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Nef Is Physically Recruited into the Immunological Synapse and Potentiates T Cell Activation Early after TCR Engagement

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The HIV-1 protein Nef enhances viral pathogenicity and accelerates disease progression in vivo. Nef potentiates T cell activation by an unknown mechanism, probably by optimizing the intracellular environment for HIV replication. Using a new T cell reporter system, we have found that Nef more than doubles the number of cells expressing the transcription factors NF-κB and NFAT after TCR stimulation. This Nef-induced priming of TCR signaling pathways occurred independently of calcium signaling and involved a very proximal step before protein kinase C activation. Engagement of the TCR by MHC-bound Ag triggers the formation of the immunological synapse by recruiting detergent-resistant membrane microdomains, termed lipid rafts. Approximately 5–10% of the total cellular pool of Nef is localized within lipid rafts. Using confocal and real-time microscopy, we found that Nef in lipid rafts was recruited into the immunological synapse within minutes after Ab engagement of the TCR/CD3 and CD28 receptors. This recruitment was dependent on the N-terminal domain of Nef encompassing its myristoylation. Nef did not increase the number of cell surface lipid rafts or immunological synapses. Recently, studies have shown a specific interaction of Nef with an active subpopulation of p21-activated kinase-2 found only in the lipid rafts. Thus, the corecruitment of Nef and key cellular partners (e.g., activated p21-activated kinase-2) into the immunological synapse may underlie the increased frequency of cells expressing transcriptionally active forms of NF-κB and NFAT and the resultant changes in T cell activation. The Journal of Immunology, 2005, 175: 6050–6057.

Nef is a 25- to 34-kDa myristoylated viral protein that plays a major role in the pathogenesis of HIV. Among its many recognized functions, Nef modulates the surface expression of different transmembrane proteins, including CD4 and MHC-I (for review, see Ref. 1). Down-regulation of CD4 increases virion release and infectivity, whereas down-regulation of MHC-I enables HIV-1-infected cells to escape immune detection. Nef also increases the infectivity of HIV-1 virions by a CD4-independent mechanism (2) and inhibits apoptosis in infected cells (3). The effect of Nef on the cellular response after TCR activation is controversial; some reports indicate no effect (4), and others found either a represive (5–7) or a potentiating (8–13) effect. The molecular mechanisms by which Nef acts on T cell signaling pathways are unclear. Many interactions between Nef and cellular partners involved in T cell signaling have been described in the past. Nef has been proposed to interface with the calcium signaling pathway by interacting with the cellular inositol 1,4,5-trisphosphate receptor (14, 15). The N-terminal region of Nef also interacts with a cellular complex containing the Src kinase Lck (16). Using its Src homology 3 (SH3)3 ligand, proline-rich (PxxP) motif, Nef interacts with the Src kinase Hck (17, 18), the guanine nucleotide exchange factor Vav (19), and the N-associates kinase, recently identified as p21-activated kinase 2 (PAK2) (20–22). Interestingly, PAK2 does not have an SH3 domain. However, a recent study has shown that the Nef PxxP motif is essential for Rac activation and PAK2 recruitment by Nef, through its direct interaction with the dedicator of cytokinesis 2 (DOCK2)-engulfment and cell motility 1 (ELMO1) complex (4).

PAK family members play key roles in cytoskeleton remodeling, cell polarization, and apoptosis (23), and PAK2 has been implicated in T cell activation (24). Indeed, a dominant negative PAK2 protein blocks TCR-induced up-regulation of CD69, activation of NFAT, and calcium flux. Nef-induced activation of PAK also appears to be required for the effects of Nef on cellular activation and HIV production (21, 25–29). Nef interacts only with the autophosphorylated form of PAK2, and this Nef-PAK2 complex is detectable exclusively in lipid rafts, membrane microdomains that are enriched in cholesterol and sphingolipids (30, 31).

Lipid rafts appear to be important in intracellular trafficking and signal transduction. In T cells, engagement of the TCR by MHC-bound Ag triggers the lateral recruitment and reorganization of membrane proteins present in lipid rafts, resulting in the formation of a larger signaling platform, called the immunological synapse (IS) (32). We investigated the dynamic localization of Nef in lipid rafts during IS formation and the effect of Nef on the efficiency of T cell signaling at the single-cell level. Our findings suggest that a small

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pool of Nef, present in lipid rafts, may potentiate T cell signaling, leading to increased numbers of cells effectively inducing NF-κB and NFAT transcriptional responses.

Materials and Methods
Reagents and Abs
Polystyrene latex microspheres were purchased from Interfacial Dynamics. The following mAbs were used: anti-CD3 (OKT3; University of California, San Francisco pharmacy) and anti-CD28 (clone 15E8; Caltag Laboratories). Fura 2 acetoxyethyl ester, detergent Pluronic F-127, and Alexa Fluor 594 and Alexa Fluor 647-conjugated cholera toxin β subunit (CT-B) were obtained from Molecular Probes. PMA was purchased from Sigma-Aldrich.

Plasmids
The construction of the empty lentiviral vector PPT-MCS, or containing the NRL-3 Nef-GFP wild-type or various NRL-4 Nef mutants have been described (33). The PPT-Nef-internal ribosomal entry site (IRES)-GFP vector was constructed as follows: the IRES-GFP sequence was obtained by PCR from the vector pIRE2-GFP (BD Clontech) and inserted into PPT-MCS by digestion with XhoI and SalI. Subsequently, coding sequences of wild-type or mutant NRL-4 Nef obtained by PCR were inserted into the PPT-IRES-GFP vector cut with BamHI-Xhol. PPT-NFAT-DrRed2 expressing DrRed2 under the control of the NFAT promoter was obtained after digestion of pNFAT-Luc with ClaI and HindIII, digestion of pDrRed2 (BD Clontech) with HindIII and Xhol, and ligation of these two DNA fragments into PPT-MCS cut with CluI-Xhol. PPT-xB-DrRed2 expressing DrRed2 under the control of the xB promoter was obtained by inserting the DrRed2 coding sequence under the control of the 5′ xB promoter from pGL3-xB-DrRed2 (provided by Dr. H. Kwon, Gladstone Institute of Virology and Immunology, San Francisco, CA) after digestion with NotI, filling with Klenow, and cutting with Xhol, into PPT-MCS cut with XhoI and SmaI.

Cell culture
Jurkat E6-1 cells were maintained in RPMI 1640 and HEK 293T cells in DMEM supplemented with penicillin-streptomycin (Invitrogen Life Technologies) and 10% heat-inactivated FBS (Gemini Bioproducts). Stable Jurkat reporter T cell lines expressing NF-κB- or NFAT-responsive DrRed2 protein were obtained by infection with vesicular stomatitis virus glycoprotein (VSV-G)-pseudotyped PPT-NF-κB-DrRed2 or PPT-NFAT-DrRed2 and serial dilution of the cells 4 days after infection. After 2 wk, the clones were selected for their ability to express DrRed2 after stimulation with PMA/ionomycin (50 ng/ml) overnight. CD4+ T lymphocytes were isolated from PHA/IL2-activated PBMC with CD4+ T cell enrichment columns (StemCell Technologies).

Production of pseudotyped HIV particles
Pseudotyped HIV particles were produced by calcium phosphate transfection of HEK 293T cells with VSV-G envelope expression plasmid, the packaging construct CMVΔR8.2 (provided by Dr. D. Trono, University of Geneva, Geneva, Switzerland), and the proviral construct PPT-MCS containing 5′ xB-DrRed2, NFAT-DrRed2, or the different NF-κB and Nef-IRES-GFP constructs (DNA ratio, 1:1:2). Cells were washed 8 h after transfection. Viral supernatants were collected after 48 h, filtered, and frozen in aliquots at −80°C.

T cell activation
Ninety-six-well plates were coated with anti-CD3 Ab (20 μg/ml) alone or with anti-CD28 for 2 h at 37°C and washed twice with PBS. NF-κB and NFAT-DrRed2 Jurkat cells transduced with different Nef-IRES-GFP constructs were then incubated with these Abs for 24 or 4 h and transferred into a new plate without Abs for 20 h. The level of DrRed2 expression was assessed by FACS.

Real-time multicolor video microscopy
Jurkat cells (5 × 10^5) transduced with Nef-GFP fusion proteins were incubated with Alexa Fluor 594-conjugated CT-B for 30 min at 4°C, washed, mixed with 10⁷ Ab-coated beads (anti-CD3, anti-CD28, or anti-CD3/CD28), and immediately injected into a chamber system for live cell micro-observation with a thermal control system (FCS2; Biophotes). Time-lapse images were captured every 90 s with a fully automated fluorescence microscopy system (Nikon TE-300) and a Plan Fluor ×200/0.45 objective lens; a CCD camera was mounted under the microscope to capture light directly and was controlled by MetaMorph imaging software. Digital images were processed with Adobe Photoshop.

Confocal microscopy
Jurkat cells were stimulated with Ab-coated beads for 90 min, fixed in 3.7% paraformaldehyde for 10 min, washed, and seeded onto gel-mounted slides (Biomek). Two-color images were obtained with a confocal, laser-scanning Radiance 2000 (Bio-Rad) attached to an Olympus BX60 microscope with a PlanApo ×60/1.40 oil immersion objective lens. Serial 0.5-μm sections of x-y images along the z-axis were obtained and subsequently analyzed with MetaMorph imaging software.

Kinetics of IS formation and detection of cell surface lipid rafts
Jurkat cells (10⁶) transduced with Nef-IRES-GFP constructs were mixed with 10⁶ anti-CD3/CD28-coated beads at 37°C, and an aliquot of the cell suspension was analyzed by FACS every 10 min. IS formation (number of cells interacting with a latex bead) was estimated from the forward/side scatter profile. Dead cells were excluded by staining with 7-aminoactinomycin (Viaprobe; BD Pharmingen). For detection of lipid rafts, Jurkat cells were analyzed, 48 h after transduction with Nef-IRES-GFP constructs, by labeling for 20 min at 4°C with Alexa Fluor 647-conjugated CT-B, and the level of GM1 at the cell surface was estimated by FACS.

Measurement of intracellular calcium release
Jurkat cells were incubated for 45 min at room temperature with 500 μM calcium buffer (HBSS (BioWhittaker) and 1% FBS) containing 1 μM Fura red acetoxyethyl ester and 0.02% Pluronic F-127, washed with the calcium buffer, and incubated for 15 min at 4°C with anti-CD3/CD28 (5 μg/ml). Cells were washed to remove the unbound mAb, transferred at room temperature to the FACS machine, and analyzed for 10 s; after a short pause to add the secondary Ab (25 μg/ml), the analysis was continued for 5 min.

Results
Nef increases the number of cells expressing transcriptionally active NFAT and NF-κB
To clarify the role of Nef in T cell signaling, we transduced Jurkat T cells with retroviral vectors containing a minimal promoter, multimerized NF-κB or NFAT binding sites directing the expression of DrRed2, a red fluorescent protein, and followed the intracellular expression of NF-κB and NFAT transcription factor induction at the single-cell level by flow cytometry. Using IRES-GFP retroviral vectors, these reporter cell lines were transduced with wild-type Nef, nonmyristoylated 2Nef (NefG2A), or Nef with a mutant SH3 ligand motif (NefG355-358). In unstimulated cells, NF-κB and NFAT were not expressed, even in the presence of Nef, indicating that Nef expression alone is not sufficient to induce T cell activation (Fig. 1). However, activation for 4 h with 20 μg/ml plate-bound anti-CD3 and anti-CD28 increased the number of cells expressing NF-κB or NFAT by 2.5- and 4.5-fold, respectively, in the presence of Nef (Fig. 1, A and C). Similar Nef-dependent enhancement of the frequency of responding T cells was obtained when a lower concentration (2.5 μg/ml) of plate-bound anti-CD3/CD28 Abs was added for 4 h (data not shown). The mean fluorescence intensity of the DrRed2-expressing cells was unchanged, indicating that successfully activated cells contained equivalent amounts of NFAT or NF-κB in the absence or the presence of Nef (Fig. 1, B and D). Thus, Nef appears to lower the threshold required for T cell activation, but does not increase the level of activation achieved in the stimulated cells. Activation for 24 h in the absence of Nef produced results similar to those after a 4-h stimulation (not shown), but in the presence of Nef, the relative increase in the number of activated T cells was not observed (Fig. 1, A and C). These results at the single-cell level suggest some of the reasons why different studies have yielded contradictory data concerning the Nef effect on T cell signaling. It appears that a short period of activation is necessary to signal. It appears that a short period of activation is necessary to

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T cell priming induced by Nef appears to require a membrane localization signal, because the myristoylated mutant NefG2A was unable to enhance the number of cells expressing NF-κB or NFAT (Fig. 1, A and C). The proline-rich motif mutant NefAxxA was also unable to potentiate T cell activation, raising the possibility that the Nef phenotype requires interaction with a cellular partner through the SH3 interacting domain.

**Nef potentiates T cell signaling after TCR engagement, but before protein kinase C (PKC) activation**

Next, we determined the roles of the TCR and CD28 signaling in the Nef phenotype. In cells stimulated with anti-CD3 Abs alone, Nef increases the frequency of activated T cells (Fig. 1, E and F). Stimulation with anti-CD28 Abs alone did not, even in the absence of Nef (not shown), arguing that the absence of activation is not due to Nef-induced down-regulation of CD28 (34). In the presence of PMA, a direct activator of PKC, the Nef phenotype was abolished (Fig. 1E). These findings suggest that Nef alters a key step occurring before PKC activation.

**Nef is recruited into the IS after TCR engagement**

To follow the dynamic localization of Nef in lipid rafts during IS formation, we used real-time microscopy in cells expressing GFP or Nef-GFP. Of note, Nef-GFP appears as effective as wild-type Nef for all its functions studied to date (35, 36). To label the lipid rafts, which are enriched with the ganglioside GM1, a sphingolipid specifically recognized by CT-B, we incubated the cells with recombinant CT-B conjugated to Alexa Fluor 594. As a model for APC stimulation, the cells were activated with latex beads coated with anti-CD3/TCR and anti-CD28 (37). Within minutes after a GFP-expressing Jurkat cell engaged an anti-CD3/CD28-coated bead, a pool of lipid rafts was recruited to the site of contact, leading to IS formation, as expected (Fig. 2A and Supplementary Movie 1). GFP localizing in the cytoplasm and nucleus did not migrate into the IS after T cell activation (Fig. 2A and Supplementary Movie 1). When a Jurkat cell expressing Nef-GFP interacted with an anti-CD3/CD28-coated bead, lipid rafts were recruited, an IS formed, and a pool of Nef-GFP protein was recruited into the IS (Fig. 2B and Supplementary Movie 2). This dynamic, but partial, relocalization of Nef into the IS was similarly observed when purified primary CD4+ T lymphocytes were studied (Fig. 2C and Supplementary Movie 3). In multiple observations of Nef-GFP during cell-bead incubations (20 Jurkat cells and five lymphocytes), we saw no evidence that Nef either accelerated lipid raft recruitment into the IS or increased the overall size of the IS.

To quantify Nef-GFP recruited into IS, cells were fixed and analyzed by confocal microscopy at a higher magnification.

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5 The online version of this article contains supplemental material.
Analysis of a z-axis projection of the confocal images showed that 5–10% of total Nef-GFP was recruited to the IS. No recruitment of the GFP control protein was observed (Fig. 3). These findings demonstrate that a small pool of Nef colocalizing in lipid rafts is effectively recruited into the IS after TCR and CD28 engagement.
N-terminal domain of Nef, but not its PxxP motif, is crucial for recruitment into the IS

Nef is composed of an anchor domain (approximately the first 50 N-terminal amino acids) and a core, a large globular structure with many surfaces readily accessible for interactions with cellular partners. To better define the domains of Nef required for its recruitment into the IS, we used confocal microscopy to analyze the behavior of the NefAxxA mutant. Alanine substitution of prolines in the PxxP motif did not adversely affect recruitment of Nef into the IS (Fig. 3). Therefore, the absence of enhanced T cell activation observed in the presence of NefAxxA (Fig. 1) is not due to dysregulation of the Nef dynamics of this Nef mutant in lipid rafts and recruitment into the IS.

Next, we analyzed the effect of myristoylation and C-terminal truncation on recruitment of Nef into the IS. The nonmyristoylated mutant NefG2A was not recruited to the site of contact with the anti-CD3/CD28 beads, as shown by real-time microscopy (Fig. 2D and Supplementary Movie 4) and confocal microscopy (Fig. 3). We conclude that myristoylation of Nef is important for both its association with lipid rafts and its stimulus-coupled recruitment into the IS. To confirm the role of the N-terminal region of Nef in determining its movement into lipid rafts, we analyzed the properties of the Nef anchor domain directly linked to GFP (Nef1–50GFP). This short fragment of Nef mediated efficient recruitment of GFP to the site of contact with an anti-CD3/CD28 bead (Fig. 3). However, the anchor domain alone was not sufficient to heighten the frequency of activated T cells (not shown). A more complete genetic analysis will be required to determine which specific residues outside the N-terminal myristoylation signal contribute to lipid raft localization.

Nef recruitment into the IS requires TCR signaling, but not CD28 signaling

To study the specific roles of TCR signaling and CD28 signaling in the recruitment of Nef and lipid rafts into the IS, cells were activated with latex beads coated with anti-CD3 or anti-CD28 Abs, respectively. Anti-CD28 Abs did not trigger lipid raft clustering or Nef recruitment to the site of bead contact (Fig. 4A and Supplementary Movie 5), consistent with the absence of T cell activation by plate-bound anti-CD28 Abs (not shown). Conversely, stimulation of TCR alone with anti-CD3 Abs efficiently induced both the recruitment of lipid rafts to the site of contact and the inclusion of Nef into the IS (Fig. 4B and Supplementary Movie 6). Thus, TCR engagement is key for efficient Nef recruitment and IS formation.

Nef does not affect lipid raft dynamics or calcium signaling

Nef interacts with many different cellular partners, but it is not clear how this viral protein primes T cells for activation. Microscopic analysis revealed no effect of Nef on IS formation in single cells. To assess IS formation in the overall population, we incubated Jurkat cells with anti-CD3/CD28 latex beads at 37°C and determined the kinetics of bead-cell interaction by FACS. During the first 50 min of incubation, ~30% of the Jurkat cells interacted with an anti-CD3/CD28 bead, regardless of whether wild-type or mutant Nef was present (Fig. 5A).

Because Nef has been proposed to act on the cholesterol synthesis pathway and might affect the biosynthesis of lipid rafts (38, 39), a Nef-induced increase in the number of cell surface lipid rafts...
might enhance T cell signaling efficiency. To evaluate this possibility, we labeled Nef-GFP-transduced Jurkat cells with a fluorescent marker of lipid rafts (CT-B-Alexa Fluo 647) at 4°C and quantified cell surface lipid rafts by FACS. The level of cell surface lipid rafts proved indistinguishable in the absence or the presence of wild-type or mutant Nef (Fig. 5B).

Through effects on the calcium signaling pathway, Nef seems to increase NFAT expression and facilitate T cell activation (15, 40). To directly observe changes in intracellular calcium levels, we analyzed Jurkat cells by flow cytometry after incubation with the fluorescent dye Fura 2 and activation with anti-CD3/CD28 mAbs. Within seconds after clustering of TCR and CD28 at the cell surface, calcium was released from internal stores (Fig. 6). However, none of the Nef constructs tested increased calcium release. Under the experimental conditions used, we were unable to show that Nef can mimic the calcium signaling pathway (40). For instance, activation of NFAT was undetectable after incubation of Jurkat NFAT-DsRed2 cells with PMA in the absence or the presence of Nef (not shown). It is possible that the activation level obtained under these conditions is low and could be detectable only with a more sensitive reporter gene such as luciferase, but not with DsRed2 (40). However, the Nef-induced increase in the frequency of activated T cells does not appear to be the consequence of an increase in the intracellular calcium released seconds after TCR engagement.

Discussion

To explore the molecular mechanisms underlying the enhanced T cell activation induced by Nef, we assessed the effects of wild-type Nef and Nef mutants in two reporter cell lines in which changes in the induction of transcriptionally active forms of NF-kB and NFAT can be monitored at the single-cell level. We observed that Nef increases the number of activated T cells, but does not change the level of activation achieved in each stimulated cell. These results are in agreement with a previous study suggesting that HIV-1 Nef increases the number of cells secreting IL-2, but not the amount of IL-2 secreted per cell (9). This T cell hyperactivation phenotype requires the N-terminal myristoylation signal and the proline-rich motif of Nef, indicating a key role for membrane targeting of Nef in this process and its potential interaction with cellular partners through the SH3 ligand domain. We have also shown that TCR activation and clustering are necessary and sufficient for

FIGURE 4. Requirement of TCR signaling, but not CD28 signaling, for effective recruitment of Nef into the IS. Jurkat cells transduced with Nef-GFP were incubated with beads coated with anti-CD28 (A) or anti-CD3 (B). Each panel is representative of six different experiments.

FIGURE 5. Nef does not increase IS formation or the number of cell surface lipid rafts. A, Kinetics of IS formation. Jurkat cells were incubated with anti-CD3/CD28-coated beads at 37°C, and an aliquot of the cell suspension was analyzed by FACS every 10 min. The data are representative of three different experiments. B, Quantification of cell surface lipid rafts. Jurkat cells were incubated at 4°C with CT-B conjugated to Alexa-Fluo 647 to identify lipid rafts by FACS analysis. The data are representative of two experiments.
The recruitment of Nef in lipid rafts into the IS may well be linked to the Nef-induced T cell hyperactivation phenotype. PAK2 was recently described as a critical regulator of T cell function (24). Nef mutations previously reported to disrupt its association with activated PAK (P72A and P75A) did not affect the recruitment of Nef into the IS, but dramatically impaired the ability of Nef to induce an increased frequency of activated T cells. Like Nef, the ability of PAK2 to enhance T cell activation is forfeited when PMA is used instead of anti-CD3/CD28 (24). Both PAK2 and Nef appear to enhance T cell signaling very early after TCR ligation.

It is interesting to note that NL4–3 Nef is associated with less PAK2 activity than HIV SF2 Nef (20) and that SF2 Nef has been shown to induce a higher level of T cell activation than NL4–3 Nef (9). Furthermore, recent studies have shown that the specific interaction between Nef and autophosphorylated PAK2 occurs only in the lipid rafts, because autophosphorylated PAK2 is exclusively localized in these membrane microdomains (30, 31). Therefore, the small Nef fraction present in the lipid rafts that is recruited into the IS represents a specific subpopulation of this viral protein that is capable of interacting with this key kinase implicated in T cell signaling. The study of other factors, such as the Rac activators DOCK2 and ELMO1, in the Nef-PAK2 complex and mutual analysis of PAK2 regulatory domains will be required to further test whether PAK2 is the key and sole target of Nef in the IS. It is possible that Nef acting on PAK2 enhances T cell signaling after IS formation, leading to an improved intracellular environment for effective HIV replication.

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Disclosures

The authors have no financial conflict of interest.

References


FIGURE 6. Nef does not increase calcium release in the cytoplasm after CD3/CD28 cross-linking. Jurkot cells transduced with GFP constructs were loaded with the Ca2+-binding agent Fura 2 and activated by TCR and CD28 cross-linking. Levels of intracellular free Ca2+ were monitored by FACS analysis as the cells were warmed to room temperature to induce lipid raft patching. The data are representative of four different experiments.

Nef-induced T cell hyperactivation in the absence of CD28 ligation. T cell activation induced with the PKC activator PMA was not altered by the presence of Nef, suggesting that Nef acts at a very proximal step in the TCR activation pathway preceding PKC activation.

Protein trafficking has long been recognized as an important factor in T cell activation. Numerous studies demonstrating cytoskeletal reorganization, lipid raft clustering, and protein polarization in response to stimulation have led to the concept of the formation of an IS between the T lymphocyte and the APC (32). This structure is thought to be essential for generating and maintaining signals that determine the fate of a T cell upon antigenic stimulation. Our data strongly suggest that a fraction of HIV-1 Nef within the lipid rafts is recruited into the IS within minutes after TCR engagement and clustering. This recruitment required the highly conserved membrane-targeting sequences at the N terminus of Nef. Notably, grafting the N-terminal anchor domain of Nef onto the GFP is sufficient to observe the inclusion of Nef1–50-GFP into the IS. The efficient recruitment of lipid rafts and Nef after TCR engagement in the absence of CD28 stimulation is not altogether surprising, because the lipid raft enrichment in the IS persists in CD28-deficient mouse T cells (41). The presence of Nef does not appear to accelerate the formation of the IS or alter its overall size. Through an extensive set of experiments, we assessed whether Nef modulates the integrity of lipid rafts. In the absence or the presence of T cell stimulation, we observed the same amount of cellular proteins associated with the lipid rafts in Nef-expressing T cells, such as the TCR, Lck, PKCθ, or linker for activation of T cells (not shown). These results are in sharp contrast with a recent report that Nef increases the association between the TCR-signaling molecules and T cell rafts (42). In contrast to this previous study, we used a gradient of iodixanol instead of sucrose to isolate ing molecules and T cell rafts (42). In contrast to this previous report that Nef increases calcium levels in unactivated cells (15, 40), but cannot induce T cell activation by itself (9, 10). In our studies, we were unable to demonstrate that Nef alters calcium signaling within minutes after TCR and CD28 ligation. We conclude that it is unlikely that Nef-induced increases in cytoplasmic calcium levels (15, 40) are responsible for the T cell hyperactivation phenotype we observed at the single-cell level.


