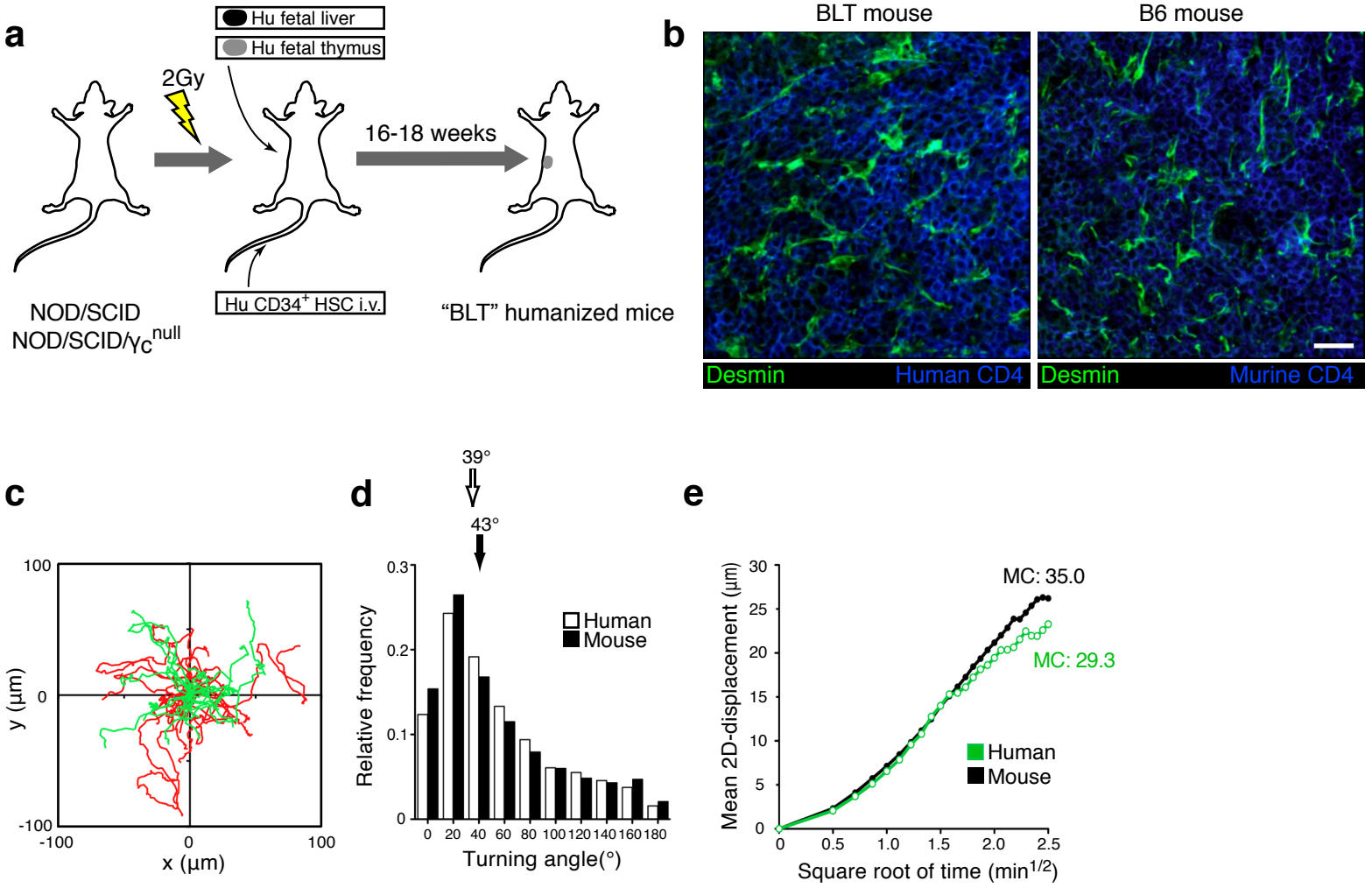
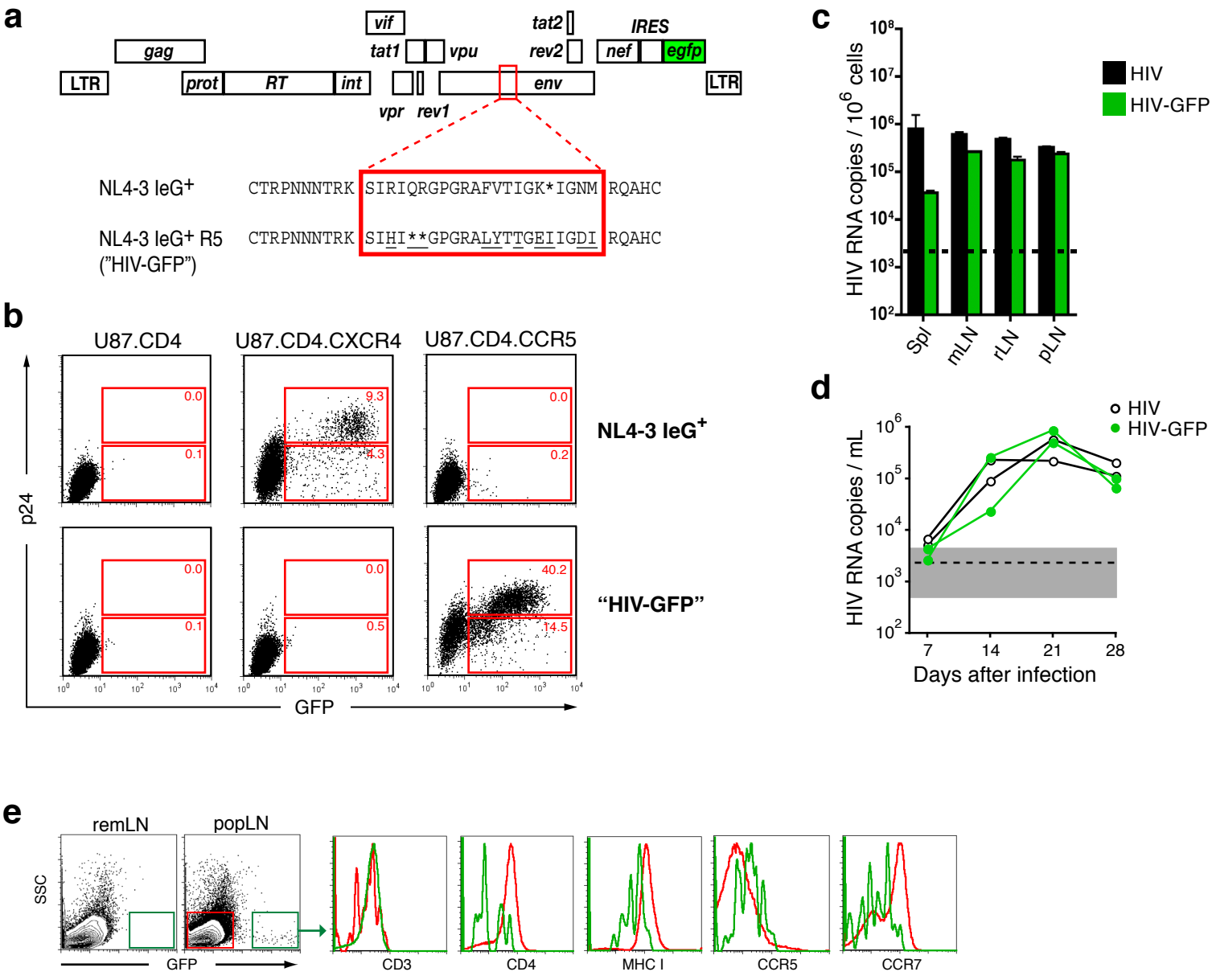
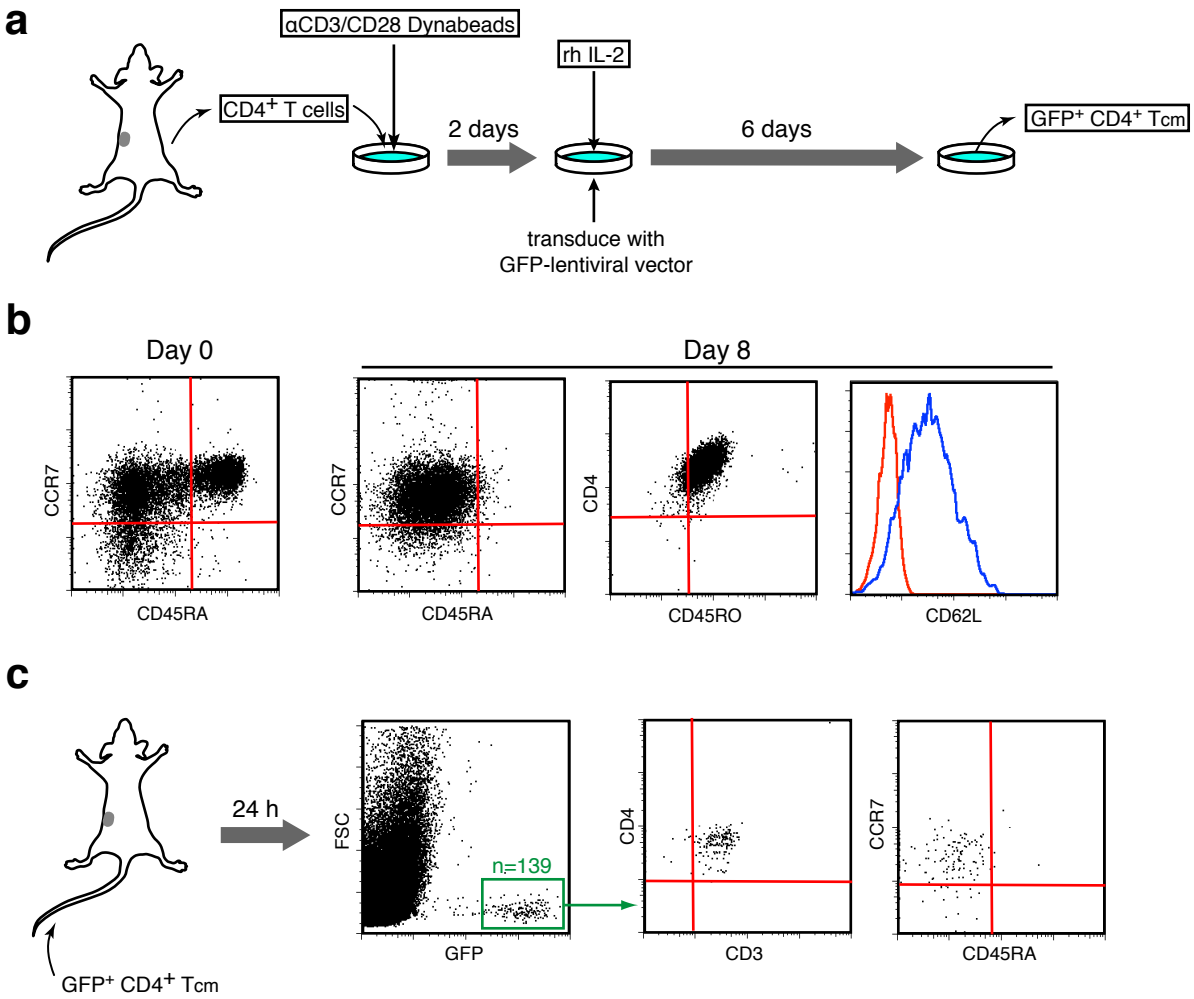


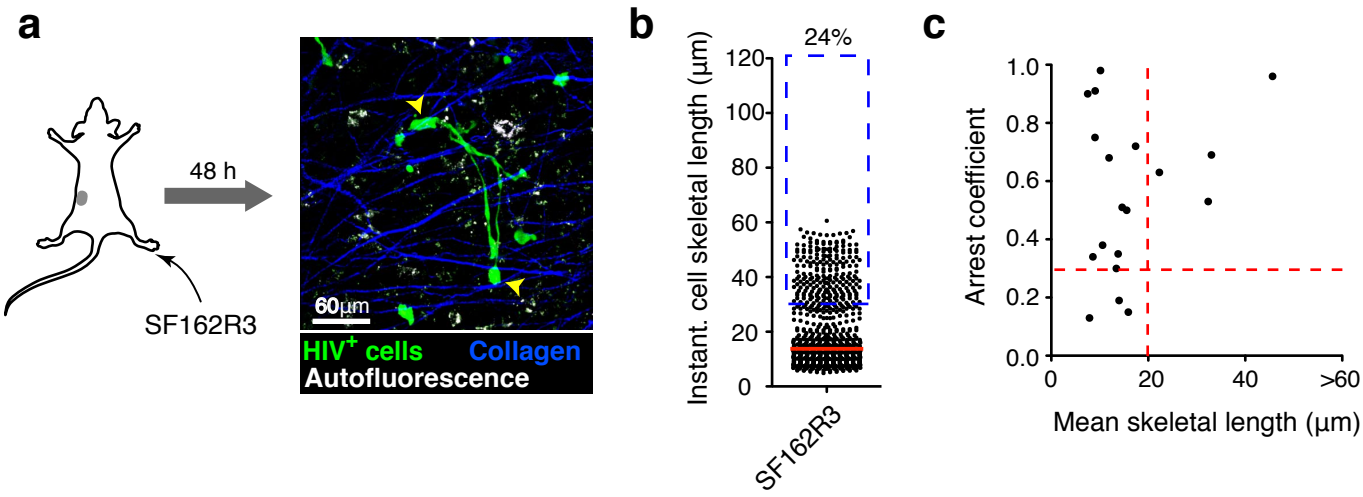
Supplementary figure 1: Human T cell migration in BLT lymph nodes. a. Generation of BLT humanized mice through transplantation of human fetal thymus and fetal liver tissue under the kidney capsule and infusion of hemato-poeitic stem cells (HSC) in sublethally irradiated NOD/SCID or NOD/SCIDγ_c^{null} mice. **b.** Popliteal LN sections of BLT and C57BL/6 mice were stained with anti-Desmin and anti-human or anti-mouse CD4 antibodies to delineate the paracortical stromal network and assess the density of $CD4^+$ T cells. Scale bar = 25 µm. **c.** Tracks of human (green) and mouse (red) T cells migrating in a BLT lymph node, normalized to their starting positions. **d.** Frequency distribution of human and mouse T cell turning angles. **e.** Mean displacement plots of human and mouse T cells. Numbers indicate T cell motility coefficients (MC). Arrows in (d) indicate medians.



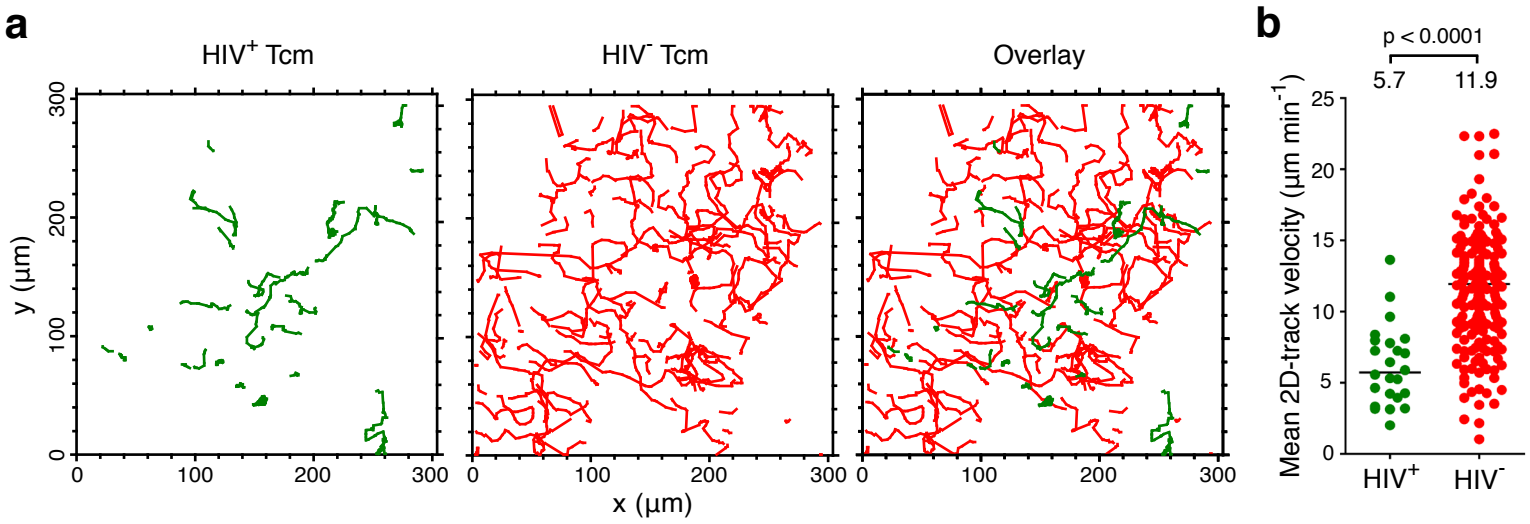
Supplementary Figure 2: Structure and tropism of the R5-tropic GFP-expressing reporter HIV. **a.** The V3 loop region of *env* of pNL4-3 IeG+ was mutagenized to the corresponding sequence of BaL (red box). **b.** GFP and p24 expression after infection of CXCR4- or CCR5-expressing U87 cell lines with R5-tropic HIV-GFP or the parental NL4-3 IeG+ (MOI = 0.5). **c.** BLT mice were infected by footpad injection with 4 x 10^4 infectious units of either HIV-GFP or HIV lacking GFP. After 45 days, spleen and lymph nodes were harvested and analyzed for HIV RNA by RT-qPCR. Data are normalized to 10^6 cell equivalents as determined by GAPDH expression. Values are means±s.e.m. Detection limit for the assay is 2.3 x 10^3 copies/mL (dotted line). Spl = spleen, mLN = mesenteric LN, rLN = remote LN, popLN = popliteal LN. **d.** BLT mice were infected by intraperitoneal injection of 4 x 10^4 infectious units of either HIV-GFP or HIV lacking GFP and plasma was analyzed for HIV RNA by RT-qPCR for 4 weeks at weekly intervals. Dashed line and grey-shaded area indicate mean and 95% confidence interval of background signal from uninfected BLT mice. **e.** Draining popliteal and remote (axillary) LNs were analyzed for the presence of GFP^+ cells 6 days after footpad infection with HIV-GFP. Expression of various markers by GFP^+ cells is shown in comparison to $CD3^+$ $CD4^+$ uninfected T cells.



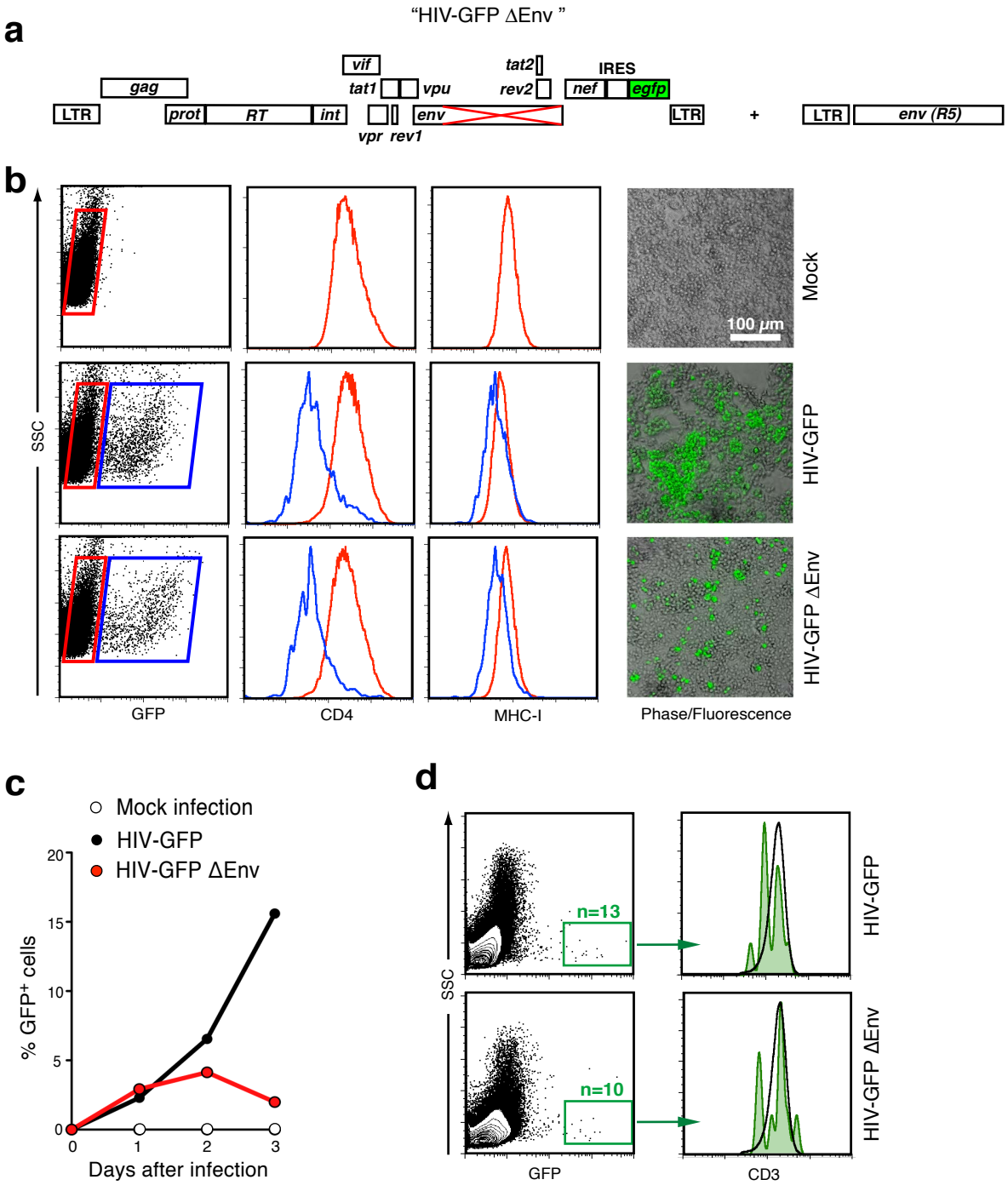
Supplementary Figure 3: Motility of in vitro generated central memory-like T cells (Tcm) in lymph nodes. a. Purified CD4+ T cells from spleen and LN of BLT mice are activated with dynabeads coated with anti-CD3/CD28 mAbs, transduced with VSV-G-pseudotyped, GFP-expressing lentivirus, and expanded with recombinant human IL-2. **b.** CD4+ T cells from either day 0 or 8 were analyzed for cell surface markers. Tcm were CD45RA$^{neg/low}$, CCR7+, CD62L+ at day 8. Red histogram represents unstained control. **c.** 5 x 10^6 GFP+ Tcm were adoptively transfered into BLT mice by tail vein injection. After 24 hours, the spleen and lymph node cells were analyzed for expression of GFP, CD3, CD4, CD45RA and CCR7. **d.** Frequency distribution of turning angles of in situ infected GFP+ LN cells and uninfected Tcm in the LN of a BLT mouse. **e.** Mean displacement (MD) plots of control GFP+ LN cells and Tcm. Numbers indicate T cell motility coefficients (MC) derived from the linear segment of the MD plot. Arrows in (d) indicate medians.



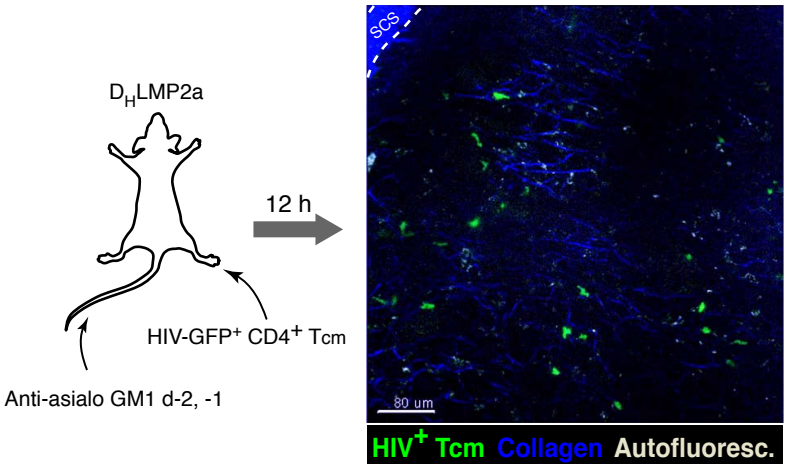
Supplementary Figure 4: Infection with the primary R5-tropic HIV-1 clone SF162R3 induces an elongated phenotype *in vivo*. a. Mice were footpad injected with 1.2 x 10^5 infectious units of SF162R3, a clone from a primary HIV isolate modified to express GFP. A representative intravital micrograph of two elongated cells recorded two days after infection is shown (yellow arrows). **b.** Instantaneous cell skeletal length of HIV-infected LN cells. Red line indicates median. Percentages refer to cells >30 µm, highlighted by dashed blue box. **c.** Relationship between the mean cell skeletal length and arrest coefficient of individual cells. Dashed lines indicate threshold values based on measurements of uninfected Tcm.



Supplementary Figure 5: Distribution and motility of HIV-infected and uninfected Tcm in LNs. **a.** HIV-GFP-infected and uninfected, CMTMR-labeled Tcm were adoptively transferred via the footpad and 12 hours later their migration was recorded by MP-IVM in the LN cortex. **a**. Tracks of infected and uninfected Tcm recorded from 125-165 µm below the LN capsule. **b.** Corresponding Mean 2D-track velocities. Numbers represent medians.

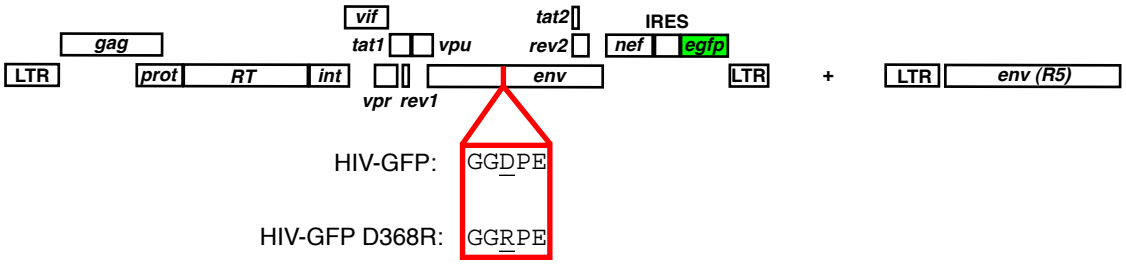


Supplementary Figure 6: Construction and validation of the HIV-GFP ΔEnv reporter virus. a. A premature STOP codon in the *env* ORF of HIV-GFP leads to the production of a non-functional Env. To produce virus capable of one round of infection, HIV-GFP ΔEnv was packaged with an exogenous intact Env. **b.** MAGI.CCR5 cells were infected with either HIV-GFP or HIV-GFP ΔEnv at an MOI of 0.5. Cells were analyzed by flow cytometry for expression of GFP, CD4 and MHC-I after 24 hours, and by fluorescence microscopy for expression of GFP, 3 days after infection. The absence of colony formation with HIV-GFP ΔEnv suggested the lack of contact-dependent spread. **c.** Quantification of data in (b) by flow cytometry. Note the plateau of infectious spread of HIV-GFP ΔEnv after day 1, indicating lack of secondary infections. **d.** HIV-GFP and HIV-GFP ΔEnv were injected into the right and left footpads of BLT mice, respectively, and GFP^+ LN cells were analyzed after 48 hours.



Supplementary Figure 7: HIV-infected Tcm do not elongate in murine LNs. HIV-GFP-infected central memory human T cells were injected into the footpad of D_HLMP2A mice, in which B cells do not express cell-surface B cell receptors and can not secrete antibodies, that were depleted of NK cells through 2 injections of anti-asialo GM1 antibody to prevent rapid rejection of transfered xenogeneic T cells. 12 hours later, the draining popliteal LNs were prepared for MP-IVM analysis of transferred T cells. SCS: Subcapsular sinus

a



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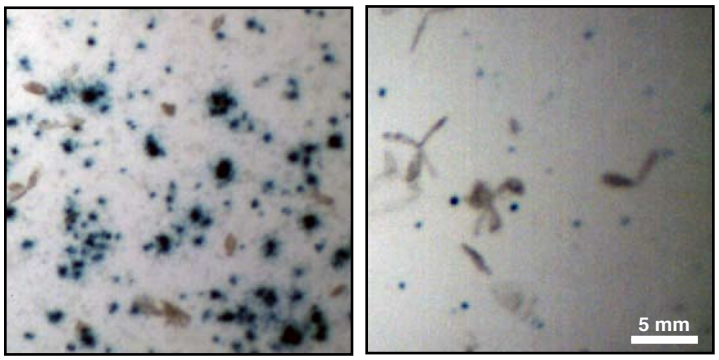
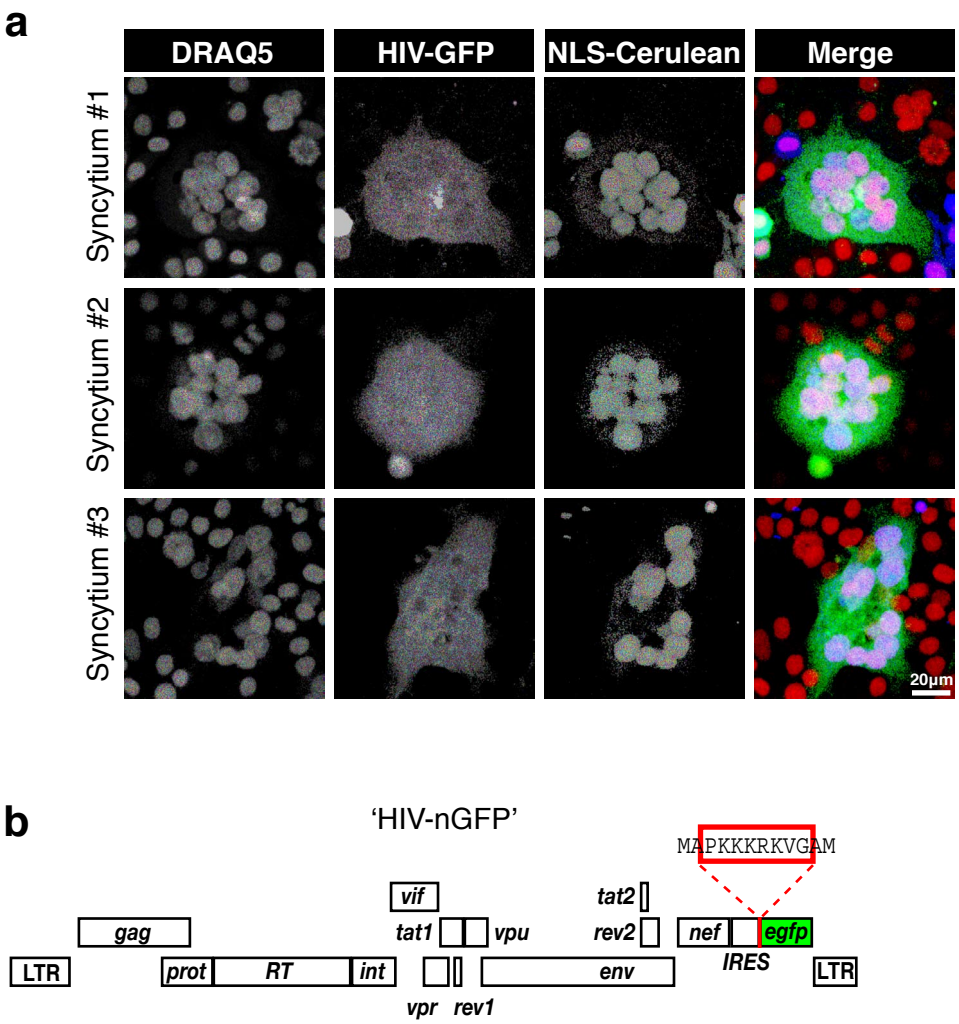
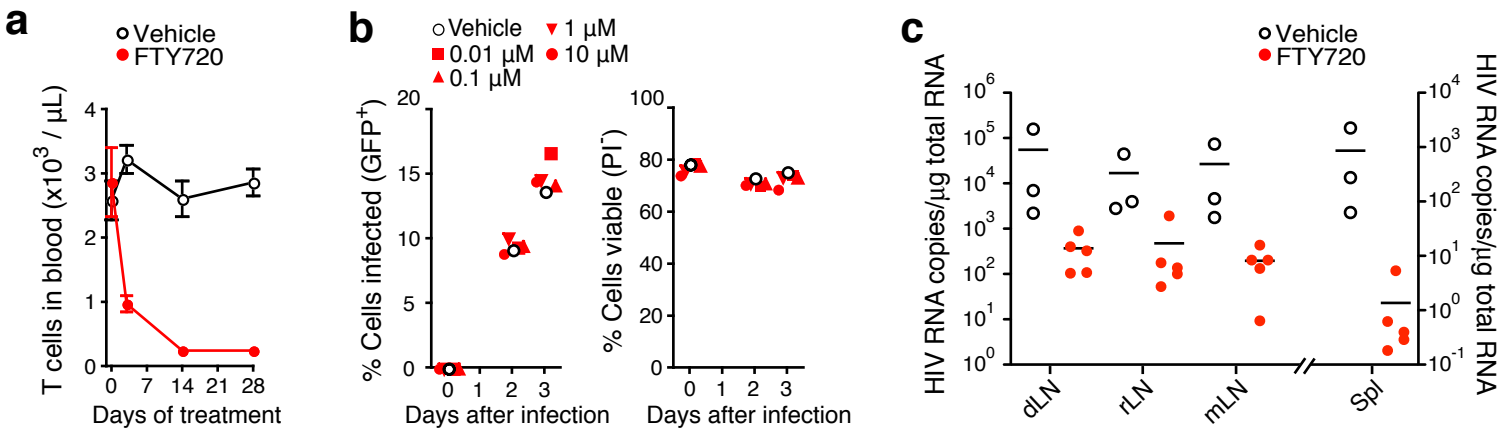


HIV-GFP HIV-GFP D368R

Supplementary Figure 8: Construction of the HIV-GFP D368R reporter virus. a. A single amino acid mutation in Env of HIV-GFP (D368R according to the HXB2 numbering system) abrogates CD4-binding capacity of Env. To produce virus capable of one round of infection, HIV-GFP D368R was packaged with an exogenous intact Env. **b.** MAGI.CCR5 cells with an LTR-driven β-galactosidase expression casette were infected with supernatants of either HIV-GFP or HIV-GFP D368R. After 48 hours, infected cells were visualized through addition of X-Gal substrate (blue color). Note that while HIV-GFP forms clusters of infected cells, HIV-GFP D368R forms only one cell colonies, indicating a lack of secondary infections due to the lack of CD4-binding by the mutant Env expressed by primary infected cells.



Supplementary Figure 9: HIV-nGFP highlights all nuclei in HIV-induced syncytia. a. To determine if fluorescent proteins tagged with the nuclear localization signal (NLS) of SV40 are targeted to all nuclei in HIV-induced syncytia, we stably transfected MAGI.CCR5 cells with NLS-Cerulean (blue), infected them with HIV-GFP (green) for 5 hours, washed, mixed with uninfected MAGI.CCR5 at a 1:15 ratio and plated onto glass slides. After 36 hours, cells were stained with the DNA dye DRAQ5 (red), fixed, and mounted for fluorescence microscopy. Three representative micrographs illustrate the enrichment of NLS-Cerulean in all nuclei of HIV-infected syncytia. **b.** The NLS of SV40 was inserted upstream of GFP in HIV-GFP to generate HIV-nGFP.



Supplementary Figure 10: No effect of FTY720 on viral replication *in vitro* and effect of FTY720 on tissue concentration of HIV RNA after *in vivo* treatment. **a.** To test whether FTY720, which requires phosphorylation by sphingosine kinases for its activity, is active in chimeric BLT mice, we treated animals with 1 mg/kg BW FTY720 or vehicle per i.p. injection every other day and monitored the number of T cells in peripheral blood weekly. Values are means±s.e.m. **b.** MAGI cells were treated with different doses of phophorylated FTY720 and infected with HIV-GFP. Before, 2 and 3 days after infection, the fraction of GFP-expressing cells was monitored by flow cytometry (left graph). At the same times, cell viablility was monitored by exclusion of propidium iodide (PI, right graph). **c.** Tissue viral RNA loads in BLT mice 8-9 weeks after infection with HIV-GFP and treatment with FTY720 or vehicle starting at the day of infection. dLN: draining LNs, rLN: remote, non-draining LNs; mLN: mesenteric LNs, Spl: spleen

SUPPLEMENTARY VIDEO GUIDE:

Video 1: Migration of human and mouse T cells in the BLT lymph node

Video 2: Migration of *in situ* HIV-infected cells in the BLT lymph node

Video 3: Migration of non-infected, central memory-like human $CD4^+$ T cells

Video 4: Representative *in situ* HIV-infected LN cells with elongated phenotypes

Video 5: Migration of HIV-infected LN cells after *in situ* infection with SF162R3

Video 6: Footpad-injected HIV-infected Tcm cells display an elongated phenotype

Video 7: Elongated cells are not observed after infection of BLT mice with an CCR5-using, GFP-expressing lentiviral vector

Video 8: Elongation and retraction of HIV^+ T cell tethers

Video 9: Migration of HIV-GFP or HIV-GFP ΔEnv-infected cells in LNs.

Video 10: HIV-infected T cells form syncytia

Video 11: HIV-infected T cells also form tethers independent of dyssynchronous nuclear movement.

SUPPLEMENTARY VIDEO LEGENDS:

Supplementary Video 1: Migration of human and mouse T cells in the BLT lymph node. Human (green) and mouse (red) T cells were adoptively transferred into BLT mice by i.v. injection and the right popliteal lymph node (popLN) prepared for IVM after 24 hours. Each individual frame is a maximum intensity projection of 11 z-stacks spaced 5 μm apart (total thickness of 50μm). Scale bar = 100μm. Time is shown in minutes and seconds. Events are accelerated 360x over real-time.

Supplementary Video 2: Migration of *in situ* HIV-infected cells in the BLT lymph node. HIV-GFP was injected into the footpad and the popLN prepared for IVM after 2 days. Tissue autofluorescence is shown in white, second harmonic signals are shown in blue. Each individual frame is a maximum intensity projection of 11 z-stacks spaced 4 μm apart (total thickness of 40 μm). Scale bar = 70 μm. Time is shown in minutes and seconds. Events are accelerated 360x over real-time.

Supplementary Video 3: Migration of non-infected, central memory-like human $CD4^+$ T cells in a BLT mouse LN. 3.5×10^6 in vitro generated Tcm (green) were injected intravenously into an uninfected BLT mouse and a popliteal LN imaged by MP-IVM after 12 hours. Each individual frame is a maximum intensity projection of 11 z-stacks spaced 4 μm apart (total thickness of 40 μm). Scale bar = 40 μm. Time is shown in minutes and seconds. Events are accelerated 360x over real-time.

Supplementary Video 4: Representative *in situ* HIV-infected LN cells displaying elongated phenotypes. HIV-GFP was injected into the footpad and the popLN prepared for MP-IVM 2 days later. 6 GFP^+ cells (green) with an elongated morphology are shown, in comparison to an uninfected, GFP-expressing Tcm in the bottom right panel. For one elongated cell, 180° vertical rotational views and 3D surface renderings are also shown. In some experiments, mice were administered Hoechst 33342 (blue) by i.v. injection. Collagen fibers are visualized through second harmonic generation (SHG, blue) Immotile, purple fluorescent objects in some movies are auto-fluorescence tissue structures. Each individual frame is a maximum intensity projection of 11 z-stacks spaced

4 μm apart (total thickness of 40 μm). Scale bar = 40μm. Time is shown in minutes and seconds. Events are accelerated 360x over real-time.

Supplementary Video 5: Migration of HIV-infected LN cells after *in situ* infection with SF162R3. SF162R3 was injected into the footpad and the popLN prepared for IVM after 2 days. Tissue autofluorescence is shown in white, second harmonic signals are shown in blue. Each individual frame is a maximum intensity projection of 11 z-stacks spaced 4 μm apart (total thickness of 40 μm). Scale bar = 60 μm. Time is shown in minutes and seconds. Events are accelerated 360x over real-time.

Supplementary Video 6: Footpad-injected HIV-infected Tcm cells display an elongated phenotype in draining popliteal LNs. HIV-GFP-infected Tcm (green) and uninfected Tcm (red) were adoptively transferred into BLT mice by footpad injection into BLT mice pretreated with antiretroviral drugs. After 12 hours, the draining popLN was prepared for IVM. Collagen fibers are visualized by SHG (blue) and auto-fluorescence is depicted in white. Each individual frame is a maximum intensity projection of 11 z-stacks spaced 4 μm apart (total thickness of 40 μm). Scale bar = 40 μm. Time is shown in minutes and seconds. Events are accelerated 360x over real-time.

Supplementary Video 7: Elongated cells are not observed after infection of BLT mice with a CCR5-using, GFP-expressing lentiviral vector. Mice were injected into the footpad with R5 LV-GFP, and the popLN prepared for IVM after 2 days. Each individual frame is a maximum intensity projection of 11 z-stacks spaced 4 μm apart

(total thickness of 40 μm). Scale bar = 40 μm. Time is shown in minutes and seconds. Events are accelerated 360x over real-time.

Supplementary Video 8: Elongation and retraction of HIV$^+$ LN cell tethers. HIV-GFP was injected into the footpad and the popliteal LN prepared for MP-IVM 2 days later. Two representative recordings of tethering infected cells (green). In the left panel, the trailing edge of a migrating T cell (cell body highlighted with red dot, point of tethering with white circle) becomes tethered to a non-visualized structure, leading to an elongation of its uropod. In the right panel, an elongated trailing edge (red circle) suddenly retracts, suggesting release of a tethering interaction. The cell body positions of the cell are marked with a red dot. Each individual frame is a maximum intensity projection of 11 z-stacks spaced 4 μm apart (total thickness of 40 μm). Scale bar = 30 μm. Time is shown in minutes and seconds. Events are accelerated 360x over real-time.

Supplementary Video 9: Migration of HIV-GFP or HIV-GFP ΔEnv-infected cells in LNs. BLT mice were injected with equal amounts of HIV-GFP ΔEnv (right footpad) and HIV-GFP (left footpad). Both popLNs were prepared for IVM at day 2. Collagen fibers are visualized by SHG (blue) and auto-fluorescence is depicted in white. Each individual frame is a maximum intensity projection of 11 z-stacks spaced 4 μm apart (total thickness of 40 μm). Scale bar = 40 μm. Time is shown in minutes and seconds. Events are accelerated 360x over real-time.

Supplementary Video 10: HIV-infected T cells form syncytia, which develop long cell tethers as a result of intermittent dyssynchnronous movement of individual nuclei. BLT mice were injected in the footpad with HIV-nGFP, where GFP is highly enriched in cellular nuclei, and prepared for IVM at day 2. Two representative syncytia are shown (top and bottom row). For the right hand panels, green fluorescence signals above 80% of the intensity maximum was used to define cell nuclei, which are shown in pure red. Collagen fibers are visualized by SHG (blue) and auto-fluorescence is depicted in white. Each individual frame is a maximum intensity projection of 11 z-stacks spaced 4 μm apart (total thickness of 40 μm). Scale bar = 40 μm. Time is shown in minutes and seconds. Events are accelerated 360x over real-time.

Supplementary Video 11: HIV-infected T cells also form tethers independent of dyssynchronous nuclear movement. BLT mice were injected in the footpad with HIV-nGFP, where GFP is highly enriched in cellular nuclei, and prepared for IVM at day 2. The circle highlights the tip of a tether, which is slowly retracted, but that is not associated with a nucleus in this multinucleated syncytium. For the right hand panel, green fluorescence signals above 80% of the intensity maximum was used to define cell nuclei, which are shown in pure red. Collagen fibers are visualized by SHG (blue) and auto-fluorescence is depicted in white. Each individual frame is a maximum intensity projection of 11 z-stacks spaced 4 μm apart (total thickness of 40 μm). Scale bar = 40 μm. Time is shown in minutes and seconds. Events are accelerated 360x over real-time.