HIV-1 Nef interferes with T-lymphocyte circulation through confined environments in vivo

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Edited by Ronald N. Germain, National Institutes of Health, Bethesda, MD, and accepted by the Editorial Board October 1, 2012 (received for review March 14, 2012)

HIV-1 negative factor (Nef) elevates virus replication and contributes to immune evasion in vivo. As one of its established in vitro activities, Nef interferes with T-lymphocyte chemotaxis by reducing host cell actin dynamics. To explore Nef’s influence on in vivo recirculation of T lymphocytes, we assessed lymph-node homing of Nef-expressing primary murine lymphocytes and found a drastic impairment in homing to peripheral lymph nodes. Intravital imaging and 3D immunofluorescence reconstruction of lymph nodes revealed that Nef potently impaired T-lymphocyte extravasation through high endothelial venules and reduced subsequent parenchymal motility. Ex vivo analyses of transendothelial migration revealed that Nef disrupted T-lymphocyte polarization and interfered with diapedesis and migration in the narrow subendothelial space. Consistently, Nef specifically affected T-lymphocyte motility modes used in dense environments that pose high physical barriers to migration. Mechanistically, inhibition of lymph node homing, subendothelial migration and cell polarization, but not diapedesis, depended on Nef’s ability to inhibit host cell actin remodeling. Nef-mediated interference with in vivo recirculation of T lymphocytes may compromise T-cell help and thus represents an important mechanism for its function as a HIV pathogenicity factor.

Results

HIV-1 Nef Is Functional Following Retroviral Transduction and Isolation of Primary Murine T Lymphocytes. To investigate whether the inhibition of T-lymphocyte motility observed in vitro is relevant for Nef’s role in HIV pathogenesis, we established an experimental strategy that mimics aspects of acute HIV-1 infection by expression of Nef in murine T lymphocytes for subsequent adoptive transfer and functional characterization of their homing behavior (Fig. S1). Expressed upon optimized transduction with murine leukemia virus vectors (20), Nef.GFP displayed full biological activity in primary mouse T lymphocytes, including cell-surface receptor down-modulation, inhibition of chemokine-induced actin remodeling (2), induction of high levels of phosphorylated (inactive) coflin (5), targeting of Lck kinase to intracellular membrane compartments (21), and chemotaxis through 3- and 5-μm transwell membranes (22) (Fig. S2). Similar to human cells (5), the PAK2-association deficient Nef mutant F195A did not interfere with actin remodeling and host cell motility in transduced primary murine T lymphocytes. Similar results were obtained following magnetic bead selection of (>95% NGFR+) primary mouse T lymphocytes transduced with IRESΔNGFR versions of these expression constructs encoding for NGFR with cytoplasmic tail deletion and nonfusion Nef. Nef

T-lymphocyte homing | two photon intravitral microscopy

The Nef protein of HIV and simian immunodeficiency viruses promotes viral replication in vivo and rapid progression to AIDS. Numerous in vitro functions mediated by specific protein–protein interactions were assigned to Nef by which the viral protein affects host cell intracellular transport and signal transduction (1). How these functions individually contribute to the prominent role of Nef in AIDS pathogenesis remains to be established. Inhibition of dynamic host cell actin remodeling, mediated via association with the cellular kinase PAK2 that induces inactivation of the actin-severing factor coflin to reduce actin turnover, represents a conserved activity of lentiviral Nef proteins (2–8). Nef-PAK2 association depends on a critical phenylalanine at position 195 of Nef (or 191 depending on the HIV-1 nef allele analyzed) that is dispensable for other Nef activities (9). Disruption of host cell actin remodeling by Nef does not affect intrinsic replication properties of HIV-1 (10, 11) but Nef impairs T-lymphocyte chemotaxis in vitro (12–14). Whether Nef also affects T-lymphocyte motility in mammals, which is only in part driven by chemokines (15), has not yet been addressed.

Physiologically, T lymphocytes continuously traffic between blood and secondary lymphoid organs. For entry into lymph nodes, T lymphocytes use CD62L to transiently attach to high endothelial venules (HEV) that pass through the T-cell areas of lymph nodes. Subsequently, T lymphocytes are activated by the surface-binding chemokine CCL-21, which through its receptor CCR7 triggers lymphocyte function-associated antigen 1-dependent shear-resistant firm arrest. Before diapedesis, T lymphocytes acquire a polarized cell shape and actively migrate on the luminal surface of HEVs, presumably to find an appropriate egress site (16). After diapedesis, lymphocytes remain attached for a few minutes in the perivascular space before being finally released into the underlying parenchyma (16, 17). The subendothelial migration away from transmigration sites into the underlying tissue represents the last critical step and allows actual entry of T lymphocytes into the T-cell area of lymphoid organs (16, 18). Within the T-cell area, T lymphocytes crawl with an average speed of 15 μm/min along the 3D network of fibroblastic reticular cells and screen antigen-presenting cells for antigens. Notably, HEVs are tightly surrounded by matrix but the lymph-node parenchyma is less tightly packed with fibers as migration tracks, indicating that T lymphocytes face microenvironments with different densities during these migration steps (19). In the absence of cognate antigens, T lymphocytes exit lymph nodes within 12–24 h. Repetitive homing to and egress from lymph nodes thus requires active migration of T lymphocytes and represents a prerequisite for proper immune surveillance and screening of rare antigen-specific T lymphocytes for cognate antigen (18).

In this study we use a combination of in vivo and ex vivo model systems to quantify and visualize the effects of HIV-1 Nef on physiological circulation of T lymphocytes.
expression levels were identical to those in HIV-1 infected human T lymphocytes (Fig. S3). Our transduction/isolation strategy thus allows for functional analysis of homogenously Nef-expressing primary mouse T lymphocytes in which Nef exerts its activities in analogy to HIV-1 infection of primary human T lymphocytes.

HIV-1 Nef Efficiently Interferes with T-Lymphocyte Homing to Lymph Nodes. Next, transduced and sorted cell populations were labeled with CellTracker dyes ex vivo for subsequent detection and adoptively transferred into recipient mice to assess whether expression of WT or F195A Nef influences T-lymphocyte trafficking in vivo. Four and 24 h posttransfer, spleen, and peripheral (pLN) and mesenteric (mLN) lymph nodes were harvested and analyzed independently for homing efficiency of adoptively transferred cells by flow cytometry. As an internal homing control, cells expressing the empty vector were cotransferred together with equal amounts of WT or F195A Nef-expressing cells. Nef expression only slightly affected homing to spleen (Fig. 1A) (31 ± 31% and 34 ± 23% reduced homing compared with control at 4 h and 24 h, respectively), a process that does not require the crossing of an endothelial barrier (18). In contrast, Nef caused a marked reduction of T-lymphocyte homing to the pLN (Fig. 1B) and mLN (Fig. 1C). This inhibition was observed at the early 4 h homing time-point (75 ± 13% and 63 ± 20% reduced homing compared with control cells for pLNs and mLNs, respectively) as well as after 24 h when equilibrium between homing and egress is reached (62 ± 6% and 64 ± 5% reduced homing compared with control cells for pLNs and mLNs, respectively). Nef F195A was partially defective in blocking lymph node homing at the 4 h time-point. However, 24 h posttransfer, similar amounts of control and Nef F195A-expressing cells were observed in secondary lymphoid organs. Because our transduction strategy did not distinguish between CD4+ and CD8+ T lymphocytes, and only CD4+ cells are physiological targets of HIV-1 infection, homing of both populations was also assessed separately. Notably, homing of in vitro mitogen-stimulated CD4+ T lymphocytes was about fourfold less efficient compared with homing of CD4+ T lymphocytes, and homing to spleen and lymph nodes of CD4+ T lymphocyte was more potently blocked by Nef than that of CD8+ T lymphocytes (Fig. S4). In contrast to the situation in CD4+ T lymphocytes, the moderate effects of Nef on homing of CD8+ T lymphocytes did not depend on the F195 motif. HIV-1 Nef thus efficiently interferes with homeostatic trafficking of CD4+ T lymphocytes in vivo.

HIV-1 Nef Interferes with T-Lymphocyte Transmigration Under Shear. We next asked at which specific step Nef interferes with T lymphocyte trafficking during the homing process. Because transmigration across the endothelial cells lining HEVs constitutes a critical step in the homing process, we first addressed whether Nef affects crawling and diapedesis through an endothelial cell monolayer under shear flow (23, 24). Transduced and purified T lymphocytes were differentially labeled with CellTracker dyes in vitro, mixed, and perfused over a CCL-21-coated primary brain microvascular endothelial cell monolayer to image the recruitment process by video microscopy over 50 min (Fig. 24 and Movie S1). Frame-by-frame offline analysis of transendothelial migration (TEM) under shear flow enabled us to dissect which step of the TEM process is influenced by Nef. T lymphocytes were allowed to accumulate on the endothelium at low shear stress (0.15 dyn/cm²) and after the accumulation phase flow was increased to physiological shear stress (1.5 dyn/cm²). TEM is initiated by capture of the T lymphocytes to the endothelium during the accumulation phase, followed by the acquisition of a polarized phenotype and an optional crawling step to find permissive sites for diapedesis (24). In contrast to previous studies that addressed effects of Nef on cell adhesion in the absence of physiological shear flow (8, 14), WT or F195A Nef did not affect the initial accumulation of T cells on the CCL-21-coated endothelium under our shear-flow conditions (Fig. 2B). Most control cells acquired a polarized shape after binding to the endothelial cell layer (Fig. 2C). In contrast, Nef-expressing T cells were significantly less elongated at all time-points analyzed than were control cells. Defects in cell polarization were also observed in the absence of shear flow upon plating of transduced and purified T lymphocytes on fibronectin-coated surfaces and stimulation with CCL-21 to analyze efficient formation of a leading and trailing edge. This disruption of cell polarization by Nef in the absence or presence of shear flow was strictly dependent on its F195 motif (Fig. 2C and D, and Fig. S5A and B), indicating that functional actin remodeling is crucial for proper cell polarization after adhesion.

To assess whether locomotion of T lymphocytes on the endothelium subsequent to firm arrest is affected by Nef, single cells were manually tracked until they successfully transmigrated (Fig. 24, cell marked with the orange arrowhead at 900 s). After adhesion, control T lymphocytes immediately started to transmigrate and only few additional diapedesis events were observed after the first 20 min of observation (Fig. 3B). Because most cells transmigrated nearby the site of their first attachment to the endothelium and thus underwent minimal lateral locomotion before transmigration, Nef only marginally affected lateral locomotion distance and velocity of T lymphocytes before diapedesis (Fig. S6) and consistently did not exert major effects on the activity of the Rac1 GTase that is required for these early events (Fig. S5C) (23, 24). In contrast, Nef-expressing cells showed significantly less diapedesis events and transmigrated with delayed kinetic relative to control cells (Fig. 3 A and B). Quantification of transmigration efficiency revealed a twofold block in diapedesis imposed by Nef (Fig. 3A, “diapedesis”) (71 ± 14 vs. 35 ± 8%). Cells that were not transmigrating remained adhered on the surface of the endothelium during the entire observation period (Fig. 3D, “on top”). Importantly, diapedesis of Nef F195A-expressing cells was equally impaired to that of WT Nef expressing cells and Nef did not disrupt formation of potentially invasive and chemokine-sensing filopodia (25) (Fig. S7). Thus, HIV-1 Nef potently blocks lymphocyte diapedesis via a mechanism that does not involve its ability to interfere with actin remodeling.

Fig. 1. Nef interferes with T-lymphocyte homing to lymph nodes. Primary T lymphocytes were isolated, transduced, and sorted (Fig. S3), labeled with CellTracker dyes, and adoptively transferred into recipient mice. Control cells were always coinjected with Nef WT or F195A-expressing T lymphocytes as an internal control. Spleen (A), pLN (B), and mLN (C) were harvested after 4 or 24 h and single-cell suspensions were analyzed by flow cytometry. The homing ratio in each mouse was calculated relative to coinjected control cells, arbitrarily set to 1 (indicated by the dashed line). Each circle represents data from one animal. P values were calculated performing a Kruskal–Wallis test with a Dunn’s posttest. *P < 0.05; **P < 0.005; ***P < 0.0005.
Fig. 2. Nef interferes with transendothelial migration and polarization of T lymphocytes on the endothelium. Twenty-four hours posttransduction and sorting, primary T lymphocytes were labeled with one of three different CellTracker dyes (CMTMR/red, CMAC/blue, CFSE/green), mixed in equal amounts and applied to a TNF-α–stimulated, CCL-21–coated primary murine brain endothelium under low shear flow (0.15 dyne/cm²). After efficient cell attachment had occurred (about 60 s), shear flow was increased (1.5 dyne/cm²), and TEM was monitored over 50 min with one image acquired every 20 s. (A) Still images of Movie S1 showing a representative field-of-view of a TEM experiment. Shown is an endothelial cell monolayer with control and Nef expressing T lymphocytes, labeled in red and blue, respectively. Colored lines highlight migration tracks of one Nef-expressing cell (blue line) and one control cell (red line, orange following diapedesis). The Nef-expressing cell is additionally highlighted by a blue arrowhead, which turns orange following diapedesis. (B) Relative efficiency of adherence to the endothelium under shear flow. Shown is the percentage of input cells that attached to the endothelial cell monolayer at the time-point when higher shear flow rates were first applied (60 s of imaging). (C) polarization indices were calculated by dividing cell length by cell width at three different time points, one is shown here. (D) polarization indices of single cells directly after (0 s) increasing shear flow from three independent TEM experiments, measured as shown in C. Red bars indicate mean values. P values were calculated using a one-tailed ANOVA test with a Newman–Keuls posttest. n.s., not significant. Images were acquired using a 20 × objective. ***P < 0.0005.

Nef Impairs Subendothelial Migration. Following successful diapedesis, T lymphocytes proceed to crawl underneath the endothelium as an important step for dissemination into the target tissue (16, 18, 26). We therefore tracked subendothelial T lymphocyte motility after diapedesis (Figs. 2A, orange part of the track, 3C, and Movie S2). Although control lymphocytes displayed long migration paths in this highly confined microenvironment, most Nef-expressing cells were unable to move away from their initial site of diapedesis. As a result, these cells covered significantly shorter migration distances and migrated with reduced velocities (Fig. 3D) (42% of the velocity of control cells). Similar to in vitro chemotaxis (Figs. S2E and F and S3E) and lymph node homing at early time-points (Fig. 1B and C), this effect largely depended on the increased chemotactic activity of the F195 Pak2–association motif (Fig. 3C and D). Inhibition of subendothelial T-lymphocyte migration by Nef following diapedesis thus likely involves inhibition of actin remodeling. With diapedesis and subsequent subendothelial migration, Nef inhibits two distinct and essential steps for T-lymphocyte TEM by genetically separable mechanisms.

Nef Interferes with T-Lymphocyte Motility in Confined 3D Matrices. The above results revealed that Nef specifically affects diapedesis and subendothelial migration. Reflecting the need for acquiring motility in distinct physiological environments with varying degrees of confinement, leukocytes adopt different motility modes involving distinct machineries in response to select extracellular cues (27). We therefore tested whether the ability of Nef to block T-lymphocyte motility is related to the density and texture of 3D environments, using well-established collagen matrices as models for pore-size restricted migration. Transduced/isolated T lymphocytes were embedded in low-density or high-density collagen matrices in the presence of CCL-21, and migration of control and Nef-expressing cells in these 3D cultures was followed by time-lapse microscopy over a time-course of 2.5 h. In low-density collagen and thus absence of significant physical barriers, shape and length of tracks of Nef-expressing cells, as well as their velocity, were indistinguishable from those of control cells (Fig. 4A and C, Fig. S8A, and Movie S3). In contrast, the motility of Nef-expressing T lymphocytes was significantly impaired relative to control cells in the more compact environment of a high-density collagen matrix (Fig. 4B, Fig. S8B, and Movie S4), in which cells require higher contractile force generation for locomotion (27). This finding was reflected in an approximately 35% reduced velocity of Nef-expressing cells in high-density collagen (Fig. 4D). This motility inhibition by Nef strictly depended on its ability to interfere with actin remodeling (Fig. 4D) (control: 1.8 ± 0.9 μm/min, Nef: 1.2 ± 0.9 μm/min; F195A: 1.7 ± 0.8 μm/min). Nef thus specifically inhibits T

Fig. 3. Nef-expression interferes with T-lymphocyte subendothelial motility. TEM assays of transduced/sorted T lymphocytes as in Fig. 2. (A) Quantification of the percentage of cells that perform diapedesis, remain on top of the endothelium (on top) or are washed off the endothelium by the shear flow (washed off). Shown are mean values of four to five independent experiments with SD. P values were calculated using a Student t test. (B) Kinetics of diapedesis during the 50-min time course of imaging. Shown are mean values from four independent experiments with SD. P values relative to the control were calculated performing a Student t test. (C) Tracks of single cells crawling underneath the endothelium derived from one of four to five representative TEM experiments. T lymphocytes were tracked manually after successful diapedesis. (D) Mean velocity of single cells migrating underneath the endothelial cell monolayer, calculated from single-cell tracks, as shown in C from four to five independent TEM experiments. Red bars indicate mean values. P values were calculated using a one-tailed ANOVA test with a Newman–Keuls posttest. *P < 0.05; **P < 0.005; ***P < 0.0005.
lymphocyte motility modes used in high-density environments with high physical constraints, and 3D collagen matrices represent a suitable experimental system for the analysis of this process.

**Nef-Expressing Cells Are Retained in HEVs and Show Reduced Motility in pLNs.** We next analyzed whether the blocks conferred by Nef to T lymphocyte diapedesis and motility in confined environments in vitro might explain the defect observed in lymph node homing. Transduced/isolated T lymphocytes were labeled with CellTracker dyes, adoptively transferred into recipient mice and allowed to home for 30 min, followed by 3D immunofluorescence (3-DIF) analysis to determine the precise localization of transferred T lymphocytes (28). Two-times more Nef-expressing than control cells were injected to normalize for the homing defect imposed by Nef expression. Our 3D reconstructions revealed that the majority of control cells had moved away from the HEV network to enter the lymph node parenchyma. In contrast, Nef-expressing cells were often detected inside and close to HEVs (Fig. 5A). Quantification of intralymph node localization by 3-DIF revealed Nef-expressing cells to be approximately twofold-enriched over control cells in the intravascular and perivascular space in all three animals analyzed (Fig. 5B). In line with our ex vivo analysis, these results indicate that Nef impairs T lymphocyte diapedesis in vivo to reduce extravasation of T lymphocytes from HEVs into lymph nodes.

To assess whether Nef affects the dynamic in vivo motility of T lymphocytes at and inside lymph nodes, we performed two-photon microscopy (2PM)-based intravital imaging of T-lymphocyte migration. The 2PM confirmed that Nef expressing T lymphocytes were enriched inside HEVs and analysis of single cells revealed that these cells migrated slower in and near HEVs relative to control cells (Fig. 5 C, Upper, and D, and Movie S5) (median: 5.1 vs. 8.1 µm/min). Nef-expressing T cells also migrated with slightly reduced instantaneous velocities (velocity between two frames) compared with control cells inside the lymph node parenchyma (Fig. 5 C, Lower, and E, and Movie S6) (median: 11.0 vs. 8.1 µm/min). As a result, the motility coefficient that provides a measure for the ability of a randomly migrating cell to move away from its starting position was reduced about 40% upon Nef expression in HEVs, as well as in the parenchyma, compared with control cells (Fig. 5F). Taken together, these results reveal that Nef mediates a marked block in vivo to the diapedesis of T lymphocytes from HEVs into lymph nodes to reduce homing efficiencies, and exerts moderate effects on subsequent parenchymal motility.

**Discussion**

Based on quantification and visualization by 3-DIF and intravital 2PM techniques, we demonstrate here that HIV-1 Nef interferes with trafficking of CD4+ T lymphocytes in vivo and specifically impairs their homing to lymph nodes at the transmigration step from the HEV into the lymph node parenchyma. This approach revealed a potent Nef-mediated block to T-lymphocyte homing that is predominantly impeded at the diapedesis step, with Nef causing an additional but less-pronounced reduction in interstitial motility of T lymphocytes that had successfully entered the lymph node parenchyma. The significantly less-potent inhibition of lymph node homing observed with the F195A Nef mutant suggests that this block critically involves Nef-mediated inhibition of actin remodeling via PK2-dependent coflin phosphorylation. Additional mechanisms independent of actin remodeling are clearly involved in the decrease of T-lymphocyte homing to lymph nodes and might include the slight reduction of cell-surface exposure of the homing receptors CD62L and CCR7 observed in the presence of WT and F195A Nef proteins (Fig. S2B). Because Nef also inhibited T-lymphocyte chemotaxis toward sphingosine-1-phosphate (SIP), a key chemoattractant involved in lymphocyte exit from lymph nodes (Fig. S8C), the viral protein may as well affect egress of infected T lymphocytes from lymph nodes. This effect may increase the density of infected cells inside lymph nodes and facilitate virus spread by enhancing the probability for virus cell-to-cell transmission.

The use of an ex vivo cell system to analyze the influence of Nef on diapedesis through primary endothelial cells under physiological shear flow by video microscopy allowed us to recapitulate this Nef-mediated block in TEM and to dissect which step is specifically affected by Nef. Somewhat surprisingly, given its ability to disrupt triggered actin remodeling, Nef did not affect early steps of the TEM process, including shear-resistant attachment, crawling on the endothelial surface, and formation of potentially invasive and chemokine-sensing filopodia. Nef thus distinguishes between host cell protrusions to achieve simultaneous inhibition of sheet-like actin-rich protrusions, such as lamellipodia (25) and promotion of filopodia formation (8, 10). Of note, Murooka et al. recently described that HIV-1-infected T cells in lymph nodes of humanized mice display reduced motility and form long filopodia-like cell protrusions or nanotubes that facilitate virus transfer to neighboring cells in a manner dependent on the HIV glycoprotein Env (29). Our results are consistent with these observations and suggest Nef as an additional HIV protein that is directly involved in these phenomena. In this scenario, Nef likely promotes nanotube/filopodia formation via its recently described association with the exocyst complex, an established regulator of nanotube generation (30).

Potentially indicative of high selective pressure on this Nef activity, Nef inhibited two later steps of TEM via independent mechanisms. First, Nef interfered directly with the diapedesis step via a mechanism that did not involve its ability to interfere with
chemokine-induced actin remodeling. Although Nef expression reduced T-cell polarization on CCL-21-coated endothelium, the inhibition of TEM was comparable to F195A-expressing T-cells. Polarization is thus not a prerequisite for successful TEM under shear, which reflects previous observations that T lymphocytes undergo TEM despite impaired polarization because of deletion/mutations of Rac or Rac activators (16, 23, 25). Detailed analysis of single cells indicated that protrusions into the subendothelial space are readily observed for Nef-expressing cells, but translocation of the cell body to complete diapedesis was impaired, indicating that Nef does not block diapedesis initiation but completion. These later stages of diapedesis are determined by successive protein–protein interaction steps, involving VE-cadherin, JAM family proteins, and CD99 (31), and depend on the cells’ ability to dynamically adapt their overall shape as well as that of their nucleus. Because all these aspects represent potential targets of Nef, deciphering of the exact step and the molecular targets affected by Nef represents an interesting aspect for future research. Second, Nef interfered with motility of T lymphocytes underneath the endothelial cell surface. This effect strictly required the integrity of the F195 motif and thus correlated with Nef’s ability to interfere with dynamic actin remodeling. In line with this finding, diapedesis and subendothelial locomotion, but not arrest on the endothelium, strongly depend on F-actin integrity (32), and regulators of F-actin dynamics govern T-lymphocyte crawling or polarization (23, 33–36). Taken together, these findings suggest that the observed polarity defect represents the key consequences of Nef-mediated disruption of actin dynamics, which in turn determines the reduced migratory capacity of Nef-expressing T lymphocytes.

Nef only exerted mild effects on T-lymphocyte crawling on top of the endothelium but almost completely arrested locomotion in the confined subendothelial space. These results were mirrored in 3D matrices where Nef specifically inhibited T-lymphocyte motility in high-density collagen, although motility at low collagen density remained unaffected by Nef expression. It thus emerges that Nef specifically interferes with cell-motility modes that rely on the generation of contractile force to overcome physical constraints of the direct environment, including T-lymphocyte chemotaxis across transwell membranes (4, 5, 12, 14) and diapedesis (present study). This specificity would also explain why Nef-mediated inhibition is relatively moderate on T-lymphocyte homing to spleen, which does not involve the crossing of an endothelial barrier, and motility in the lymph node parenchyma, where lymphocytes are embedded in a relatively wide-spaced extracellular matrix structure (19). In contrast, motility in the confined subendothelial space depends on the formation of sheet-like protrusions (37) that are efficiently targeted by Nef to impair cell motility. Taken together, these data show that the dual mechanism by which Nef interferes with T-lymphocyte TEM likely explains, at least in part, the inhibition of homing to lymph nodes observed in vivo.

T-lymphocyte trafficking and homing is crucial for immune surveillance and rapid eliciting of adaptive immune responses (18). Based on the inhibitory effects of Nef on T-lymphocyte homing and diapedesis, the viral protein would be expected to impair mounting of a specific humoral immune response in HIV-infected patients. Indeed, B-cell dysfunction characterized already at early stages of HIV-1 infection by the lack of high-affinity antibodies is increasingly recognized as a hallmark of AIDS pathogenesis (38, 39). Nef is suggested to contribute to this phenomenon by impairing antigen presentation and prevention of B-cell class switching following transfer from infected cells (40–43). The results presented herein, in line with those from other laboratories (44, 45), suggest that Nef also suppresses B-cell help by HIV-1–infected T lymphocytes. In addition, the observed reduction of parenchymal lymphocyte motility may facilitate cell-associated virus spread in the context of HIV-1 infection. Subversion of host cell motility thus emerges as a cardinal function of Nef likely to play an important role in AIDS pathogenesis.
Materials and Methods

The 3-DIF imaging was essentially performed as described previously (16). Briefly, transfected and NGFR-sorted T lymphocytes were labeled with CellTracker dyes, as described in SI Materials and Methods. A 3 × 10^6 iRES:NGFR control cells were co-injected with 6- to 8 × 10^6 NeF WT-expressing cells into the retroorbital sinus of 6- to 8-wk-old WT C57BL/6 mice. Thirty minutes after the co-injection, each adoptive transfer, pLNs were harvested and fixed overnight in 0.5% paraformaldehyde. Lymph nodes were manually cleared from surrounding fat, embedded into 1.3% (wt/vol) agarose, dehydrated in methanol for 24 h, and cleared for at least 2 d in benzyl alcohol-benzyl benzoate (1:2 ratio). One-to-two scans were acquired per lymph node using a TrimScope 2PM (LaVisionBiotech) equipped with a Ti Sapphire laser (MaiTai HP, Spectraphysics) and 20× objective (Olympus) with a scan volume ranging from 0.4 × 0.4 to 0.4–0.6 mm (0.06–0.1 mm). The absolute number of lymphocytes labeled with each dye was counted using a 3D image analysis software program (Velocity, PerkinElmer). Visual analysis of individual x–y sections was used to determine the position of individual cells relative to the HEV network.

ACKNOWLEDGMENTS. We thank Reno Debets for the kind gift of the murine leukemia virus proviral constructs; Nadine Tibroni for expert technical help; Olivier Kepler for help with the organization of the in vivo experiments; and the Swiss National Microscopy facility of the microscopy imaging center of the University of Bern for service. This work was supported by the Deutsche Forschungsgemeinschaft SFB638, Project A11 and Grant FA 378/10-1 to O.T.F. and Fellowship GRK1188 (to B.S.); Swiss National Foundation Grants SNF 135649 (to J.V.S.) and SNF 31003A_133092 (to Britta Engelhardt and R.L.); a Marie Curie postdoctoral fellowship (to F.M.C.); a Leopoldina/Nationale Akademie der Wissenschaften fellowship (to B.S.); and a Swiss Multiple Sclerosis Society grant (to R.L.). R.G. was funded by the Olga Mayenfisch Foundation (to Britta Engelhardt).


Supporting Information

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SI Materials and Methods

Antibodies and Reagents. Antibodies and reagents were as follows: Purified NA/LE hamster anti-mouse CD3ε (145-2C11), rat anti-mouse CD8α-FITC (53-6.7), rat anti-mouse CD8α-PerCP (53-6.7), rat anti-mouse CD62L-biotin (MEL-14), rat anti-mouse CD90.2-APC (53-2.1) Streptavidin-APC (all BD Biosciences). Rat anti-mouse CD4-APC (PJ66) (ImmunoTools). Rat anti-mouse/human CD44-Biotin (IM7) (Biolegend, BIOZOL). Rabbit anti-PKCζ (C-20), mouse anti-Lck (SAS) (Santa Cruz Biotechnology). Mouse anti-mouse MHC-I-APC (H-2Kb), rat anti-mouse CXCR4-PE (2B11), rat anti-mouse CCR7-APC (4B12), eFluor 670 (eBioscience). Mouse anti-GFP (GFP-20), mouse anti-CD62L-biotin (ME14), mouse CD8a-FITC (53-6.7), rat anti-mouse CD8a-PerCP (53-6.7), rabbit anti-PKCζ, mouse CD4-APC (PJP6) (Immunotools). Rat anti-mouse/human CD62L-biotin (ME14), mouse CD8a-FITC (53-6.7), rat anti-mouse/human NGFR antibody was fixed with 4% paraformaldehyde, followed by a second permeabilization with 0.1% Triton X-100 in PBS. Brieﬂy, transduced Jurkat T cells, scoring between 50% and 70% infection/transduction efficiency and 1 × 10⁶ total cells were lysed and loaded per lane.

Flow Cytometry Analyses of Cell-Surface Receptors. For flow cytometry analyses, 5 × 10⁵ to 2 × 10⁶ lymphocytes were labeled with the following antibodies diluted in PBS: directly ﬂuorophor-coupled CD4-APC (1:20), MHC-I-APC (1:100), CXCR4-PE (1:20), CD90.2-APC (1:100), and CD8α-PerCP (1:100) for 30 min on ice; CCR7-APC (1:20) for 30 min at 37°C; CD62L-Biotin (1:100) and anti-NGFR (1:800) for 30 min on ice, followed by a washing step in PBS and incubation with the ﬂuorophor-coupled secondary reagent/antibody streptavidin-APC (1:20) and anti-mouse APC (1:250), respectively, for 30 min on ice. Following a washing step in PBS, 5,000 transduced cells were analyzed for cell-surface receptor modulation by flow cytometry. Receptor down-modulation was calculated relative to untransduced cells and GFP-transduced control cells were set to 100% as described previously (2).

Immune Fluorescence Analyses and GTPase Activity Assays. Analysis of the Lck accumulation, chemokine-induced membrane-ruffle formation and coﬁlin-phosphorylation were essentially performed as described previously (2). Approximately 5 × 10⁶ cells were seeded per 9-cm-lysine–coated glass-Well. Next, 200 ng/mL recombinant murine CCL-21 was used to induce F-actin rich membrane ruffle formation. When NGFR-sorted lymphocytes were used, Nef was detected by sheep anti-Nef (1:500) antibody followed by labeling with Alexa Fluor 488 donkey anti-sheep IgG (1:1,000). For analysis of cell polarization, transduced lymphocytes were plated onto ﬁbronectin-coated glass-wells (10 μg/mL ﬁbronectin for 30 min at 37°C) and stimulated with 100 ng/mL recombinant murine CCL-21 for 30 min at 37°C before PFA cross-linking (3.7% PFA, 15 min, room temperature). Cells were permeabilized with 0.1% triton-X 100 for 2 min, blocked with 1% BSA/PBS for 30 min, and stained with primary antibodies rat anti-CD44-biotin (1:200) and rabbit anti-PKCζ (1:50) for 2 h. Independent stainings were performed for confocal image acquisition and manual counting at a ﬂuorescence microscope. Following PBS wash, CD44 was revealed by either Alexa Fluor 568 goat anti-rat IgG (1:1,000) or streptavidin Alexa Fluor 660 conjugate (1:500). PKCζ with Alexa Fluor 550 goat-anti-rabbit IgG (1:500) or Alexa Fluor 568 goat anti-rabbit IgG (1:1,000). Phenotypes were quantiﬁed by manual counting of at least 100 transduced cells per cover glass at an Olympus IX81 microscope with CellS software. Confocal stacks of chemokine induced membrane ruffling were obtained with a Leica TCS SP5 microscope and LAS AF software. All other confocal micrographs were acquired using a Zeiss LSM 510 Axiovert microscope and LSM Meta software.

Rac1 activity of transduced and sorted primary murine lymphocytes following chemokine stimulation was analyzed performing a Rac1-G-LISA (Cytoskeleton) according to the manufacturers’ instructions. Brieﬂy, transduced and sorted lymphocytes were starved for 24 h in medium containing 1% BSA. Before chemokine stimulation, lymphocytes were cultured at 2 × 10⁶ cells/mL in a 37°C overexpression in human T-cell lines, followed by standard Nef functional analyses.

Western Blotting. Approximately 1 × 10⁶ sorted, transduced murine T lymphocytes were lysed directly in 2x SDS sample buffer and analyzed on 12% SDS-PAGE. For comparison of Nef expression in HIV-infected primary human lymphocytes and Nef transduced primary murine T lymphocytes, infected and transduced cells were sorted, respectively. Sorted cells were adjusted to 70% infection/transduction efficiency and 1 × 10⁶ total cells were lysed and loaded per lane.
water-bath for 10 min. Then, 200 ng/mL CCL-21 was added to the cells and stimulation was stopped after 1 and 10 min by transferring the cells into a large volume of ice-cold PBS; 2 × 10⁶ cells were used per sample for triplicate analyses.

**Isolation, Culture, and HIV Infection of Human Peripheral Blood Mononuclear Cells.** Human peripheral blood mononuclear cells were isolated, stimulated, and infected with HIV, as described previously (2). Proviral constructs and virus production has been described previously (2).

**MLV-Based Vector Production.** Vector production was optimized regarding use of the vector-producing cell line, transfection reagent, plasmid ratios, pseudotyping of the particles, different GagPol constructs, lentiviral vs. MLV-based vector systems and harvesting time points, resulting in the following vector production protocol (workflow shown in Fig. S1): 293T cells were maintained in DME medium with high glucose, supplemented with 10% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin. Twenty-four hours before transfection, 5 × 10⁶ 293T cells were seeded per 15-cm dish. Transfection was performed using JetPEI transfection reagent (Peglab) according to the manufacturers’ recommendations using: 20 μg pSTITCH, 20 μg pHit60, 5 μg pHit123, and 5 μg MLV-A. The transfection mix was directly added to the medium and a medium exchange was performed after 5 h. Virus supernatant was harvested after 48 h or 72 h by filtration of the cell supernatant (0.22-μm pore-size radio-sterilized filters) and used immediately for preparation of primary murine T lymphocytes.

**Preparation and Transduction of Murine T Lymphocytes.** The preparation and transduction protocol for murine T lymphocytes was optimized with respect to in vitro stimulation (anti-CD3/CD28 vs. ConA/IL-2), culture density during stimulation, duration of mitogenic stimulation before transduction, cell density during transduction, format of transduction (24- to 96-well plates), reagents used to increase transduction efficiency (polybrene vs. retrogenin), centrifugation speed, and duration of spin infection, culture density following transduction, timing of Dynabead-sorting, and read-out. The optimized protocol is illustrated in Fig. S1. Six- to 9-wk-old WT C57BL/6 mice (Charles River) were kept in the central animal facility of the University Heidelberg and were killed by CO₂ perfusion (approved by the University of Heidelberg G-152/07). Spleen, peripheral, and mesenteric lymph nodes were harvested and single-cell suspensions were maintained by squeezing organs through cell strainers (70 μm; BD Biosciences). After red blood cell lysis, lymphocytes were washed, counted, and cultured at 2 × 10⁶ cells/mL in complete mouse medium (CMM: RPMI 1640 GlutaMAX, supplemented with 15% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM Glutamine, 50 μM 2-mercaptoethanol, 1x nonessential amino acids, 1x sodium pyruvate, 1x MEM vitamins; all from Gibco, Invitrogen) containing 2 μg/mL Concanavalin A (ConA; Sigma) and 2 × 10⁶ U/mL recombinant human IL-2 (Biomed). After red blood cell lysis, lymphocytes were washed, counted, and cultured at 2 × 10⁶ cells/mL in complete mouse medium (CMM: RPMI 1640 GlutaMAX, supplemented with 15% FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM Glutamine, 50 μM 2-mercaptoethanol, 1x nonessential amino acids, 1x sodium pyruvate, 1x MEM vitamins; all from Gibco, Invitrogen) containing 2 μg/mL Concanavalin A (ConA; Sigma) and 2 × 10⁶ U/mL recombinant human IL-2 (Biomed). For transduction, 24-well plates were coated with 16 μg/mL retrogenin/PBS for 3 h at 37°C. Retrogenin (TaKaRa Bio, MoBiTec) was recycled up to 10 times and stored at −20°C. Retrogenin coated plates were stored in PBS, at 4°C for up to 2 wk. One day after lymphocyte preparation, 2 mL of fresh filtered virus supernatant per well were spun for 1 h at 4,000 rpm at room temperature onto the retrogenin-coated plates. Virus supernatant was removed subsequently and 2 mL of new virus supernatant was added per well together with 2 × 10⁶ lymphocytes. Cells were spin-infected for 1 h at 2,300 rpm at room temperature and cultivated for 5 h at 37°C 5% CO₂, before performing a medium exchange. Cells were resuspended at 1 × 10⁶ cells/mL in CMM containing 2 × 10⁵ U/mL IL-2. Sorting and experiments were performed 24 or 48 h after transduction, respectively.

**Dynabead Sorting of ΔNGFR⁺ Cells.** Twenty-four hours post-transduction, ΔNGFR-transduced cells were sorted using the CELLlection Pan Mouse IgG Kit (Invitrogen) according to the manufacturers’ recommendations. Briefly, transduction efficiency was analyzed by flow cytometry and 25 μL of Pan Mouse IgG Dynabeads were coupled with 2 μL of the purified hybrid-antibody derived anti-NGFR antibody (HB9573 hybrida; ATCC; LGC Standards) per 2.5 × 10⁶ ΔNGFR⁺ cells for 45 min at 4°C, rocking. Following extensive washing, total lymphocytes were diluted to 10⁷ cells/mL and added to the antibody-coupled beads. After incubation for 30 min at 4°C, rocking, unbound cells were removed by extensive washing and bound ΔNGFR⁺ lymphocytes were eluted from the beads by DNase I treatment. This purification protocol yielded 95–99% pure ΔNGFR⁺ lymphocytes. Cells were either incubated at 1 × 10⁹ cells/mL in CMM containing 2 × 10⁵ U/mL IL-2 for another 24 h at 37°C and 5% CO₂ before analysis, or used immediately in case of 24-h homing.

**Transwell Chemotaxis Assay.** The transwell chemotaxis assay was essentially performed as described previously (2), using 50 ng/mL recombinant murine CCL-21 as chemotactant. Where indicated, transwell inserts with 3-μm pore size (Costar #3415) were used instead of 5 μm (Costar #4321). In the case of NGFR-sorted lymphocytes, input and migrated cells were counted manually using a Neubauer counting chamber and Trypan blue to exclude dead cells, because ~100% of the cells were transduced. Chemotaxis efficiency was calculated relative to the control cells. Usually about 60% of the input cells migrated within the 2 h of the experiment. The transwell chemotaxis assay toward sphingosine-1-phosphate (SIP) was performed as previously described (5). Briefly, transduced cells were starved for 24 h in medium containing 1% fatty acid-free BSA (Sigma) before chemotaxis toward 25 nM SIP (Sigma) at 37°C 5% CO₂ for 4 h.

**Live T-Lymphocyte Migration in 3D Fibricular Collagen.** ΔNGFR transduced cells were sorted 24 h posttransduction using the CD271 MicroBead APC Kit (MiltenyiBiotec) according to the manufacturer's instructions. Briefly, 1 × 10⁷ total cells were resuspended in 90 μL MACS buffer (PBS; 2 mM EDTA, 0.5% FCS), 10 μL of FcR Blocking Reagent, and 10 μL of APC-coupled NGFR antibody were added and refrigerated for 10 min. After washing, cells were resuspended in 70 μL MACS buffer, 10 μL FcR Blocking Reagent, and 20 μL of anti-APC MicroBeads were added and refrigerated for 15 min. After washing, cells were positively separated using the autoMACS Pro Separator (program possel-d). Cells were allowed to rest in CMM for several hours before subjecting them to collagen migration experiments. This procedure yielded between 90% and 98% ΔNGFR⁺ cells and the experimental performance of cells in collagen matrix was the same as upon sorting using CELLlection Pan Mouse IgG Kit (Invitrogen).

Cell migration in 3D collagen I lattices was monitored by time-lapse microscopy within self-constructed migration chambers: A coverslip was connected to an object slide by a dental wax spacer, which was cut into pockets open to one side (approximate chamber size of 8 × 4 × 2 mm, volume of ~120 μL). Through the openings the pockets were partly filled with a collagen matrix including the cells. Cell-containing collagen gels were prepared as described (6): Low-density collagen matrix (1 mg/mL) was prepared by mixing 750 μL bovine collagen I (PureCol, Nutacon) with bicarbonate-buffered MEM [50 μL 7.5% NaHCO₃ and 100 μL 10x MEM (Gibco)]. One-hundred microliters of matrix were subsequently combined with 50 μL cell-suspension (3 × 10⁶ cells/mL in 0.5% FCS-reduced media) and allowed to polymerize at 37°C. High-density collagen matrices (4.6 mg/mL) were prepared correspondingly using highly concentrated rat collagen I (BD Biosciences) and the bicarbonate-buffered MEM described above. After polymerization, gels were overlaid with FCS reduced media containing 100 ng/mL CCL-21 (Peprotech) and were sealed with melted dental wax.
The samples were monitored via bright-field of an inverse light microscope (10x objective, Axiovert 200M; Zeiss) coupled to an EM-CCD camera (Roper Scientific), equipped with a clatimization control maintaining 37 °C. For the duration of 2.5 h, pictures were taken in 3-min intervals. Cells were scored as migrating if they once left their initial position during the time-lapse monitoring. Migrating cells were then tracked manually using the ImageJ manual tracking plugin and analyzed with the chemotaxis tool plugin. Collagen matrix consistency was visualized by confocal reflection microscopy using the 488-nm laser and 40x oil immersion objective of a Leica TCS SP5 microscope. A 10-μm stack of optical sections (z-stack) was acquired in 0.5-μm slices, which were subjected to maximum projections using the LAS AF software.

Homing to Lymphoid Organs. Transduced and NGFR-sorted T lymphocytes were labeled with CellTracker dyes (Invitrogen) at 1 × 10^7 cells/mL at 37 °C; CMTMR 5 μM 20 min, CFSE 2.5 μM 15 min, eFluor670 2.5 μM 20 min. Dyes were exchanged between samples for successive replicates to exclude effects of the dyes on homing behavior. Subsequently, cells were washed with CMM and resuspended at 1 × 10^6 cells/mL 5 × 10^5 IRES-NGFR control cells were co-injected with an equal number of Nef wt or F195A expressing cells into the retrobulbar sinus of 6- to 8-wk-old WT C57BL/6 mice. Four and 24 h after adoptive transfer spleen, peripheral, and mesenteric lymph nodes were harvested separately and single-cell suspensions were obtained as described above. Cells were either subjected immediately to flow cytometry to analyze the relative abundance of CellTracker-positive cells or labeled with anti-CD90.2 and anti-CD8a antibodies (BD Biosciences) to distinguish between the CD8+ T lymphocytes. Between 500,000 and 750,000 cells were acquired per sample. Homing ratios of Nef WT or F195A expressing cells were calculated relative to control cells for each organ. All experiments were approved by the Kanton of Bern and comply with cantonal and federal animal experimentation regulations.

Transendothelial Migration Assay, Single-Cell Tracking, and Scanning Electron Microscopy. Primary mouse brain microvascular endothelial cells (pMBMECs) were isolated from sex-matched 5- to 8-wk-old C57BL/6 mice, cultured in DMEM, 20% FCS, 1 mmol/L sodium pyruvate, 1% nonessential amino acids, 50 ng/mL gentamicin, and 1 ng/mL basic fibroblast growth factor (7, 8). pMBMECs were stimulated with TNF-α (25 ng/mL; PeproTech) for 16–18 h. All experiments were performed in migration assay medium (DMEM, 5% calf serum, 25 mM Hepes) at 37 °C.

For live-cell imaging, a parallel flow chamber connected to an automated syringe pump (Harvard Apparatus) was mounted on TNF-α-stimulated pMBMECs previously overlaid with 1 μM CCL-21 and placed on the heating stage of an inverted microscope (AxioObserver Z1; Carl Zeiss). Shear stress (0.15 dyn/cm^2) were allowed to accumulate for around 1 min at low shear stress (0.15 dyn/cm^2). The instantaneous 3D velocity is the velocity between two frames. The motility coefficient, a measure of the ability of a cell to move away from its starting position, was calculated from the gradient of a graph of mean displacement against √(time).

**Statistical Analysis and Software.** Statistical significance on parametrically distributed datasets was calculated performing a Student t test (or Mann–Whitney test for not normally distributed data) or a one-tailed ANOVA test with a Newman–Keuls posttest when comparing two or several datasets, respectively (**P < 0.005, *P < 0.05, *P < 0.05). The nonparametric Kruskal–Wallis test was used for evaluation of the lymph node homing because of the limited available sample size. Calculations were done with Microsoft Excel and GraphPad Prism; image-editing was performed using Adobe Photoshop and Illustrator CS5.

*Fig. S1.* Scheme of the optimized protocol for efficient transduction of primary murine T lymphocytes.
Fig. S2. Nef function is recapitulated in primary murine T lymphocytes. Primary murine T lymphocytes were isolated from spleen and lymph nodes of donor mice, stimulated and retrovirally transduced in vitro, as described in Fig. S1. Analyses were performed 24 h posttransduction. Shown are mean values with SD from three independent experiments. P values have been calculated performing a Student t test. (A) Schematic drawing of the pSTITCH-based packaging constructs used to achieve expression of GFP, Nef.GFP, or NefF195A.GFP in primary murine T lymphocytes. (B) Relative receptor down-modulation by Nef of the cell-surface receptors CD4, MHC class I, CXCR4, CCR7, and CD62L. Down-modulation was calculated relative to untransduced control cells in each sample with GFP expressing controls set to 100%. (C) Representative confocal micrographs of transduced T lymphocytes plated onto poly-L-lysine–coated cover-glasses. Cells were analyzed for membrane ruffling upon stimulation with 200 ng/mL CCL-21, cofilin phosphorylation, and Lck accumulation, respectively. Cells were fixed and stained for F-actin, phosphorylated cofilin (p-cofilin) or Lck as indicated. Arrows highlight transduced GFP+ cells. (Scale bars, 10 μm.) (D) Quantification of the cells shown in C. Cells showing F-actin rich membrane ruffles, high p-cofilin levels, or Lck accumulation were quantified by manual counting of at least 100 transduced cells per condition. (E and F) Analysis of chemotaxis toward CCL-21 in a transwell assay using a membrane with 5 μm (E) or 3 μm (F) pore size. Migration of transduced cells was calculated relative to GFP-expressing cells. *P < 0.05; **P < 0.005; ***P < 0.0005.
Fig. S3. Sorting of transduced T lymphocytes by their NGFR cell-surface marker results in homogenous cell populations with Nef expression levels and function comparable to HIV-infected primary human T lymphocytes. (A) Schematic drawing of the pSTITCH-based packaging constructs used to achieve expression of ΔNGFR alone or together with Nef or its F195A mutant in primary murine T lymphocytes. (B) Primary murine T lymphocytes were isolated from spleen and lymph nodes of donor mice, stimulated and retrovirally transduced in vitro as described in Fig. S1. Twenty-four hours posttransduction successfully transduced cells were sorted using dynabeads coated with an anti-NGFR antibody. Shown are transduced cells before (Input) and after (unbound cells first wash) dynabead-dependent depletion of NGFR+ cells as well as DNase I-treated dynabeads (eluted dynabeads) and the obtained pure NGFR+ cell population (eluted cells). The numbers in the gates represent the percentage of NGFR+ T lymphocytes in the sample. Following sorting, T lymphocytes were cultured for another 24 h before analysis. (C) Representative confocal micrographs of transduced, sorted T lymphocytes, plated onto poly-l-lysine coated cover-glasses and stimulated with 200 ng/mL CCL-21 to induce membrane ruffling or left untreated for analysis of cofilin phosphorylation. Cells were fixed and stained for F-actin and p-cofilin, as indicated. All cells shown are ΔNGFR+ because of previous sorting. (Scale bars, 10 μm.) (D) Quantification of the cells shown in C that display F-actin rich membrane ruffles or cofilin hyperphosphorylation, respectively. Shown are mean values with SD of three independent experiments with at least 100 transduced cells manually counted per condition. P values have been calculated performing a Student t test. (E) The cells shown in C were subjected to a transwell chemotaxis assay toward CCL-21. Chemotaxis efficiency was calculated relative to control cells. Shown are mean values with SD of three independent experiments with at least 100 transduced cells manually counted per condition. P values have been calculated performing a Student t test. (F) Representative Western blot of the cells shown in C, probed for Nef and tubulin as a loading control. (G) Western blot of HIV-infected primary human T lymphocytes and primary murine T lymphocytes transduced with the constructs shown in A. Infected/transduced cells were enriched and cell populations adjusted to contain 70% infected or transduced cells. Next, 1 × 10^6 were lysed and subjected to Western blot analysis for expression of Nef and MAPK (loading control). Intensities of the Nef bands was quantified and normalized to the corresponding MAPK signal. Numbers below the panels indicate Nef expression levels relative to that observed in human peripheral blood mononuclear cells infected with HIV-1 WT, which was arbitrarily set to 1. *P < 0.05; **P < 0.005.
Fig. S4. Nef interferes with T lymphocyte homing to lymph nodes. Primary T lymphocytes were isolated, transduced, and sorted as described in Fig. S3. Cells were labeled with CellTracker dyes and adoptively transferred into recipient mice. Control cells were always coinjected with Nef or F195A-expressing T lymphocytes as an internal control. Spleen (A), peripheral (pLN) (B), and mesenteric (mLN) (C) lymph nodes were harvested after 4 or 24 h and single-cell suspensions were analyzed by flow cytometry following cell surface labeling with anti-CD90.2 and anti-CD8α antibodies to distinguish between CD4+ (round symbols) and CD8+ (square symbols) T lymphocytes. The homing ratio in each mouse was calculated relative to the coinjected control cells that was arbitrarily set to 1. Each symbol represents data observed from one animal. *P values have been calculated performing a Kruskal-Wallis test with a Dunn’s posttest. *P < 0.05; **P < 0.005.

Fig. S5. Nef interferes with T lymphocyte polarization but not Rac1 activity. (A) Representative confocal micrographs of primary mouse T lymphocytes expressing GFP, Nef wt.GFP or Nef F195A.GFP following retroviral transduction. Cells were plated onto fibronectin-coated cover-glasses, stimulated with 100 ng/mL CCL-21 for 30 min, fixed, and stained for the trailing edge marker CD44 (blue) and the leading edge marker PKCζ (red). (Scale bar, 10 μm.) (B) Quantification of the cells shown in A with polarized localization of CD44 to the trailing edge. Shown are mean values with SD of three independent experiments with at least 100 transduced cells manually counted per condition. *P values have been calculated performing a Student t test. (C) Primary murine T lymphocytes were transduced with the constructs shown in Fig. S3, sorted based on NGFR expression, and starved for 24 h. Rac1-activity following CCL-21 stimulation for 1 and 10 min was analyzed by a Rac1-GLISA. Shown are the mean values of triplicate measurements of one representative of two experiments. **P < 0.005; ***P < 0.0005.
Fig. S6. Nef-expression does not exert pronounced effects on the locomotion of T lymphocytes on top of the endothelium. Transduced and sorted T lymphocytes were subjected to transendothelial migration (TEM) assays as in Fig. 2. (A) Single-cell tracks of cells crawling on top of the endothelium before successful diapedesis. The tracks are derived from one of four to five representative TEM experiments. Adhered T lymphocytes were tracked manually, starting when shear flow rates were first increased. (B) Mean velocity of single cells until successful diapedesis calculated from single cell tracks as shown in A from four to five independent TEM experiments. Red bars indicate mean values. P values have been calculated using a one-tailed ANOVA test with a Newman–Keuls posttest. *P < 0.05.

Fig. S7. Nef does not interfere with filopodia formation in transduced murine T lymphocytes and HIV infected human T lymphocytes. (A) Representative 3D maximum projections of confocal z-stacks of primary murine T lymphocytes transduced with the constructs shown in Fig. S2 for expression of GFP, Nef wt, GFP, NefF195A. Cells were plated onto poly-L-lysine–coated cover-glasses and analyzed for membrane ruffle and filopodia formation with and without stimulation by CCL-21. Cells were fixed and stained for F-actin. Red asterisks highlight transduced GFP* cells. Arrows and arrow heads indicate filopodia and F-actin rich membrane ruffles, respectively. (Scale bars, 10 μm.) (B) Quantification of the cells shown in A. Cells showing F-actin rich membrane ruffles or filopodia were quantified by manual counting of at least 100 transduced cells per condition. Cells showing showing F-actin–rich membrane ruffles and filopodia were counted as membrane ruffle-positive. Shown are mean values with SD of three independent samples. (C) Primary murine T lymphocytes were transduced and sorted as in Fig. S3 to achieve expression of Nef or NefF195A.GFP, and subjected to transendothelial migration as shown in Fig. 2. Five minutes after adherence to the endothelium, cells were fixed and processed for SEM. Shown are representative SEM pictures of T lymphocytes interacting with endothelial cells. Arrows indicate filopodial structures. (Scale bars, 10 μm.) Note that Nef does not suppress formation of filopodia but appears to induce the generation of numerous, microvilli-like short-cell protrusions in a F195-dependent manner. (D) Primary human lymphocytes were infected with HIV WT, a virus lacking Nef expression (HIVΔNef) or an isogenic virus encoding for NefF195A. Cells were stimulated with human CCL-21 identical to the murine lymphocytes shown in A. Shown are representative 3D maximum projections of confocal z-stacks. Red asterisks highlight HIV infected/CA* cells. Arrows point toward filopodia, arrow heads toward F-actin rich membrane ruffles. (Scale bars, 10 μm.) (E) Quantification of filopodia and F-actin–rich membrane ruffles of HIV-infected lymphocytes as in B. Shown are mean values with SD from triplicate infection of lymphocytes from two independent donors.
Fig. S8. Nef interferes with T-lymphocyte motility in 3D matrices dependent on the pore size. Shown are still images of additional time points of the 3D collagen migration assay shown in Fig. 4. (A) Low-density collagen (1.6 mg/mL) showing stills of Movie S3. (B) High-density collagen (4.6 mg/mL) showing stills of Movie S4. The panels on the left show the fibrillar structures of the collagen preparations used visualized as maximum projections of 10-μm stacks using confocal reflection microscopy. Moving cells are labeled with colored dots and their tracks are indicated over time. (Scale bars, 50 μm.) (C) Primary murine T lymphocytes were transduced with the constructs shown in Fig. S2 to achieve expression of GFP, Nef wt.GFP or Nef F195A.GFP and subjected to transwell chemotaxis assays toward S1P using 5-μm pore transwell inserts. Shown is the absolute migration efficiency of GFP+ cells as mean values with SD of one representative experiments performed in triplicates.
Movie S1. Nef interferes with transendothelial migration. Representative field of view of a TEM experiment. Shown is a TNF-α–stimulated, CCL-21–coated primary murine endothelial cell monolayer with control and Nef transduced T lymphocytes, labeled in red and blue, respectively. Cells were imaged over a time-course of 50 min with one image acquired/20 s. The movie starts when shear flow rates were first increased. Images were acquired using a 20× objective.

Movie S2. Single-cell tracks of T lymphocytes migrating underneath an endothelial cell monolayer. Control, WT Nef and Nef F195A transduced T lymphocytes were tracked following successful diapedesis while migrating underneath an endothelial cell monolayer until the end of the experiment. Different lengths of the tracks are result from diapedesis after different time points.

Movie S3. T lymphocytes migrating in low-density collagen matrices. Control, WT Nef and Nef F195A transduced T lymphocytes were embedded into low-density (1.6 mg/mL) collagen matrices in the presence of 100 ng/mL CCL-21. Migration of cells was monitored by bright-field microscopy over 2.5 h with one image acquired every 3 min. Moving cells are labeled with colored dots and their tracks are indicated over time. Images were acquired using a 10× objective.
Movie S4. T lymphocytes migrating in high-density collagen matrices. Control, WT Nef and Nef F195A transduced T lymphocytes were embedded into high-density (4.6 mg/mL) collagen matrices in the presence of 100 ng/mL CCL-21. Migration of cells was monitored by bright-field microscopy over 2.5 h with one image acquired every 3 min. Moving cells are labeled with colored dots and their tracks are indicated over time. Images were acquired using a 10× objective.

Movie S4

Movie S5. Nef interferes with diapedesis of T lymphocytes across HEVs in vivo. The 2PM intravital imaging of a surgically exposed popliteal lymph node 24 h postadoptive transfer of transduced, sorted, and CellTracker-labeled, T lymphocytes. Migration of cells close to a HEV was monitored over 20 min with one 3D stack acquired every 20 s. The HEV is labeled by injection of a fluorophor-coupled MECA-79 antibody and displayed in brown. Control cells are displayed in red, Nef-expressing cells in blue. Images were acquired using a 20× objective.

Movie S5
**Movie S6.** Nef reduces T-lymphocyte motility in vivo. The 2PM intravital imaging of a surgically exposed popliteal lymph node 24 h post adoptive transfer of transduced, sorted and CellTracker-labeled, T lymphocytes. Migration of cells in the lymph node parenchyma was monitored over 20 min with one 3D stack acquired every 20 s. Control cells are displayed in red, Nef-expressing cells in blue. Images were acquired using a 20× objective.