HIV-1 Nef Disrupts CD4+ T Lymphocyte Polarity, Extravasation, and Homing to Lymph Nodes via Its Nef-Associated Kinase Complex Interface

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HIV-1 Nef is a multifunctional protein that optimizes virus spread and promotes immune evasion of infected cells to accelerate disease progression in AIDS patients. As one of its activities, Nef reduces the motility of infected CD4+ T lymphocytes in confined space. In vivo, Nef restricts T lymphocyte homing to lymph nodes as it reduces the ability for extravasation at the diapedesis step. Effects of Nef on T lymphocyte motility are typically mediated by its ability to reduce actin remodeling. However, interference with diapedesis does not depend on residues in Nef required for inhibition of host cell actin dynamics. In search for an alternative mechanism by which Nef could alter T lymphocyte extravasation, we noted that the viral protein interferes with the polarization of primary human CD4+ T lymphocytes upon infection with HIV-1. Expression of Nef alone is sufficient to disrupt T cell polarization, and this effect is conserved among lentiviral Nef proteins. Nef acts by arresting the oscillation of CD4+ T cells between polarized and nonpolarized morphologies. Mapping studies identified the binding site for the Nef-associated kinase (NAKC) as critical determinant of this Nef activity and a NAKC-binding–deficient Nef variant fails to impair CD4+ T lymphocyte extravasation and homing to lymph nodes. These results thus imply the disruption of T lymphocyte polarity via its NAKC binding site as a novel mechanism by which lentiviral Nef proteins alter T lymphocyte migration in vivo. The Journal of Immunology, 2018, 201: 2731–2743.

N egative factor (Nef) is a 25–34-kDa myristoylated accessory protein encoded by the primate lentiviruses HIV-1, HIV-2, and SIV. Although Nef is not needed for virus replication in cell culture, it optimizes virus replication in the infected host and thus significantly contributes to disease progression to AIDS. To mediate this critical function in AIDS pathogenesis, Nef acts as versatile protein interaction adaptor that manipulates a remarkable range of host cell processes, including signal transduction and vesicular transport pathways, by genetically separable but incompletely defined molecular mechanisms (1–5). Nef mediates its functions via various interactions with host cell proteins, thereby inducing changes in central intracellular transport and signaling pathways of HIV-infected cells (2, 6). This includes reducing cell surface densities of transmembrane receptors and peripheral membrane proteins by molecular mechanisms that affect endocytosis, anterograde transport, and/or protein stability (7–12). By modulating surface exposure of cell-surface receptors such as MHC class I and II, CD4, chemokine receptors, costimulatory molecules such as CD80 and CD86, tetraspanins, and NK cell ligands, Nef acts to evade host cell immune responses and prevents superinfection of infected cells (8, 10–17). In addition, Nef affects activation state and responsiveness of T lymphocytes to TCR signaling by modifying their vesicular transport and actin remodeling pathways (18–26). These alterations reduce activation-induced cell death and thus prolong the survival of productively infected cells (27). Finally, Nef enhances the infectivity of HIV particles by antagonizing the restriction factors SERINC 3 and 5 via yet to be determined mechanisms (28–31).
In addition to activities that are directly linked to the replicative abilities of the virus, HIV-1 Nef was also shown to interfere with the motility of infected CD4+ T lymphocytes (32–34), which, together with macrophages, constitute the main target cells of the virus. We previously identified that the mechanism by which Nef affects T lymphocyte motility involves its ability to inhibit dynamic remodeling of host cell actin. At least in part, this reduction of actin remodeling results from the association of Nef with cellular PAK2 kinase that induces phosphorylation, and thus inactivation, of the actin-severing factor cofilin (35). Subsequent work suggested that this block to T lymphocyte motility in vitro translates into a potent block to lymph node homing of T lymphocytes when Nef-expressing CD4+ T lymphocytes were adoptively transferred into recipient mice (36, 37). The multistep homing process of T lymphocytes via extravasation through high endothelial venules initiates with the selectin-mediated trapping and rolling with the direction of blood flow subsequent engagement of CCR7 by its endothelium-presented chemokine ligands CCL21 and CCL19 result in integrin activation and firm arrest of T lymphocytes on the inner vessel wall. Following acquisition of a polarized phenotype and an optional crawling step (irrespective of the direction of blood flow) to find permissive sites for diapedesis, T lymphocytes enter the lymph node parenchyma and resume migration (38, 39). Three-dimensional visualization and intravital microscopy demonstrated that the Nef-mediated block in lymph node homing was primarily due to a strong inhibition of extravasation, whereas subsequent parenchymal motility was only slightly reduced. Consistently, ex vivo analyses of T lymphocyte extravasation across an endothelial cell monolayer under physiological shear stress revealed that Nef potently disrupts T lymphocyte polarization and interfered specifically with the diapedesis step. The use of an Nef mutant (F195A) that is specifically deficient in PAK2 association, and thus inhibiting actin remodeling, but exerts all other known Nef activities (37, 40–43) demonstrated that Nef affects extravasation via a dual mechanism: although inhibition of subendothelial migration appears to be achieved via the known deregulation of actin dynamics, this mechanism was dispensable for the effects of Nef on T cell polarization during diapedesis (37). Because it remains unknown how the viral protein induces these effects, the goal of this study was to gain insight into the mechanisms and relevance of Nef-mediated disruption of T cell polarization and diapedesis.

Materials and Methods

Expression constructs

The plasmids encoding GFP fusion proteins of HIV-1SF2 Nef wild type (WT) and mutants thereof as well as the panel of expression constructs encoding for GFP or YFP fusion proteins of HIV-1, HIV-2, and SIV Nef alleles were described previously (10, 22, 27, 35, 44–47). The panel of expression constructs encoding for GFP or YFP fusion proteins of HIV-1, HIV-2, and SIV Nef (WT) and mutants thereof as well as the proviral plasmids inserting the respective alleles were described previously (10, 22, 27, 35, 44–47). The proviral plasmids encoding for GFP or YFP fusion proteins of HIV-1, HIV-2, and SIV Nef (WT) and mutants thereof as well as the proviral plasmid DNA into 293T cells, which were transfected with 15-cm2 culture dishes 1 day before transfection. Cells were centrifuged at 2000 rpm for 90 min at 37˚C and incubated 4–6 h at 37˚C and 300 µL of 2×SDS sample buffer. Forty microliters of the cell lysate were loaded per sample on a 14% SDS-PAGE. Membranes were probed against Nef.GFP to detect expression of Nef.GFP fusion constructs and transferrin receptor as an internal loading control.

HIV-1 production and infection

Virus stocks were generated by transfection (jetPEI, PEQLAB - Life Science) of proviral HIV-1 plasmid DNA into 293T cells, which were seeded in 15-cm2 culture dishes 1 day before transfection. Cells were centrifuged at 2000 rpm for 90 min at 37˚C and incubated 4–6 h at 37˚C and 5% CO2. Virus stocks were generated by transfection (jetPEI, PEQLAB - Life Science) of proviral HIV-1 plasmid DNA into 293T cells, which were seeded in 15-cm2 culture dishes 1 day before transfection. Cells were centrifuged at 2000 rpm for 90 min at 37˚C and incubated 4–6 h at 37˚C and 5% CO2. Virus titers were evaluated by p24 ELISA or SYBR Green I-based PERT (SG-PERT) assay, and infectivity was determined by flow cytometry (BD FACSVerses), and numbers of transfected cells, as well as total cell numbers, were adjusted by addition of untransfected cells. Cells were pelleted and directly lysed in 300 µL of 2×SDS sample buffer. Forty microliters of the cell lysate were loaded per sample on a 14% SDS-PAGE. Membranes were probed against Nef.GFP to detect expression of Nef.GFP fusion constructs and transferrin receptor as an internal loading control.

Polarization assay

Six hours posttransfection or 72 h postinfection, 3×10^5 A3.01 cells or monocyte-depleted, standard activated PBMCs were analyzed for polarization. To exclude dead cells from the analysis, they were stained with CMAC CellTracker dye (1 µM for 15 min at 37˚C) and discriminated from viable cells by the characteristic loss of the dye. Cells that were seeded in 100 µL of supplemented RPMI 1640 onto fibronectin-coated cover glasses (30 µg/mL 1 h at room temperature) and placed in the incubator for 2 h to allow polarization, followed by fixation with 3% paraformaldehyde in PBS (w/v) and incubated for another 15–30 min at room temperature. Cells were then permeabilized with 0.1% Triton X-100 for 2 min and blocked in 1% BSA in PBS (w/v) for 30 min at room temperature. Cover glasses were washed three times with 1× PBS, and incubation with the secondary goat anti-mouse Ab coupled to Alexa 647 (1:2000, in 1× PBS; Invitrogen) was carried out for 1 h at room temperature protected from light. After washing with 1× PBS, cover glasses were mounted using Mowiol, dried for 2 h at room temperature protected from light, and stored at 4˚C until analyzed by microscopy. Polarization of cells was typically quantified based on overall cell morphology [polymorphized cells have a polarization index (cell length/cell width) ≥ 2 (37)]. Analysis based on the enrichment of the uropod marker CD44 at one pole of the cell gave similar results (see Supplemental Fig. 1), and CD44 staining was included in most experiments as quality control.
**Mice**

C57BL/6 (JANVIER LABS) and LifeAct-GFP (50) mice were used in this study, and all the experiments were carried out in accordance with the standards approved by central animal facilities of the Universities of Heidelberg (G252/14 and T45/14) and Bern. Mice were used for the experiments at 5–10 wk of age.

**T lymphocyte homing to lymph nodes**

Effects of Nef on T lymphocyte homing to lymph nodes in mice were investigated by adoptive transfer of mouse T cells following transduction and sorting as described previously (37). Briefly, for virus production, 293T cells were transfected using jetPEI transfection reagent (PQELAB - Life Science) according to manufacturer’s instructions with 20 μg pSTITCH construct, 20 μg pHITα0, 5 μg pHIT123, and 5 μg amphotropic M-ENV. Viral supernatant was collected after 48 h, filtered (0.45-μm pore size filter; Roth), and used immediately for transduction of murine T cells.

Single-cell suspensions were obtained from spleen and peripheral lymph node by mechanical disruption (70-μm pore size filter; Roth), and used immediately for transduction of murine T cells.

**Ex vivo extravasation assay**

Transendothelial migration or extravasation assays were essentially performed as described previously (37). Isolation and culture of primary mouse brain microvascular endothelial cells (pMBMECs) from LifeAct-GFP mice was performed exactly as described before (51, 52). Cytokine stimulation of pMBMECs was done for 16–20 h with 10 ng/ml TNF-α. All experiments were performed in migration assay medium (DMEM, 5% calf serum, and 25 mM HEPES) at 37˚C. For live 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 CCL21 and placed on the heating stage of an inverted microscope (Axio Observer Z1; ZEISS) (53).

Primary murine T lymphocytes were activated, transduced, and NGFR-sorted as described above, and 1 × 10^6 cells/ml were labeled with Cell-Tracker dyes (CMTMR, 5 μM; CMAC, 20 μM; Deep Red, 0.5 μM; for 20 min at 37˚C). Dyes were exchanged between samples for successive replicates to exclude adverse effects of the dyes. The three differentially labeled cell populations were mixed 1:1:1 and allowed to accumulate at low shear stress (0 dyn/cm^2) for 4 min, then shear stress was increased to physiological stress (1.5 dyn/cm^2), and T lymphocytes were recorded over 20 min using a 10× objective with phase contrast and fluorescent illumination. Time-lapse videos were created from one frame every 10 s. Adherence to the endothelium was calculated based on the ratio of input cells, precisely defined by flow cytometry (FACSCalibur; BD Biosciences), relative to the ratio of cells adhered to the endothelium immediately after the increase of shear stress. Cells were tracked and phenotypes counted manually using a customized ImageJ Annotation Plugin developed by J.-P. Bergeest (Karl Rohr Laboratory, Heidelberg), which combines the ability of the Cell Counter plugin and the Manual Tracking plugin. We differentiated the following: 1) cells crawling on top of the endothelium, 2) diapedesis, 3) migration under the endothelium, 4) cells that detached, and 5) cells crawling out of view. Exported tracks were filtered according to the phenotype annotations using Microsoft Excel to calculate relative frequencies of phenotypes. Percentage of cells staying on top of the endothelium were calculated by dividing the number of cells defined as action 1, which do not show other behaviors during the course of the experiment (cells that migrate out of view on top of the endothelium were not taken into account), by the total number of tracked cells ([no. 1 – no. 2 – no. 4 – no. 5]/ total no. tracks). Percentages of cells that were washed off and that undergo diapedesis were calculated by dividing the number of tracks with actions 4 and 2 (cells that migrate out of view afterward are also counted) by the total number of tracked cells, respectively. Polarization index of cells migrating on top of the endothelium was calculated by dividing the cell length by the cell width, measured manually with Fiji (37).

**Image acquisition, analysis, and quantification**

Cell polarization was assessed using an epifluorescence microscope (IX81; Olympus). Cells were considered polarized when cell length exceeded cell width by at least a factor of 2. At least 100 transfected cells or 30 infected cells were evaluated per experiment and condition. Polarization of an equal number of untransfected or infected neighboring cells on the same coverslip was also determined, and values of transfected/injected cells were normalized to this internal control.

For time-lapse microscopy videos, A3.01 cells were transduced and 6 h later, stained with the PKH26 membrane dye and seeded on fibronectin-coated, eight-well chambered cover glasses (Lab-Tek). Live microscopy was performed with a Nikon Ti PerkinElmer UltraView VoX Spinning Disc Confocal Microscope equipped with a perfect focus system, a 60× oil numerical aperture = 1.49 objective, Hamamatsu ORCA Flash.4.0 scientific complementary metal-oxide-semiconductor camera, and an environmental control chamber (37˚C, 5% CO2). Each frame contained a Z-stack with 1 μm of spacing. Acquisition of approximately 1 frame/min resulted in time-lapse videos of ~1 h length. For representation of change in cell morphology over time, all single frames of one video were color coded and projected into one image.

**Power spectrum analysis**

Morphological dynamics and modes of deformation have been analyzed by binarized time-lapse videos according to previous power spectrum analyses assessing the energy consumption by shape change of mononuclear cells (54–56) and anuclear cells (57, 58). The peripheral edge of the cells was defined in each single frame and plotted in polar coordinates with the center of mass being the origin and r the radial distance to the edge of the cell. Morphological dynamics of each target cell were analyzed through the radial distance r as a function of angle θ = 0–360˚ and time t.

The deformation was defined as the normalized radial distance:

$$R(\theta, t) = \frac{R_t(\theta, t)}{R_t(0, t)}$$

The characteristic spatiotemporal patterns were identified by calculating the autocorrelation function (ACF) of R(0,0):$

$$\Gamma_{RR}(\Delta \theta, \Delta t) = \frac{\langle R_t(\theta + \Delta \theta, t + \Delta t) \cdot R_t(\theta, t) \rangle}{\langle R_t(\theta, t) \rangle^2}$$

Note that isotropic expansion/contraction (m = 0) and translational motion (m = 1) were excluded from the deformation analysis because of the normalization to $(R_t(0, t))_0$ and the center of mass being the origin in the inertial frame. Thus, $\Gamma_{RR}$ is presented in units of min$^{-1}$, as a dimensionless average over time. Because the power spectral density is proportional to the mechanical power dissipated by morphological dynamics (59), this approach allows the comparative analysis of cell deformation under different conditions. To assess the energy consumed by these processes, the power spectral density $\Gamma_m$ was calculated via spatial frequency domain analysis. In this equation, the deformation R(θ, t) was expanded in a Fourier series over spatial modes

$$\Gamma_m = \langle FT_\theta FT_t(R_t(\theta, t)) \cdot FT_t(R_t(\theta, t)) \rangle.$$

The deformation of cells involves active, energy-consuming processes such as the bending of membranes, the rearrangements of lipids and proteins, and, most importantly, the remodeling of cytoskeletal structures. To assess the energy consumed by these processes, the power spectral density $\Gamma_m$ was calculated via spatial frequency domain analysis. In this equation, the deformation R(θ, t) was expanded in a Fourier series over spatial modes

$$\Gamma_m = \langle FT_\theta FT_t(R_t(\theta, t)) \cdot FT_t(R_t(\theta, t)) \rangle.$$
cells were spin-inoculated for 90 min in the presence of polybrene with sucrose cushion–concentrated, vesicular stomatitis virus glycoprotein G–pseudotyped lentiviral particles produced in 293T in saturating amounts [see (61) for details]. Transduced cells were directly subjected to Seahorse measurement 48 h after transduction. Transduction/transfection efficiency at the time of the Seahorse measurement was analyzed by FACS and ranged between 30–60%. Ninety-six–well plates for Seahorse measurement were coated with fibronectin (12 µg/ml in PBS equals the same final concentration as on the cover glasses) and air dried overnight. 7.5 × 10^5 transduced/transfected cells were seeded per well in Seahorse medium supplemented with 10 mM glucose and 2 mM L-glutamine and measured in a Seahorse XF96 Analyzer (Agilent Technologies) using the standard Agilent Seahorse Instrument Protocol settings. Total duration of measurement was ∼140 min. After three successive measurements of baseline oxygen consumption rate (OCR; basal respiration) and extracellular acidification rate (ECAR; glycolysis), 2 µM of oligomycin (inhibits ATP synthase/complex IV) were injected (block of mitochondrial respiration) and measured three times, followed by injection of 1.5 µM carbonyl cyanide-4 (trifluoromethoxy)phenylhydrazone (FCCP; uncoupling agent that collapses the proton gradient and disrupts the mitochondrial membrane potential, resulting in maximal oxygen consumption, which equals maximal respiration). After three measurement steps, 1.5 µM of rotenone were injected (complex I inhibitor, shuts down mitochondrial respiration, which equals non mitochondrial respiration), followed by six measurement steps. Four to eight replicate wells were analyzed per experiment.

Software and statistical evaluation
Data analysis and statistical calculations were performed using Microsoft Excel and GraphPad Prism. Image editing was carried out using Adobe Photoshop and Illustrator CS4 or CS6. Video editing was performed with Fiji. Statistical significance was calculated by Mann–Whitney U test analysis (***p < 0.001, **p < 0.01, *p < 0.05).

Results
Nef affects polarization of HIV-1–infected primary human CD4+ T lymphocytes
Our previous results had identified that isolated expression of HIV-1 Nef impairs the polarization of mouse CD4+ T cells during the extravasation process (37). To test the potential relevance of this observation for HIV infection, we infected activated primary human CD4+ T lymphocytes with HIV-1 WT (HIV-1 NL4.3 carrying the nef gene of HIV-1NL4.3) and monitored for 10 min (Fig. 1A, top panel) were considered to be nonpolarized, polarization state of cells was typically assessed by the overall cell morphology. Cells were considered polarized when formation of CD44-rich uropod and leading edge structures were apparent in elongated cells (polarized cells have a polarization index [cell length/cell width] > 2; Fig. 1A, middle panel), whereas cells of round shape (Fig. 1A, top panel) were considered to be nonpolarized. Because the overall percentage of uninfected polarized cells slightly varied between donors but also between individual samples of cells from the same donor (typically ranging between 40 and 60% of cells), the percentage of polarized cells positive for p24CA was determined relative to that of uninfected neighboring cells (Fig. 1B). Polarization of cells productively infected with HIV-1 WT was markedly reduced for all donors analyzed with differences in the magnitude of this effect between cells from individual donors (60–60% of uninfected cells) (Fig. 1B, 1D). Although scoring slightly divergent overall amounts of polarized cells, quantification of cell polarization based on by the distribution of the uropod marker CD44 confirmed this pronounced interference of HIV-1 infection with CD4 T cell polarization (Supplemental Fig. 1). Notably, disruption of T cell polarization was markedly less pronounced for cells infected with HIV-1 ΔNef (Fig. 1B, 1D). Depending on the donor (D), the lack of Nef in cells infected with HIV-1 ΔNef resulted in partially (e.g., D1, D2, D5, D6, and D7) or fully restored polarization rates (D3 and D4) compared with uninfected control cells. These results identify Nef as a key viral determinant for disruption of polarization of HIV-1–infected CD4+ T lymphocytes but also suggest that additional viral parameters that can affect cell polarization may exist. Because Nef and Vpu share a number of biological activities (15, 16, 62), we tested the involvement of Vpu in polarization disruption (Fig. 1C, 1D). In these experiments, cell polarization was unaffected by the presence of Vpu, and a lack of Nef expression was associated with polarization frequencies that were virtually indistinguishable from uninfected cells. We conclude that Nef is the central viral determinant governing the disruption of CD4+ T cell polarization in the context of HIV-1 infection.

Disruption of CD4+ T cell polarization is a conserved activity of lentiviral Nef proteins
We next tested if expression of Nef alone is sufficient to disrupt CD4+ T cell polarization and transiently expressed GFP or an Nef, GFP fusion proteins that was previously characterized as functional equivalent of nonfusoin Nef (22, 35, 63, 64) in A3.01 T cells (Fig. 2). Although <50% of GFP-expressing control cells polarized, polarization efficiency was markedly reduced for cells expressing HIV-1ΔNef.GFP (Fig. 2A, 2B). This experimental system allowed us to screen a panel of HIV-1, HIV-2, and SIV Nef.YFP proteins for their ability to disrupt T cell polarization. When segregated into Nef proteins with the ability to downregulate cell-surface CD3 (group 2) versus Nef proteins that lack this activity (group 1) (27, 46), T cell polarity disruption was pronounced and well conserved among group 2 Nef proteins. Most group 1 Nef proteins also significantly reduced T cell polarization, albeit with slightly lower efficacy (Fig. 2C). Nef from HIV-1 YBF30, which is also deficient in rerouting the TCR proximal kinase Lck and SIVcpz Tan 3, which is defective in most Nef activities, including TCR-induced actin remodeling (46, 65), were impaired in affecting CD4+ T cell polarization. Nef from SIVcpz Gab2 also displayed weak polarity disrupting activity but is fully active for other Nef activities (27, 46, 65). Besides these exceptions, the ability to disrupt CD4+ T cell polarization appears to be a conserved feature of functional lentiviral Nef proteins.

Disruption of CD4+ T cell polarization requires the interaction surface with the Nef-associated kinase complex
Next, we sought to determine the molecular surfaces in Nef required for disruption of CD4+ T cell polarity. To this end, we tested a panel of HIV-1ΔNef.GFP proteins in which individual functional motifs are disrupted (Fig. 3A, 3B). Residues required for phosphorylation of Nef (serine 6 to alanine, S6A) (47), downregulation of cell surface MHC class I molecules (M20A) (66), or with the clathrin endocytosis machinery (LL1586/1609AA, ED1767/1770AA) (67) were dispensable for T cell polarity disruption and amino acid exchanges that reduce the ability of Nef to interact with SH3 domains (E4616/4620A, VGF70.72AA) (40, 45, 68) only moderately reduced the disruption of polarization as the activity of these mutants was not statistically significant from WT Nef. As for most Nef activities (2), myristoylation of glycine 2, and thus efficient interaction with cellular membranes, was essential for T cell polarity disruption (G2A). In line with our previous study, mutation of the PAK2 interaction motif (F195A) resulted in loss of polarity interference by Nef (37). Similarly, mutation of the SH3 domain interaction surface in Nef, which also disrupts NefPAK2 association but also causes defects in many other Nef functions (P76xxP79 mutated to alanine, AxxA) (7, 69), impaired T cell polarization disruption by Nef. Importantly, the deletion of the interaction surface with the Nef-associated kinase...
complex (NAKC; Nef mutant Δ12–39) also abrogated the ability of Nef to interfere with T cell polarization. This relevance of PAK2 and NAKC interaction surfaces was also observed in the context of HIV-1 infection of activated primary human CD4⁺ T lymphocytes (Fig. 3C, 3D). Because Nef Δ12–39 reduces TCR-induced actin remodeling (22) and abrogates T cell chemotaxis (data not shown), the requirement for the NAKC interaction for Nef-mediated disruption of T cell polarity suggests that inhibition of actin remodeling and cell polarization involve distinct mechanisms.

Nef prevents oscillation of CD4⁺ T cells between polarized and nonpolarized states

To gain more insight into how Nef affects T cell polarization, we recorded the morphological dynamics of A3.01 T cells transiently expressing GFP or HIV-1ΔNef.GFP on fibronectin-coated surfaces by live-cell spinning-disc microscopy (Fig. 4). Over the 60-min recording time, GFP-expressing control cells underwent frequent morphological changes and thus oscillated between polarized and nonpolarized states (Fig. 4A, also see Supplemental Video 1). These morphological dynamics were quantitatively assessed by the spatiotemporal analysis of active deformation (54). As presented in Fig. 4B, the peripheral edges of a GFP-expressing cell monitored over time imply that the cell undergoes a rigorous shape deformation after receiving a polarization cue. The ACF of the GFP-expressing cell exhibits several characteristic features, as indicated in Fig. 4C: ① indicates ACF that shows two distinct minima that are separated by $\Delta \theta = 180^\circ$, suggesting an elliptic deformation; ② indicates the position of minima that shows a drift over time, corresponding to the continuous rotation of an elliptic cell; and ③ indicates the positions of minima “jump” (e.g., $\Delta \theta_{\text{jump}} = 90^\circ$) with a
characteristic time interval of $\Delta t \approx 10$ min, indicating that the direction of elliptic deformation periodically changes. In contrast, T cells expressing Nef.GFP exhibit no major shape deformation (Fig. 4D). In fact, the calculated ACF (Fig. 4E) is featureless, and the uniformly high correlation over $\Delta t$ and $\Delta \theta$ clearly implies that cells expressing Nef.GFP adopt a round shape over time.

In general, the deformation of cells is an active, nonequilibrium process accompanied by energy consumption (54–56). To quantitatively assess energy consumption by cell deformation in individual cells, we calculated power spectra (Fig. 4F) following Eq. 2. The power spectrum of GFP-expressing cells (black) exhibits a clear peak at $m = 2$. The contribution of higher modes, such as $m \approx 5$, is negligibly small. This confirms that polarizing, GFP-expressing cells predominantly undergo an elliptic deformation. In contrast, the power spectrum in the presence of Nef.GFP (red) is much less pronounced, suggesting Nef.GFP-expressing cells do not consume the energy required for shape deformation of GFP-expressing control cells. In fact, the calculated total power $\sum_{m=1}^{\infty} P_m$ demonstrated that the median energy dissipated through shape deformation of Nef.GFP-expressing cells is 4.5-fold lower compared with that of GFP-expressing T lymphocytes over the 1-h observation period (Fig. 4G). The disruption of dynamic morphological T lymphocyte polarization by HIV-1 Nef is, therefore, paralleled by a reduction in energy consumption at time when cell polarization would typically be initiated.

To test whether this reduced energy consumption of individual Nef.GFP-expressing cells reflects a global effect of the viral protein on T cell metabolism, we analyzed the OCR of mitochondrial respiration and the ECAR of glycolysis performing a mitochondrial stress test in bulk cultures of GFP-expressing control and Nef.GFP-expressing cells (Fig. 4H, Supplemental Fig. 2). A3.01 cells were cotransfected with expression plasmids for GFP or Nef.GFP and pDisplay.YFP, sorted using GFP microBeads and plated onto Fibronectin-coated Seahorse microplates. OCR and ECAR were measured at various time points in bulk cells before treatment, upon oligomycin, FCCP, and rotenone treatment, as highlighted in Fig. 4H and Supplemental Fig. 2 (arrows), to assess basal respiration, ATP production, proton leak, maximal respiration, spare capacity, and nonmitochondrial respiration, as indicated (Fig. 4H).

No significant differences between GFP- and Nef.GFP-expressing cultures were detectable in mitochondrial respiration (Fig. 4H) and glycolysis (Supplemental Fig. 2). We conclude that although the disruption of cell polarization by Nef is accompanied by reduced energy consumption in individual cells at the initiation of cell polarization, Nef does not imprint general metabolic changes in CD4 $^+$ T lymphocytes.

Plotting the duration of each phase of polarized/nonpolarized morphology revealed that GFP-expressing control cells displayed polarized morphology for the majority of the time analyzed (Fig. 5A, 5C, 5G). In contrast, Nef.GFP-expressing cells almost played polarized morphology for the majority of the time analyzed upon oligomycin, FCCP, and rotenone treatments (Fig. 5B), cells expressing Nef.G12–39 had a tendency to display more expanded periods of polarization; however, this difference was not statistically significant to Nef/F195A (Fig. 5G). Expression of Nef, hence, reduces both the frequency with which T cells undergo polarization as well as the duration of intervals with polarized cell morphology in a manner that depends on the interaction surface with NAKC and PAK2. Disruption of CD4 $^+$ T cell polarity by Nef correlates with the ability to interfere with T cell extravasation

In our previous work, we showed that the inability of Nef-expressing T cells to polarize was associated with their failure to undergo diapedesis (37). Therefore, we tested how the NAKC-binding-deficient mutant Nef.D12–39 would score in these assays. To this end, we made use of a previously established experimental system in
which mouse CD4+ T lymphocytes are transduced with MLV vectors with nefRESΔngfr expression cassettes, sorted via NGFR cell-surface expression, and labeled with different CellTracker dyes. This approach has previously been validated to allow for functional analysis of homogeneously Nef-expressing primary mouse T lymphocytes in which Nef is expressed to similar levels and exerts its activities comparably to Nef expressed in the context of HIV-1 infection of primary human T lymphocytes (37). Cells were mixed and perfused over a pMBMEC monolayer overlaid with CCL21 to image the extravasation process by video microscopy over 20 min (Fig. 6A, Supplemental Video 2). Frame-by-frame offline analysis of cell behavior under flow enabled us to dissect which step of the extravasation process is influenced by Nef. As observed previously (37), Nef expression had no effect on the cells’ ability to arrest on CCL21-overlaid pMBMECs and Nef-expressing cells resisted detachment equally well during an observation period of 20 min (Fig. 6A, 6E [detached]) but resulted in a marked reduction of cell polarization on the endothelium (Fig. 6C, 6D). Nef expression also induced a mild, but statistically significant, reduction in the number of overall diapedesis events (Fig. 6E, diapedesis) and a delay in transmigration kinetics (Fig. 6F). Interestingly, polarization and diapedesis of cells expressing NefΔ12–39 were virtually indistinguishable from that of control cells (Fig. 6C–F), indicating that disruption of the NAKC-binding site in Nef results in a loss of its ability to interfere with CD4+ T cell polarization and diapedesis.

Disruption of CD4+ T cell polarity by Nef correlates with the ability to interfere with T cell homing to lymph nodes

Finally, we sought to assess whether the disruption of T cell polarization and diapedesis may be linked to the ability of Nef to restrict T lymphocyte motility in vivo. To this end, mouse CD4+ T lymphocytes were transduced, sorted via NGFR cell-surface expression, and then adoptively transferred into recipient mice as established previously (37). Transduced and sorted cell populations were labeled with cell-tracing dyes, and control T cells were mixed with WT or mutant Nef-expressing T cells. Nef WT and NAKC-binding deficient NefΔ12–39 were compared with Nef F195A, which is deficient in preventing T cell actin remodeling and lymph node homing (37). Nef-expressing cells were mixed with control cells and adoptively cotransferred into C57BL/6 recipient mice, and a small fraction of cells from each sample were analyzed by flow cytometry to estimate the input ratio that was used to normalize the homing efficiency. Four and twenty-four hours after cell transfer, spleen, peripheral, and mesenteric lymph nodes were harvested and analyzed independently for homing efficiency by flow cytometry. In line with our previous results (37), Nef expression did not have any statistically
significant effect on T cell recruitment to spleen, which does not require the crossing of an endothelial barrier (70) (data not shown). In contrast, homing of Nef-expressing T lymphocytes to peripheral and mesenteric lymph nodes was markedly reduced at 4 h (42 ± 16% or 40 ± 16% residual homing compared with control) and 24 h (45 ± 20% and 41 ± 23% residual homing compared with control) posttransfer. As shown previously, T lymphocytes expressing Nef F195A only displayed a mild reduction of homing efficiencies 4 h posttransfer, whereas homing at 24 h was slightly enhanced as compared with control cells (37). In contrast, T lymphocytes expressing Nef Δ12–39 displayed full homing potential as early as 4 h posttransfer and were indistinguishable from control cells at both time points analyzed. NAKC and PAK2 interaction surfaces are thus both critical for the ability of Nef to interfere with CD4+ T lymphocyte trafficking in vivo.

Discussion
The goal of this study was to gain insight into the actin-remodeling independent mechanism by which the HIV-1 pathogenesis factor Nef impairs extravasation of CD4+ T lymphocytes at the diapedesis step. We find that Nef potently restricts the polarization of infected primary, as well as immortalized-transfected, CD4+ T lymphocytes on adhesive substrates and results in rounded, instead of elongated, cell morphology. Because this effect was not observed in cells with nef-deficient HIV, and isolated expression of Nef alone impaired cell polarization, Nef is necessary and sufficient for disruption of CD4+ T lymphocyte polarity. Polarity disruption resulted from arrest of dynamic changes in T lymphocyte morphology and was paralleled with a reduced need for energy to support these alterations in cell morphology. In contrast, mitochondrial respiration was not globally altered in Nef-expressing CD4+ T cells. Mapping studies revealed the binding site for NAKC as important molecular determinant for this Nef activity. NAKC-binding–deficient Nef, hence, did not disrupt T cell polarization of CD4+ T lymphocytes primed for

**FIGURE 4.** Interference of Nef with T cell polarity oscillation is associated with significant reduction in energy consumption. (A) A3.01 cells were transfected with the GFP or Nef.GFP expression plasmids, stained with the PKH26 membrane dye 6 h later, and seeded on fibronectin-coated MatTek dishes for live-cell spinning-disc microscopy. Acquisition of one frame every 1 min results in time-lapse videos of ~1 h in length. Time projection of Supplemental Video 1 shown as temporal color code. Scale bar, 5 μm. (B) Tracking of the cell periphery and (C) the corresponding autocorrelation map of a GFP-expressing T cell, showing a pronounced shape deformation. (D and E) Corresponding data set for a T cell expressing Nef.GFP, showing a drastic suppression of shape deformation. (F) Power spectra of T cells expressing GFP (black) or Nef.GFP (red). GFP-expressing cells predominantly undergo an elliptic deformation (m = 2). (G) Total power of deformation for T cells expressing GFP (black) and Nef.GFP (red). In case of Nef.GFP, the median energy consumption by active cell deformation decreased by a factor of 4.5 compared with GFP-expressing cells. Each dot represents one cell. Shown are box plots with median values and range. (H) Mitochondrial stress test of transfected and GFP microBead–sorted A3.01 cells seeded onto fibronectin-coated Seahorse microplates and measured in a Seahorse XFe analyzer to assess mitochondrial respiration. Time points of drug injection (arrows), as well as the respective parameters that are assessed by the drug treatments, are indicated. Shown are the mean OCR with SD from four to five replicate wells derived from one out of three representative experiments. Transfection efficiencies were 80 and 56% for GFP and Nef.GFP, respectively.
FIGURE 5. Determinants in Nef for association with NAKC as well as PAK2 reduce T cell polarity oscillations. (A) Time projection of live-cell spinning-disc microscopy videos of A3.01 cells transiently expressing the indicated proteins as in Fig. 4A. Scale bars, 5 μm. (B) Western blot analyses of the cells shown in (A) adjusted for similar amounts of GFP-positive cells and probed for GFP and transferrin receptor (TIR). (C–F) Polarization plots of A3.01 cells analyzed as in (A) over a 1-h observation period. Polarization (black) and no polarization (white) periods for individual cells are depicted over the acquisition time (in minutes). Fifteen cells were analyzed per condition. (G) Relative time in a polarized morphology of the cells shown in (C)–(F). Shown are mean values with SD. The p values have been calculated using a one-way ANOVA with Holm Sidak multiple comparison test. **p < 0.01, ***p < 0.001.
FIGURE 6. The NAKC interaction site in Nef is critical for Nef-mediated interference of T-cell extravasation and homing to lymph nodes. Twenty-four hours posttransduction and sorting, primary T lymphocytes were labeled with one of three different CellTracker dyes (CMTMR, red; Deep Red, green; CMAC, blue), mixed in equal amounts, and applied to a TNF-α-stimulated, CCL21-overlaid primary murine brain endothelium under low shear stress (0.1 dyne/cm²). After 4 min, cell attachment had occurred, shear stress was increased (1.5 dyne/cm²), and extravasation was monitored over 20 min with one image acquired every 10 s. (A) Still images of Supplemental Video 2 showing a representative field of view of an extravasation experiment. Shown is an endothelial cell monolayer with control, Nef WT, and Nef Δ12–39-expressing T lymphocytes, pseudocolored in green, red, and blue, respectively. Colored lines highlight migration tracks of an Nef-expressing cell (red line), two control cells (dark green line, light green following diapedesis), and an Nef Δ12–39-expressing cell (dark blue line, light blue following diapedesis). Scale bar, 10 μm. Images were acquired using a 10× objective. (B) Relative efficiency of arrest on the endothelium under shear stress. Shown is the percentage of input cells that attached to the endothelial cell monolayer at the time point when higher shear stress rates are first applied. (C) Still image of a representative extravasation time-lapse video with indicated measurements of cell length and width as used for calculation of polarization indices (calculated by dividing cell length by cell width). (D) Polarization (Figure legend continues)
extravasation under shear stress, did not restrict subsequent diapedesis, and failed to affect T lymphocyte homing to lymph nodes. These results suggest disruption of T cell polarization as a novel mechanism by which HIV-1 Nef impairs T lymphocyte motility in vivo and identifies the NAKC binding site as critical determinant for this activity.

A central finding of this study was that disruption of T cell polarity by Nef correlates with the inability of these cells to undergo efficient extravasation and homing to lymph nodes. Considering that 1) morphological plasticity and polarization is a well-established prerequisite for T cell diapedesis (39), and 2) extravasation represents an essential step in the homing process (38), it is conceivable that polarity disruption directly contributes to the reduced homing potential of Nef-expressing CD4+ T lymphocytes. Conceivably, Nef-induced polarity disruption results in impaired diapedesis, and this effect likely synergizes with the reduction of host cell actin dynamics, which in turn, limits intralymph node motility of cells that successfully underwent diapedesis (37).

Consistently, protein interaction motifs essential for interaction of Nef with NAKC (Δ12–39) and for interference with actin remodeling (F195A) are both required for Nef’s ability to interfere with lymph node homing. However, cells expressing NAKC-binding–deficient Nef Δ12–39 homed to lymph nodes with efficiencies comparable to that of control cells already early after adoptive transfer (4 h), whereas Nef F195A–expressing cells reached full homing efficiency, or are even slightly enriched, after 24 h. Temporally altered homing efficiencies may result from a concerted residual impairment of homing and egress by the F195A, but not Δ12–39, mutant of Nef. Of note, mutation of either the PAK2 or NAKC interaction motif in Nef abrogated its ability to disrupt T cell polarization, but only Nef Δ12–39 had lost the ability to block diapedesis. This indicates that Nef, via its PAK2 as well as NAKC binding sites, targets essential, but distinct, aspects of morphological T lymphocyte polarization. However, only interactions occurring via the NAKC binding surface lead to functional disruption and thus compromise diapedesis.

These findings raise the question about the molecular mechanism by which the NAKC binding site enables Nef to disrupt T cell polarity and extravasation. An obvious hypothesis is that this reflects the association of Nef with NAKC. NAKC assembly is best known to mediate the enhancement of HIV transcription (19, 47, 71–73) as well as the release of extracellular vesicles with proinflammatory molecules (74, 75) by Nef. These effects are likely unrelated to T lymphocyte polarization and diapedesis. However, with Lck, PI3-K, and PKC θ (19, 47, 76), NAKC contains at least three kinases with known roles in T lymphocyte polarization toward the immunological synapse (77, 78), and the association with NAKC enables Nef to affect integrin signaling (72, 79) and thus a central pathway–regulating cell polarity. Our attempts to test the relevance of individual NAKC components for the disruption of T cell polarization by silencing their expression and/or pharmacologically inhibiting their activity were hampered by the interference of these measures with cell polarization even in the absence of Nef, which precluded meaningful analysis (data not shown). Dissecting whether NAKC is directly involved in these effects of Nef will therefore require the identification of mutants that uncouple intrinsic roles in T cell polarity from Nef-mediated disruption thereof. Because the NAKC binding site is recognized as an important determinant for an increasing number of Nef activities, including antagonism of the host cell restriction factor SERINC5 (29) and promoting CD4+ T cell depletion in ex vivo cultures of human tonsils (80); the 27-aa stretch deleted in Nef Δ12–39 may also mediate additional, yet unknown, interactions with cellular ligands other than NAKC.

An additional observation made in the course of this study is that the perceived disruption of cell polarization by Nef at any given time point reflects the inhibition of frequent morphological oscillations of T lymphocytes between elongated and rounded shapes, which expression of Nef arrests at the rounded stage. Our measurements revealed that this reduction in morphological alterations is associated with a reduced requirement for energy in individual cells when shape changes are initiated, which, however, does not translate into appreciable differences in the metabolism of the corresponding cell culture when analyzed in bulk. This seeming discrepancy likely reflects that cell polarization of individual cells in the culture occurs in an asynchronous manner, and differences imprinted by Nef in the few cells in which polarization is initiated per time point cannot be detected on the background metabolism of the bulk of cells in the culture. Importantly, however, this indicates that effects of Nef on energy consumption are limited to phases of cell shape transition, suggesting that Nef is not a major determinant of HIV-induced alterations of T lymphocyte metabolism (81–84). Whether Nef induces specific mechanisms to deplete energy to prevent morphological oscillations, or the reduced energy consumption at phases of shape transition is a consequence of the inhibition of host cell vesicular trafficking and cytoskeleton dynamics by the viral protein remains to be determined.

In sum, this study defines disruption of T cell polarity and extravasation as a novel activity of lentiviral Nef proteins. This mechanism is essential for the ability of the viral protein to interfere with T lymphocyte homing to lymph nodes. The fact that Nef contains two molecular surfaces that interfere with lymph node homing via independent mechanisms and the evolutionary conservation of this activity suggests that this activity may be critical for the role Nef plays in AIDS pathogenesis. Interference of Nef with homing by T cell polarity disruption may reduce the frequency of HIV-infected cells in distal areas of the lymph node and thereby locally enrich HIV-infected cells in the T cell–rich paracortex to facilitate viral spread and/or immune evasion of infected cells (36).
The authors have no financial conflicts of interest.

References


