumes were matched by the addition of kinome lysis buffer. Samples were spotted onto kinome peptide arrays (JPT Peptide Technologies GmbH, Berlin, Germany) and incubated for 2 h at 37 °C and 5% CO2. Following incubation, arrays were washed once with PBS containing 1% Triton X-100, followed by a single wash in deionized H2O. Arrays were stained with PRO-Q Diamond phosphoprotein stain (Invitrogen, Carlsbad, CA, USA) for 1 h with gentle agitation. Arrays were subsequently destained (20% acetonitrile, 50 mM sodium acetate, pH 4.0) 3 times × 10 min each with the addition of fresh destain each time. A final 10 min wash was performed with deionized H2O. Arrays were dried by gentle centrifugation. Array images were acquired using a PowerScanner microarray scanner (Tecan, Morrisville, NC, USA) with a 580-nm filter to detect dye fluorescence. Signal intensity values were collected using Array-Pro Analyzer version 6.3 software (Media Cybernetics, Rockville, MD, USA).

Kinome data analysis

The specific responses of each peptide were calculated by subtracting background intensity from foreground intensity. Signal intensities induced by *ΔEnv* HIV-iGFP were subtracted from the intensities from the time-matched, wildtype HIV-iGFP biological conditions, and test statistics were calculated. Average intensities were then taken over the three replicate intensities, and these values were subjected to hierarchical clustering analysis using InnateDb (www.innatedb.com) and ingenuity pathway analysis (IPA) software (Ingenuity Systems, Redwood City, CA). For our investigations, input data were limited to peptides that showed consistent responses across the biological replicates ($p<0.05$) as well as statistically significant changes between the wildtype and ΔEnv HIV conditions to identify biological pathways that are specifically induced through Env:CD4 interactions. In Figure 6E, we used the IPA regulation z-score algorithm to identify biological functions that were above a z-score >1 (black bars), and p-values (red line), calculated with the Fischer's exact test, reflects the likelihood that the association between a set of active kinases and a biological function is significant [p-value < 0.05].

MATERIAL AVAILABILITY

HIV reporter plasmids generated in this study will be available to researchers upon request.

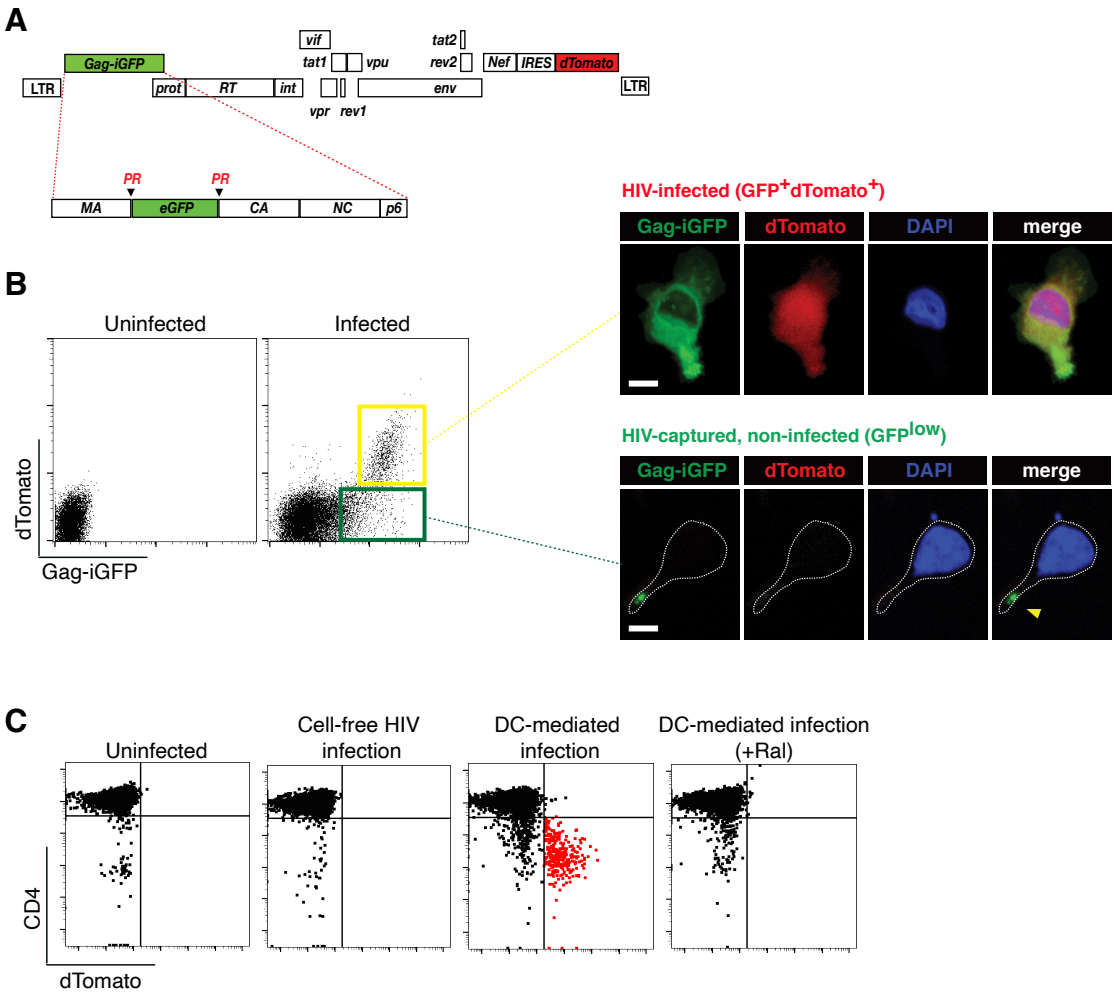
QUANTIFICATION AND STATISTICAL ANALYSIS

Unpaired Student's t test and Mann-Whitney U test were used for comparisons of datasets with normal and non-normal distribution, respectively, using Prism 5 (GraphPad). Median and p

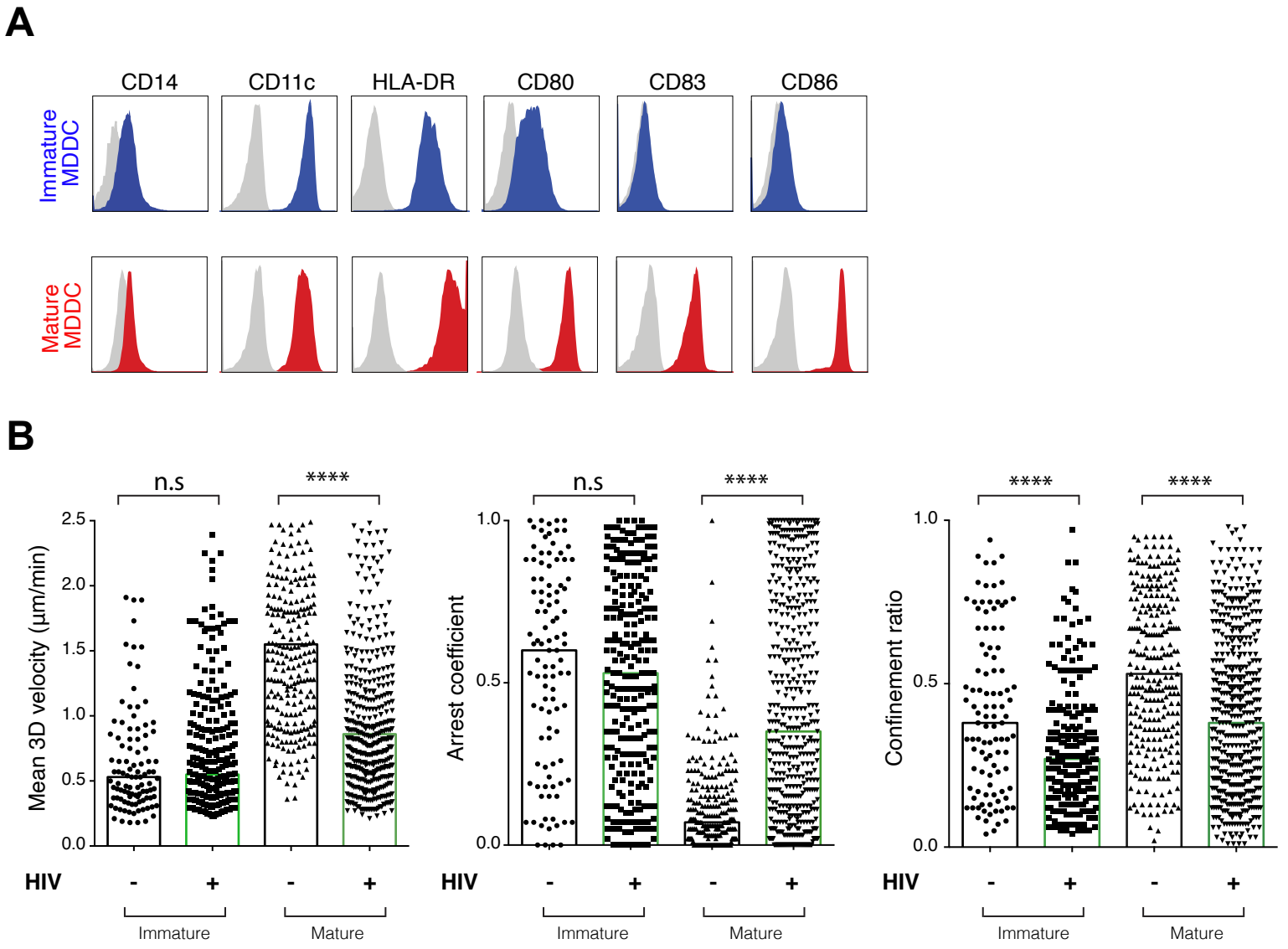
values from statistical analyses are indicated in each graph. When p values were higher than 0.05, differences were considered as not significant.

DATA AND SOFTWARE AVAILABILITY

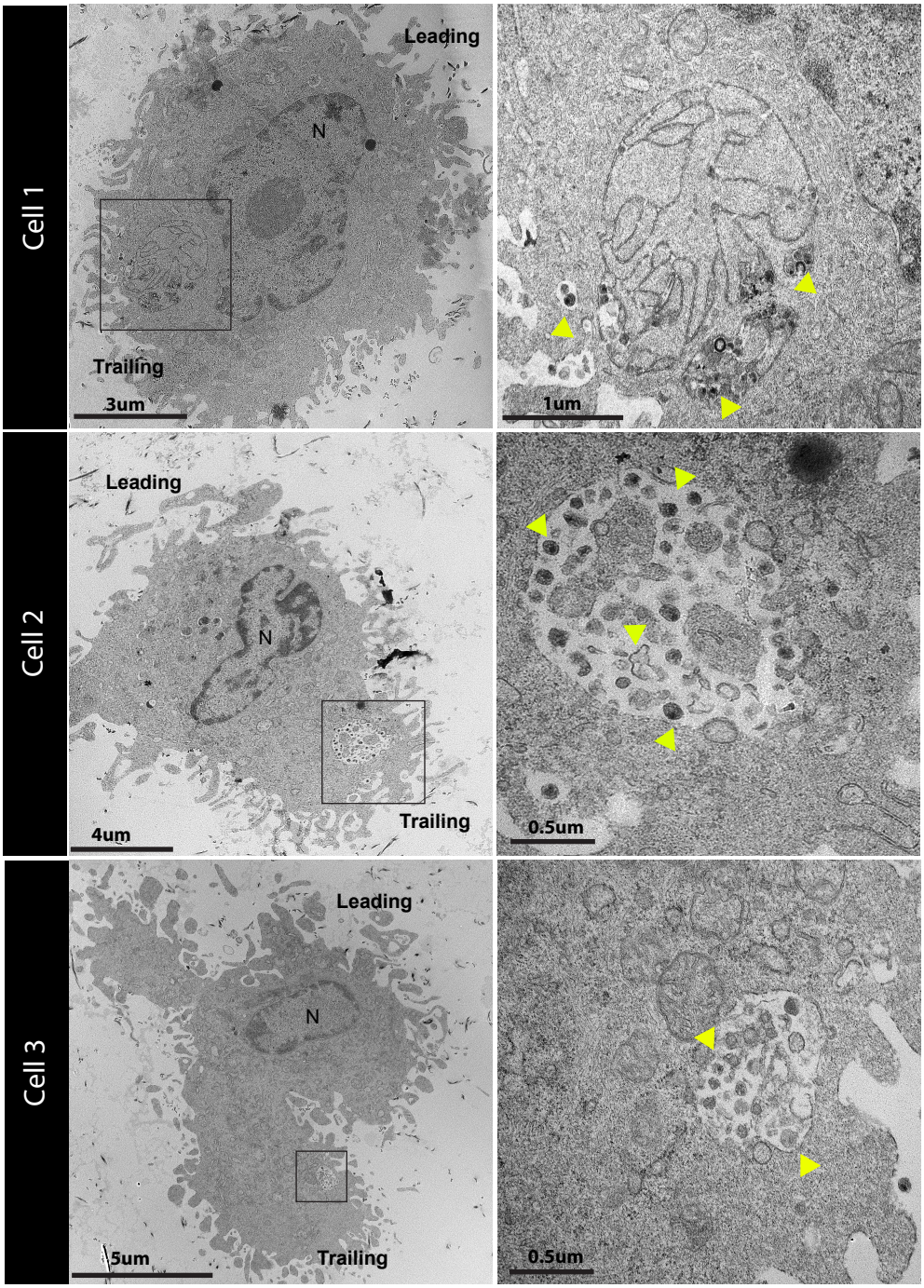
MATLAB cell motility analysis scripts will be made available upon request. Motility parameters were calculated as described by Beltman et al. (2009). Briefly, mean velocities were defined as the average of the distance a cell traveled over the time period between the two consecutive frames. Confinement ratio is calculated based as the ratio of the displacement a cell has traveled over the total length of the path that cell has travelled. Figures and illustrations were prepared using Adobe® Illustrator and BioRender.com.



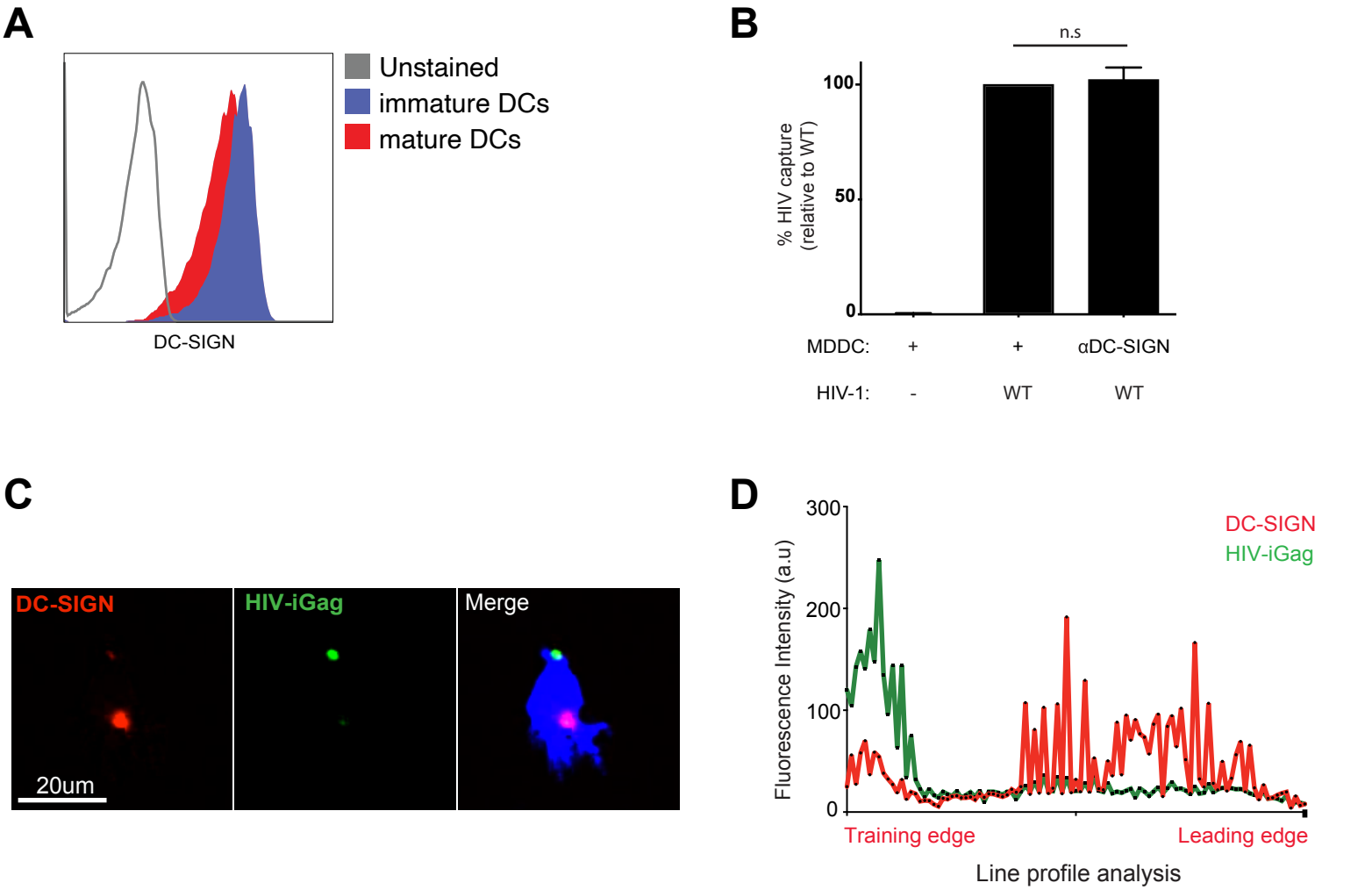
Supplementary Figure 1: Schematic and validation of the HIV Gag-iGFP/dTomato reporter, Related to Figure 1 and 4. (A) Schematic illustration of the reporter proviral vector. PR = protease cleavage sites. **(B)** T cells were infected with HIV-iGFP and characterized by flow cytometry and confocal microscopy. **(C)** T cells were infected with HIV-iGFP in the presence or absence of DCs, and assessed for infection after 48 hours. Raltegravir was added at the time of infection to serve as control. The presence of DCs enhanced T cell infection, and productively infected dTomato+ T cells (red dots) downregulate CD4 expression, as expected. Representative data from 3 independent experiments are shown.



Supplementary Figure 2: 3D migration behavior of mature DCs after HIV exposure, Related to Figure 1.
(A) Phenotypic analysis of monocyte-derived dendritic cells (MDDCs) before and after stimulation with LPS for 24 hours. **(B)** Immature or mature DCs were either left alone or exposed to HIV-iGFP for 4 hours, washed, and embedded into collagen chamber for live-cell microscopy studies. Each data point represents a single cell. Bar graph indicate median values. Cumulative data from 3 independent experiments are shown. n.s. = not significant. **** $p \leq 0.001$



Supplementary Figure 3: Transmission electron micrograph of polarized HIV-captured DCs in collagen matrix, Related to Figure 2. Mature HIV particles are found in membrane invaginations close to the cell surface (yellow arrowheads in enlarged insets). The leading and trailiing edge are indicated, based on the location of the nucleus (N).



Supplementary Figure 4: DC-SIGN does not facilitate HIV capture by motile DCs in 3D collagen, Related to Figure 3. (A) Phenotypic expression of DC-SIGN between immature and mature MDDCs. (B)% HIV capture by DCs, relative to DCs treated with vehicle prior to incubation with virus. Representative data from two independent expeirments is shown. Mean +/- SEM. n.s. = not significant. (C) Representative micrograph of a HIV-captured DC in collagen matrix stained for DC-SIGN. (D) Representative line profile analysis of HIV-Gag-iGFP and DC-SIGN fluorescence intensity in a polarized HIV-captured DC shown in (B) (n=13).