The Nef protein is a key determinant of human immunodeficiency virus (HIV) pathogenicity that, among other activities, sensitizes T-lymphocytes for optimal virus production. The initial events by which Nef modulates the T-cell receptor (TCR) cascade are poorly understood. TCR engagement triggers actin rearrangements that control receptor clustering for signal initiation and dynamic organization of signaling protein complexes to form an immunological synapse. Here we report that Nef potently interferes with cell spreading and formation of actin-rich circumferential rings in T-lymphocytes upon surface-supported TCR stimulation. These effects were conserved among Nef proteins from different lentiviruses and occurred in HIV-1-infected primary human T-lymphocytes. This novel Nef activity critically depended on its Src homology 3 domain binding motif and required efficient association with Pak2 activity. Notably, whereas overall signaling microcluster formation immediately following TCR engagement occurred normally in Nef-expressing cells, the viral protein inhibited the concomitant activation of the actin organizer N-Wasp. During the subsequent maturation phase of the stimulatory contact, Nef interfered with the translocation of N-Wasp to the cell periphery, the overall induction of tyrosine phosphorylation, and the selective recruitment of phosphorylated LAT to stimulatory contacts. Consistent with such a critical role of N-Wasp in this process, Nef also blocked morphological changes induced by the known N-Wasp regulators Rac1 and Cdc42. Together, our results demonstrate that Nef facilitates immune evasion of infected cells and substantially increases virus replication in vivo (1–4). Whereas the underlying molecular mechanisms remain to be fully elucidated, Nef facilitates immune evasion of infected cells and directly boosts virus spread. These effector functions probably reflect the ability of Nef to serve as a protein-interaction adaptor that modulates cellular vesicle transport and signal transduction machineries (5, 6). In CD4+ T-lymphocytes, one of the major HIV-1 target cell populations in vivo, Nef lowers the threshold of TCR activation possibly to induce an intermediate activation state that is permissive for HIV-1 replication (7–12). Several protein assemblies, including the association with the Nef-associated kinase complex, the guanine exchange factors Vav and DOCK2-ELMO1, and the TCR 𝜖 chain, are involved in the modulation of TCR signaling by Nef (13–16). However, it has not been addressed how Nef affects early events of TCR signal initiation. Physiologically, TCR triggering occurs in the context of a close contact between a T-cell and antigen-presenting cell referred to as the immunological synapse (IS). Within this highly dynamic structure, spatial redistribution of select cell surface receptors leads to receptor clustering as well as subsequent recruitment and activation of receptor-proximal signaling machineries, and this then initiates signal transduction (17, 18). Whereas stable synapse formation is not an absolute prerequisite for signaling (19), the profound actin rearrangements that parallel IS formation conceivably drive segregation of surface receptors and receptor-proximal signaling machineries to modulate, amplify, and stabilize output signals.

In a well established experimental system for studies of early TCR signal transduction events, incubation of T-cells on TCR-stimulatory surfaces leads to the formation of lamellipodia and filopodia cell protrusions that contact the substratum to cause rapid cell spreading. This process is paralleled by marked rearrangements of F-actin into a pronounced circumferential actin-rich ring (20–22). Initial contact with the stimulatory surface triggers immediate clustering of TCR molecules and proximal machinery into microclusters at the contact sites in the cell center, leading to rapid induction of tyrosine phosphorylation and increase of cytoplasmic Ca2+ levels. This experimental system thus reflects the hallmarks of TCR signal transduction, closely parallels the dynamic events triggered by major histocompatibility class I-mediated antigen presentation on planar bilayers, and is particularly well suited for the time-resolved analysis of actin dynamics upon TCR engagement (23–25).
utes, maturation of cell-substratum contacts by cell spreading is paralleled by the continuous induction of such clusters at the cell periphery. Recent data suggest an important role of peripheral as well as central microclusters for initiation and duration of TCR signals (26). Presumably reflecting the physical association of the TCR with the actin cytoskeleton, microcluster formation requires intact F-actin filaments. Experimental disruption of F-actin prior to TCR stimulation blunts signal transmission (20, 27, 28). Consistent with such a role of actin dynamics, the Wave2 actin-organizing complex was recently demonstrated to play a critical role in morphological changes and signal transduction on TCR-stimulatory surfaces (29, 30).

**EXPERIMENTAL PROCEDURES**

**Cells and Reagents**—293T and Jurkat TAg cells were cultivated in Dulbecco’s modified Eagle’s medium and RPMI 1640 medium, respectively, supplemented with 10% fetal calf serum, 1% 1-glutamine, and 1% penicillin-streptomycin (all from Invitrogen). For T-cell spreading, the mononclonal antibodies anti-CD3 (clone HIT3a) and anti-CD28 (clone CD28.2) were used (both from BD Pharmingen). Further analyses were performed with the following antibodies: polyclonal rabbit anti-p24CA (31), rabbit anti-phosphotyrosine (BD Pharmingen), rabbit anti-phospho-Zap70, rabbit anti-Pak1/2/3 (both from Cell Signaling Technology), rabbit anti-phospho-LAT (Upstate Technology, Inc.), rabbit anti-phospho-N-Wasp (Abcam), and monoclonal antibodies phycoerythrin and allophycocyanin-conjugated anti-CD3 (clone SK7 and HIT3a, respectively) (BD Pharmingen). The allophycocyanin-conjugated anti-mouse antibody was from Jackson ImmunoResearch, and all other secondary fluorescent antibodies were purchased from Molecular Probes. For F-actin stain tetramethylrhodamine isothiocyanate-conjugated (Sigma and Cytoskeleton) or Alexa Fluor 660-conjugated (Molecular Probes), phalloidin was used. The N-Wasp inhibitor wiskostatin was obtained from Calbiochem.

**Expression Constructs**—Most expression constructs for Nef.GFP fusion proteins and the bicistronic Nef expression vectors as well as the HIV-1 wild type and Δnef proviruses along with the vesicular stomatitis virus glycoprotein expression plasmid were described recently (32). The expression constructs for GFP fusion proteins for the Δ12–39 and Δ12–39/EDAA Nef variants were generated accordingly. To create the expression construct for Nef.RFP, the gene encoding for mRFP1 was amplified using two newly introduced 5’/H11032 and 3’/H9262 NotI sites. The amplifed pRSETB mRFP1 as template (kindly provided by Roger Tsien) (33). The amplified nef gene was used to replace the egfp gene in the pEGFP-N1 vector (Clontech) by using two newly introduced 5’ BamHI and 3’ NotI sites. The resulting pmRFP-N1 expression vector has intermediate sequences between the multiple cloning site and the fluorescent gene open reading frame identical to those of the pEGFP-N1 vector, as has been confirmed by sequence analysis. The HIV-1Δ52 nef gene was subsequently introduced via BglIII and EcoRl, resulting in the vector pmRFP-Nef. Correct expression of RFP and Nef.RFP was confirmed using confocal microscopy. Co-expression of Nef.GFP and Nef.RFP in CHO cells resulted in a very pronounced colocalization of both proteins (Fig. S5). The expression constructs for GFP fusion proteins of activated Rho, Rac1, and Cdc42 have been described (34).

The expression construct for GFP-tagged N-Wasp was kindly provided by Dr. Michael Way.

**Analysis of TCR-mediated Cell Spreading and Actin Ring Formation**—T-cell spreading on stimulatory surfaces was analyzed essentially as described (20). Briefly, microscope cover glasses (Marienfeld) were cleaned with 1 M HCl, 70% ethanol for 30 min and dried at 60°C for 30 min before treating with a 0.01% poly-L-lysine (Sigma) solution for 5 min. For antibody coating, dried cover glasses were then covered with anti-CD3 and anti-CD28 antibodies diluted in PBS (each 7 μg/ml) for 3 h at 37°C. After washing with PBS, the cover glasses were stored in PBS at 4°C. 5 × 10⁶ Jurkat TAg cells were transfected via electroporation (15–40 μg of total plasmid DNA, 960 microfarads, 250 V; Bio-Rad Gene Pulser). 24 h post-transfection (48 h in the case of the bicistronic Nef expression vectors and associated controls; 17 h after infection), 3 × 10⁶ Jurkat cells were plated on the treated cover glasses and incubated for various time periods at 37°C and were subsequently fixed for 10 min by directly adding 3% paraformaldehyde. After permeabilization with PBS, 0.1% Triton X-100 for 1 min, cells were blocked with PBS, 1% bovine serum albumin for 30 min. Direct immunofluorescence was performed by incubating cells with 1:50–1:800 diluted primary antibodies for 2 h (overnight for anti-phosphotyrosine, anti-phospho-Zap70, and anti-phospho-LAT, which were diluted in Tris-buffered saline). After washing with PBS, fluorochrome-labeled secondary antibodies (1:2000) were added for 30 min. For F-actin staining, cells were treated with fluorochrome-conjugated phalloidin (1:400–1:1000) in combination with the secondary antibodies or directly after the blocking step. Nuclei were stained with 2.5 ng/μl Hoechst (Sigma). Cover glasses were mounted in Histomount (Linaris) and analyzed with a LSM 510 confocal laser-scanning microscope (Zeiss). Images were taken using a ×100 oil immersion objective and processed using Adobe Photoshop. For real-time imaging, eight-chambered cover glasses (Lab-Tek) were coated with anti-CD3 and anti-CD28 antibodies as described above. 24 h post-transfection, 5 × 10⁶ Jurkat cells in 50 μl of complete RPMI 1640 medium were injected into the chambers that were preloaded with 100 ml of medium. Real-time imaging was performed at 37°C with 5% CO₂ using the ×100 oil objective (numerical aperture 1.4) of an Axiovert 200M epifluorescent microscope (Zeiss) equipped with a CCD camera (Cascade II; Roper Scientific). Time lapse images were taken every 15 s, edited using Metamorph Software (Molecular Devices), and exported as Quick Time videos.

**HIV-1 Production and Infection**—Virus stocks were generated by co-transfection of proviral HIV plasmids into 293T cells as described (32). For infection of Jurkat cells, virus stocks were generated in the presence of the vesicular stomatitis virus G protein. Two days after transfection, culture supernatants were harvested. The HIV-1 p24 antigen concentration was determined by a p24 antigen enzyme-linked immunosorbent assay. 250–9000 ng of p24 were used to infect 1 × 10⁶ Jurkat cells. 17 h postinfection, cells were subjected to actin ring formation analysis as described above, except that the cells were stained for F-actin and p24CA to identify productively infected cells. Human peripheral blood mononuclear cells (PBMCs) were isolated as described previously (35). Cells were cultured in com-
Modulation of T-cell Actin Dynamics by Nef

Nef on TCR-induced actin rearrangements in Jurkat T-lymphocytes that were plated on glass surfaces coated with anti-CD3 and anti-CD28 antibodies. Most GFP-expressing control Jurkat T-lymphocytes spread on the cover glass surface within 10 min and displayed thick circumferential F-actin rings that contained multiple pronounced F-actin bundles with perpendicular orientation (Fig. 1A, GFP, bottom). Very similar phenotypes were induced by stimulation with anti-CD3 antibodies alone (data not shown). Uncoated surfaces allowed cell adhesion and some spreading including the accumulation of some F-actin at the contact site, at cell-cell contacts, and at the periphery, where sometimes membrane ruffling and the formation of microspikes was observed. However, no formation of F-actin rings was observed under these conditions (Fig. 1A, GFP, top). In sharp contrast, Jurkat T-cells expressing a fusion protein of Nef from HIV-1SF2 with GFP (Nef.GFP) were markedly impaired in the formation of actin-rich rings and in spreading in response to TCR engagement despite the accumulation of F-actin at the cell periphery (Fig. 1A, Nef.GFP, bottom). Actin rings also did not appear at later time points in Nef.GFP-expressing cells (data not shown). Whereas Nef.GFP was present at cell-substratum contacts, the viral protein was not particularly enriched in distinct clusters and was found most prominently at the plasma membrane and at a membranous organelle in the cytoplasm. The disruption of actin organization by Nef was most prominent upon specific TCR engagement and did not significantly affect the behavior of cells on uncoated cover glasses (Fig. 1A, Nef.GFP, top) in most experiments. Occasionally, however, we observed that, even on uncoated cover glasses or cover glasses coated with an irrelevant isotype-matched control antibody, Nef.GFP-expressing cells appeared less efficiently spread and with less pronounced F-actin structures than GFP-expressing controls (data not shown). Consistent with this, Nef.GFP-expressing Jurkat T-lymphocytes in suspension displayed ~20% reduced steady-state F-actin levels relative to GFP-expressing control cells (Fig. S1). A quantification of the inhibitory effect of Nef.GFP on TCR-induced actin rearrangements revealed that only 10–15% of cells expressing Nef.GFP were able to induce the formation of pronounced actin rings, whereas 65–70% of GFP-expressing (GFP) or untransfected (mock) control cells readily responded to TCR stimulation (Fig. 1B). Of note, steady-state cell surface levels of the CD3 and CD28 receptors required for stimulation were unaffected by Nef.GFP expression (Fig. S2). To monitor the impact of Nef on the dynamics of actin polymerization in the process of contact maturation, live cell imaging was performed on cells expressing actin fused to GFP together with mRFP1 (RFP) or a fusion protein of Nef with mRFP1 (Nef.RFP) (see Fig. S5 for the characterization of Nef.RFP fusion proteins). Individual cells were monitored starting from the initial contact with the stimulatory surface. Selected frames from the indicated time points are presented in Fig. 1C, the corresponding movies can be viewed as supplemental material. In RFP expressing control cells, initial contact immediately resulted in the increase of actin polymerization in particular at the cell periphery resulting in the formation of a circumferential actin ring and pronounced cell spreading within the first 2 min. Subsequently, cell spreading was maintained, and local waves of actin polymerization induced
highly dynamic lamellipodia at the cell periphery over the entire period of observation. In sharp contrast, the induction of actin polymerization and cell spreading was potently suppressed in cells expressing Nef.RFP (bottom). While these cells remained attached to the initial contact site, only localized bursts of actin polymerization at the periphery were observed that sometimes led to formation of individual protrusions (see 180 s time point). These Nef.RFP-expressing cells, however, failed to develop mature circumferential actin rings and did not spread. More detailed analysis of this phenomenon on fixed cells after a 10-min incubation on stimulatory surfaces corroborated a net effect of Nef on cell spreading; diameters of Nef.GFP-expressing cells (26.0 ± 8.0 μm (n = 32)) were significantly reduced relative to the GFP-expressing control cells (43.6 ± 8.6 μm (n = 32)) (Fig. 2, A and B). Despite this reduction in cell spreading, Nef.GFP had no detectable effect on the ability of cells to contact and adhere to the stimulatory surface (Fig. S3). To distinguish whether the Nef-induced disruption of F-actin ring formation was due to a failure in the distribution of F-actin to the contact site or caused by an overall reduction in F-actin levels, z-sections of the cells were taken from top to bottom, and the overall F-actin fluorescence was quantified (Figs. 2, C and D). Expression of Nef.GFP significantly reduced F-actin levels in cells following TCR stimulation relative to the GFP control (35,870 ± 16,070 arbitrary units (n = 36) versus 81,710 ± 30,630 arbitrary units (n = 30)). Thus, Nef.GFP efficiently disrupts actin rearrangements and T-lymphocyte spreading in response to TCR engagement, and this is paralleled by a reduction of overall cellular F-actin levels.

Circumferential Actin Ring Disruption Occurs in HIV-1-infected Primary Human T-lymphocytes and Is a Conserved Function of Lentiviral Nef Proteins—To test whether this novel Nef phenotype could also be observed in the context of HIV-1 infection, human PBMCs were activated with phytohemagglutinin/
interleukin-2 for 2 days, infected with HIV-1NL4–3 expressing Nef from HIV-1SF2 (HIV) or its nef-deleted counterpart (HIV\^Nef) (Fig. 3, A and B) and cultured in the presence of interleukin-2. One week postinfection, formation of TCR-induced actin rings was analyzed. Whereas most cells infected with HIV\^Nef readily formed actin rings that were indistinguishable from uninfected neighboring cells, infection with HIV-1 consistently interfered with these morphological changes. The magnitude of this Nef effect varied between cells from the four different donors analyzed, ranging from 30 to 75% inhibition (Fig. 3B). Similar results were obtained upon infection of Jurkat T-lymphocytes (Fig. S4). Thus, the disruption of TCR-induced actin rings observed upon transfection of Nef expression constructs mirrors Nef-dependent processes in the context of HIV-1 infection in T-cell lines and primary T-lymphocytes. We next analyzed whether Nef proteins from primates encoded by lentiviruses other than HIV-1SF2 (HIV) or its nef-deleted counterpart (HIV\^Nef) were also active in our assay. We next analyzed whether Nef proteins from primate lentiviruses other than HIV-1SF2 (HIV) or its nef-deleted counterpart (HIV\^Nef) were also active in our assay. Nef interferes with cell spreading and actin polymerization following TCR engagement. A, dot plot of cell diameters of Jurkat T-cells expressing GFP (n = 32) or Nef.GFP (n = 32) following a 10-min incubation on TCR-stimulatory surfaces. B, histogram of the experiment depicted in A. Values are the arithmetic means ± S.D. and are representative of three independent experiments. C, total cellular F-actin levels were measured in Jurkat T-cells expressing GFP (n = 36) or Nef.GFP (n = 36) after a 10-min incubation on TCR-stimulatory surfaces by integrating the F-actin pixel intensity of 2-sections spanning the cells from top to bottom. Plotted are individual arbitrary units of F-actin intensities. D, histogram of the experiment depicted in C. Values are the arithmetic means ± S.D.

FIGURE 3. Disruption of TCR-induced actin rearrangements by Nef occurs in HIV-1-infected primary human T-lymphocytes and is a conserved function of lentiviral Nef proteins. A, analysis of cell spreading and actin ring formation of HIV-1-infected PBMCs. PBMCs infected with wild type HIV-1 (HIV) or its nef-deleted counterpart (HIV\^Nef) were incubated at 1 week postinfection for 5 min on TCR-stimulatory surfaces and subsequently analyzed for cell spreading and actin ring formation. Infected cells were identified by staining for the viral structural protein p24CA, and the phalloidin stain of cytoskeletal actin. White bar, 10 μM B, quantification of actin ring formation on TCR-stimulatory surfaces in HIV-1-infected PBMCs from four donors. C, quantitative analysis of actin ring formation with Jurkat T-cells expressing the indicated Nef.GFP fusion proteins or Nef.GFP expressed from bicistronic vectors for 48 h. Values are the arithmetic means of at least three independent experiments ± S.D., in which over 100 cells were counted per condition. SIV, simian immunodeficiency virus.

Actin Ring Disruption Requires Nef Myristoylation and the SH3 Binding Motif—Analysis of a panel of well characterized mutants of HIV-1SF2 Nef (6, 32) identified determinants in the Nef protein that mediate interference with TCR-induced actin rearrangements; whereas the EDAA Nef mutant (ED178/179AA) that fails to interact with the AP-2/clathrin endocytic machinery displayed full potential to interfere with actin ring formation, the G2A Nef variant, which lacks membrane associ-
activity of which causes well characterized F-actin rich structures such as stress fibers, lamellipodia/membrane ruffles, and microspikes/filopodia, respectively, in fibroblasts (38). We tested whether Nef affects cytoskeletal rearrangements triggered by specific small GTPases. In the absence of additional stimulatory antibodies, actin organization and cell morphology was analyzed in Jurkat T-lymphocytes that transiently co-expressed Nef.RFP or RFP together with GFP-tagged small GTPases (Fig. 5A). Co-expression of activated RacL61.GFP with the RFP control caused pronounced cell spreading and the formation of a thick F-actin ring reminiscent of the phenotype observed for untransfected or GFP-expressing control cells on TCR-stimulatory surfaces. Cdc42L61.GFP triggered the formation of numerous extended F-actin-rich filopodia, whereas RhoV14.GFP induced an overall increase in F-actin underneath the plasma membrane and the formation of several very short microspikes per cell. Remarkably, expression of Nef.RFP potently abrogated the morphological changes triggered by active Rac1 and Cdc42. In contrast, whereas Nef largely interfered with the formation of Rho-induced cell protrusions, the viral protein did not alter the increase in subcortical F-actin induced by active Rho. Whereas Nef partially co-localized with the GTPases at the plasma membrane and at intracellular structures, the viral protein did not introduce appreciable changes in the subcellular distribution of the GTPases. In contrast, expression of RacL61 consistently increased the diffuse distribution of Nef in this experimental system. The Nef-mediated disruption of actin rearrangements induced by active Rac1 and Cdc42, but not by active Rho, was also observed in adherent HeLa (Fig. S6) and NIH3T3 cells (data not shown). Together, Nef specifically interferes with cellular actin remodeling machineries, the activity of which is regulated by the Rac1 and/or Cdc42 GTPases. 

Nef Interferes with the Translocation of N-Wasp to the Periphery of Mature T-lymphocyte Contacts—N-Wasp represents a key actin organizer that activates Arp2/3-mediated actin nucleation in lymphocytes downstream of Cdc42 and Rac and is recruited to TCR-stimulatory contacts (39). We therefore analyzed the effects of Nef.RFP on the dynamic distribution of N-Wasp “early” (2 min) and “late” (10 min) after contact of transfected Jurkat T-lymphocytes with TCR-stimulatory surfaces (Fig. 5B). Besides RFP alone, the AXXA Nef mutant RFP fusion protein (AXXA.RFP) that is deficient in binding to SH3 domains and does not interfere with TCR-induced actin nucleation in lymphocytes downstream of Cdc42 and Rac and is recruited to TCR-stimulatory contacts (39). We therefore analyzed the effects of Nef.RFP on the dynamic distribution of N-Wasp “early” (2 min) and “late” (10 min) after contact of transfected Jurkat T-lymphocytes with TCR-stimulatory surfaces (Fig. 5B). Besides RFP alone, the AXXA Nef mutant RFP fusion protein (AXXA.RFP) that is deficient in binding to SH3 domains and does not interfere with TCR-induced actin remodeling (see Fig. 4) was used as a specificity control. Interestingly, N-Wasp was present at early microclusters as well as at the tips of lamellar protrusions of mature actin rings and markedly co-localized with F-actin-rich structures in virtually all cells that were activated by contact with the stimulatory surface (Fig. 5B, Nef.RFP). Activation and Contact Site Recruitment of N-Wasp Is Inhibited by Nef Early after TCR Engagement—To address the mechanism by which Nef prevented the translocation of N-Wasp to the cell periphery and actin ring formation, activation of endogenous N-Wasp was monitored by staining with an antibody...
that specifically recognizes the active, phosphorylated form of the protein (pN-Wasp). pN-Wasp was efficiently induced at contact sites after 2 min, where it accumulated in pronounced clusters in ~50% of the control cells (Fig. 6, A (left) and B). In the presence of Nef, however, the early induction of pN-Wasp was markedly inhibited, and less than 20% of the cells displayed appreciable pN-Wasp clusters (Figs. 6, A and B). To quantify the effects of Nef on the induction and distribution of pN-Wasp, overall pN-Wasp pixel intensities and their relative accumulation at the contact site were determined from stacks of confocal sections spanning from top to bottom of the cells. This analysis revealed that Nef reduced the immediate induction of overall pN-Wasp in response to TCR engagement approximately 3-fold (Fig. 6C). Moreover, recruitment of the residual pN-Wasp population to the contact site was also significantly inhibited by Nef (Fig. 6D). Over extended periods of observation, contact maturation in control cells progressively resulted in the appearance of pN-Wasp at the cell periphery, resulting in a distribution across the contact site with some enrichment in the circumferential actin ring (Fig. 6A, right). In contrast, pN-Wasp remained almost undetectable in Nef-expressing cells. Together, these results demonstrate that Nef interferes with the activation of N-Wasp and its recruitment to contact sites early following TCR induction on stimulatory surfaces.

Nef Modulates Overall Tyrosine Phosphorylation and Microcluster Composition Late after TCR Engagement—Since actin rearrangements induced by TCR engagement are required for signal initiation and transmission (17, 40), we analyzed the induction of overall tyrosine phosphorylation as a correlate for
activation of the TCR cascade in our experimental system (Fig. 7, A–C). As expected, stimulation of GFP and AXXA.GFP expressing control cells resulted in the rapid appearance of central Tyr(P) (pTyr) microclusters at the contact site (Fig. 7A), indicating successful initiation of signal transduction. Similar Tyr(P) microclusters were observed in Nef.GFP-expressing cells, demonstrating that Nef did not significantly affect overall tyrosine phosphorylation and initial microcluster formation early following TCR triggering. When analyzed after 10 min, Tyr(P) microclusters were distributed over the entire contact area and were somewhat enriched in the meanwhile matured actin ring of GFP- and AXXA.GFP-expressing control cells (Fig. 7B). In contrast, Nef.GFP-expressing cells displayed less prominent Tyr(P) microclusters. Quantification of these results demonstrated that late after TCR engagement, Nef efficiently interfered with the overall induction of tyrosine phosphorylation and moderately affected the recruitment of Tyr(P) to the cell-substratum contact (Fig. 7C). Of note, these effects were specific to late events following contact with the stimulatory surface, since, using the same quantitative approach, no significant effects of Nef on tyrosine phosphorylation early after stimulation or on uncoated surfaces were observed (data not shown). We next analyzed the fate of activated, tyrosine-phosphorylated forms of two specific markers for signaling clusters induced on TCR-stimulatory surfaces (24, 25): the adaptor protein LAT (pLAT) and the Zap70 kinase (pZap70). Whereas no effects of Nef on both phosphotyrosine species were observed at early contacts (data not shown), Nef significantly reduced the overall tyrosine phosphorylation of both molecules after 10 min of stimulatory contact (Fig. 7, D and F, as well as E and G (top panels)). Nef also interfered with the contact site recruitment of pLAT but not pZap70 (Figs. 7, E and G, bottom panels). Importantly, despite this reduction in overall levels, clusters of all Tyr(P) species were detectable at the center and the periphery of Nef.GFP-expressing cells, indicating that Nef did not per se abrogate the induction of signaling microclusters. Quantitative analysis revealed that the relative distribution of these microclusters between the cell center and periphery was not significantly different between T-lymphocytes expressing Nef.GFP or GFP (data not shown). Thus, whereas Nef appears to selectively target phosphorylation of N-Wasp at early contacts, expression of the viral protein leads to an overall reduction of tyrosine phosphorylation late following TCR engagement and alters the composition of the tyrosine-phosphorylated (active) protein

FIGURE 6. Nef interferes with N-Wasp activation early following TCR engagement. A, Jurkat T-cells expressing RFP, Nef.RFP, or AXXA.RFP were incubated on TCR-stimulatory surfaces, fixed after the indicated time points, and stained for pN-Wasp. Results depict confocal images representative of at least three independent experiments. White bar, 10 μm. B, quantification of effects of Nef on induction of pN-Wasp clusters at contact sites at 2 min. Values are the arithmetic means of at least three independent experiments ± S.D. in which over 100 cells were counted per condition. C, quantification of overall pN-Wasp levels. Values are mean overall pN-Wasp pixel intensities at 2 min from at least 10 cells representing the phenotypes depicted in A. D, quantification of accumulation of pN-Wasp at contact sites. Values are mean relative percentages of total pN-Wasp at the contact site z-section at 2 min from at least 10 individual cells representing the phenotypes depicted in A. Statistical significance is indicated by the p values derived from Student’s t test analysis.
FIGURE 7. Nef modulates overall Tyr(P) (pTyr) induction and recruitment of select Tyr(P) species to the stimulatory contact late after TCR engagement. Jurkat T-cells expressing GFP, Nef.GFP, or AXXA.GFP were incubated on TCR-stimulatory surfaces, fixed after the indicated time points, and analyzed for the subcellular distribution of Tyr(P)-positive signaling complexes by confocal microscopy. Results depict images representative of at least three independent experiments. White bar, 10 μm. A, distribution of Tyr(P), GFP, and F-actin early (2 min) after TCR stimulation. B, distribution of Tyr(P), GFP, and F-actin late (10 min) after TCR stimulation. C, quantification of overall Tyr(P) levels (top) and relative amounts of Tyr(P) at late contact sites (bottom). Values represent the mean ± S.D. from at least 10 cells representing the phenotypes depicted in B. Statistical significance is indicated by the p values derived from Student’s t test analysis. D, subcellular localization of phosphorylated LAT (pLAT) and GFP late (10 min) after TCR stimulation. E, quantification of total and contact site pLAT analogous to the analysis in C. F, subcellular localization of pZap70 and GFP late (10 min) after TCR stimulation. G, quantification of total and contact site pZap70 analogous to the analysis in C.
population at the contact sites. Whereas signaling microclusters are generated at normal subcellular localizations, their frequency is reduced, and they are not embedded in a matured circumferential actin ring structure.

**Inhibition of N-Wasp Prevents T-lymphocyte Contact Maturation in a Manner Analogous to Nef**—The above results suggested that Nef prevents the maturation of stimulatory T-lymphocyte contacts by inhibition of N-Wasp. To address this hypothesis, we made use of the selective cell-permeable N-Wasp inhibitor wiskostatin (41). Similar to expression of Nef, preincubation of Jurkat T-lymphocytes with wiskostatin (40 μM, 30 min) potently disrupted cell spreading and circumferential actin ring formation on TCR-stimulatory surfaces in virtually all cells under these experimental conditions (Fig. 8). In some cells, intracellular and plasma membrane F-actin aggregates were observed. Again similar to effects induced by Nef, wiskostatin also efficiently prevented the induction of Tyr(P), pLAT, and pZap70 in these cells (Figs. 8, A–C). Moreover, and consistent with its activity in preventing N-Wasp activation, wiskostatin abrogated induction of pN-Wasp after TCR engagement (Fig. 8, D and E). Together, direct inhibition of N-Wasp resulted in a block of contact maturation and modulation of TCR-induced signaling events in a manner comparable with Nef.

**Involvement of Nef-associated Pak2 Activity in the Inhibition of Contact Maturation**—We have previously reported that Nef induces a loss of actin stress fibers in mouse fibroblasts via its association with Pak kinase activity (42). Since Pak2 has been identified as the predominant Pak isoform that accounts for Nef-associated Pak activity in T-lymphocytes (43) and the PXXP motif of Nef is critical for both kinase association and inhibition of stimulatory contact maturation, we addressed the role of Pak2 in the modulation of actin dynamics by Nef by RNAi-mediated knockdown in our experimental system. As shown in Fig. 9, RNAi against Pak2, but not an unspecific control RNAi oligonucleotide, caused a robust reduction of cellular Pak2 levels, whereas expression of Pak1 was not affected (Fig. 9A). The analysis of Nef-associated Pak activity in these cells demonstrated that this reduction in Pak2 levels significantly reduced (to approximately 60% of the control) but did not abrogate the association of Nef with Pak activity (Fig. 9B). Incubation of aliquots of these cells on stimulatory surfaces revealed efficient cell spreading in mock-transfected cells irrespective of treatment with control or Pak2-specific RNAi oligonucleotides. However, the inhibitory effect of Nef on contact maturation was decreased upon knockdown of Pak2. Importantly, the degree of reduction in inhibition of actin ring formation/cell spreading by Nef corresponded well to the magnitude of loss of

**FIGURE 8.** Pharmacological inhibition of N-Wasp prevents maturation of TCR-stimulatory contacts. Jurkat T-lymphocytes were incubated with 40 μM wiskostatin (Wisk) or an Me2SO control (solvent) for 30 min, incubated on TCR-stimulatory surfaces for the indicated time periods, fixed, and stained for F-actin together with the indicated phosphoproteins. Results depict confocal images representative of at least three independent experiments. White bar, 10 μM. A, Tyr(P) (pTyr). B, pLAT. C, pZap70. D, pN-Wasp at 2 min. E, pN-Wasp at 10 min.
Modulation of T-cell Actin Dynamics by Nef

**DISCUSSION**

This study reveals interference with actin dynamics as a mechanism of the HIV pathogenicity factor Nef to modulate early steps of TCR signal transduction in a model system of physiologic stimulation of HIV target T-cells. Such effects are generally consistent with previous reports on the disassembly of F-actin structures by Nef in fibroblasts (14, 33). Interestingly, Nef is known to activate Rac1 and Cdc42 in T-lymphocytes (15, 44) but, as shown herein, disrupts cytoskeletal structures induced by these GTPases. This might reflect that both, seemingly opposing, activities of Nef are independently exerted by distinct Nef subpopulations in cells. Alternatively, Nef may induce GTPase activity to selectively channel downstream signals toward actin depolymerization events (45). Finally, we cannot exclude at present the possibility that, under the experimental conditions used herein, Nef directly interferes with the activity of these GTPases.

Consistent with its role in actin organization at the IS (39), the presented data identify N-Wasp as one critical factor that is, via direct or indirect mechanisms, targeted by Nef to interfere with stimulatory contact maturation. Like the activities described here, previously reported effects of Nef on the host cytoskeleton were mediated via the association of Nef with Pak2 kinase activity, and these effects were also dependent on the Nef SH3 domain binding motif (14, 42). Indeed, the reduction in Nef-associated Pak2 activity caused by RNAi-mediated knockdown of Pak2 correlated with the decrease of the ability of Nef to interfere with the maturation of stimulatory T-cell contacts. Since Pak2 was dispensable for circumferential actin ring formation per se, Nef conceivably redirects Pak2 signaling to alter TCR-induced actin dynamics. The involvement of both Pak2 and N-Wasp could thus reflect the direct modulation of N-Wasp activity by Pak2. Alternatively, both molecules may act independently at distinct critical steps to mediate the interference of actin ring formation by Nef (46).

The observed consequences of Nef expression on TCR-induced contact formation could in principle be explained by nonspecific effects of Nef on the reception and/or transmission of the signal or by the specific modulation of actin remodeling. Several aspects of the time-resolved analysis of actin dynamics and signaling microcluster formation on TCR-stimulatory surfaces lead us to propose that the specific inhibition of actin remodeling by Nef causes the modulation of TCR-induced contact maturation and thus subsequent signal transduction. First, whereas Nef did not impact on the ability of the cell to contact the stimulatory surface, the immediate induction of actin polymerization that results in cell spreading and formation of the circumferential actin ring was potently inhibited. Thus, Nef affects a very early event following TCR engagement. Second, at these early time points, Nef had no significant effect on the ability of the cell to contact actin remodeling by Nef causes the modulation of TCR-induced contact maturation and thus subsequent signal transduction. First, whereas Nef did not impact on the ability of the cell to contact the stimulatory surface, the immediate induction of actin polymerization that results in cell spreading and formation of the circumferential actin ring was potently inhibited. Thus, Nef affects a very early event following TCR engagement. Second, at these early time points, Nef had no significant effect on the ability of the cell to contact the stimulatory surface, the immediate induction of actin polymerization that results in cell spreading and formation of the circumferential actin ring was potently inhibited. Thus, Nef affects a very early event following TCR engagement. Second, at these early time points, Nef had no significant effect on the ability of the cell to contact the stimulatory surface, the immediate induction of actin polymerization that results in cell spreading and formation of the circumferential actin ring was potently inhibited. Thus, Nef affects a very early event following TCR engagement. Second, at these early time points, Nef had no significant effect on the ability of the cell to contact

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relaxation following receptor engagement or incomplete synapse maturation can positively modulate strength, duration, and specificity of select signal outputs (19, 47–49). Importantly, Nef-mediated disruption of cell spreading and actin ring formation did not abrogate early contact-induced microcluster formation and thus signal initiation. However, at later time points, overall Tyr(P) levels and the selective recruitment of pLAT to stimulatory contacts were reduced in the presence of the viral protein. Nef therefore modulates the amount and composition of signaling clusters and thus provides a valuable experimental tool for future studies on the role of actin remodeling for the dynamic organization of TCR-stimulatory contacts. Of note, positive effects of Nef on HIV-1 replication in vitro are most pronounced in cell culture systems where permissivity to HIV-1 replication is induced in the target T-lymphocyte by direct contact of antigen-presenting cells with where permissivity to HIV-1 replication is induced in the target T-lymphocyte by direct contact of antigen-presenting cells with resting T-cells expressing Nef following infection (50–52). These target T-cells are fully permissive for HIV replication despite the lack of classical T-cell activation markers, suggesting that Nef selectively induces a low state of activation. Importantly, mutation of the Nef PXXP motif blunts its ability to boost HIV-1 replication in co-cultures of immature dendritic cells and autologous T-lymphocytes (51) and prevents the modulation of TCR-induced actin remodeling (this study). We therefore hypothesize that this newly identified modulation of IS maturation helps Nef to generate a moderate TCR signal optimized for HIV-1 production. This scenario is consistent with a recent report on the Nef-mediated enhancement of TCR downstream effector functions, such as NF-κB and NF-AT activation after stimulation with anti-CD3-coated latex beads (10). Moreover, the protein interaction surfaces of Nef required for full actin disruption activity also mediate the effects of Nef on cell surface receptors such as major histocompatibility class I and CCR5, as well as the ability of Nef to interfere with T-lymphocyte chemotaxis (15, 32, 53). Given the cardinal role of actin dynamics in vesicular transport and cell motility (45, 54), the modulation of actin rearrangements mediated by N-Wasp might represent a general strategy of the HIV-1 pathogenicity factor Nef to optimize virus replication.

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