Single-domain antibody-SH3 fusions for efficient neutralization of HIV-1 Nef functions

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Abstract

HIV-1 Nef is essential for AIDS pathogenesis, but this viral protein is not targeted by antiviral strategies. The functions of Nef are largely related to perturbations of intracellular trafficking and signaling pathways, through leucine-based and poly-proline motifs required for interactions with clathrin-associated adaptor protein complexes and SH3 domain-containing proteins such as the phagocyte-specific kinase Hck, respectively. We previously described a single-domain antibody (sdAb) targeting Nef and inhibiting many but not all of its biological activities. We now report a further development of this anti-Nef strategy through demonstration of the remarkable inhibitory activity of artificial Nef ligands, Neffins, comprised of the anti-Nef sdAb fused to modified SH3 domains. The Neffins inhibited all key activities of Nef, including Nef-mediated CD4 and MHC-I cell surface down-regulation and enhancement of virus infectivity. When expressed in T-lymphocytes, Neffins specifically inhibited the Nef-induced mislocalization of the Lck kinase which contributes to alteration of the formation of the immunological synapse. In macrophages, Neffins inhibited Nef-induced formation of multinucleated giant cells and podosome rosettes, and counteracted the inhibitory activity of Nef on phagocytosis. Since we show here that these effects of Nef on macrophage and T cell functions were both dependent of the leucine-based and poly-proline motifs, we confirmed that Neffins disrupted interactions of Nef with both AP complexes and Hck. These results demonstrate that it is possible to inhibit all functions of Nef, both in T lymphocytes and macrophages, with a single ligand that represents an efficient tool to develop new antiviral strategies targeting Nef.
Introduction

The Nef protein of HIV-1 promotes virus replication and is essential for pathogenesis of AIDS. This essential role of Nef results from its ability to disrupt certain intracellular trafficking and signaling pathways in infected cells (for review, 1, 9). Nef is indeed a multifunctional protein able to interact with components involved in vesicular transport between membrane compartments of the endocytic pathway and in the control of intracellular signaling pathways. These interactions are related to the presence of specific motifs, reminiscent of specific interaction motifs found in cellular proteins, within the primary sequence of HIV-1 Nef. Two types of motifs of Nef have been extensively analyzed: a leucine-based motif (ED160xxxLL165), found in a C-terminal flexible loop of HIV-1 Nef, and a poly-proline (P72xxP75) motif. While the leucine-based motif allows recruitment of clathrin-associated adaptor protein (AP) complexes that participate in the vesicular transport within the endocytic pathway, the poly-proline motif is required for interactions with cellular proteins containing SH3 domains, such as tyrosine kinases of the Src family (for review, 22). Therefore, some functions of Nef, such as cell surface down-modulation of certain surface receptors including CD4, are specifically dependent of the Leu-based motif, whereas the integrity of the poly-proline motif is required for some other Nef effects, such as the intracellular redistribution of the Src kinase Lck observed in HIV-1 infected T cells (12). Interestingly, Nef-mediated enhancement of HIV-1 infectivity depends on the integrity of both Leu-based and poly-proline motifs (24, 35).

Nef is abundantly expressed early after virus infection and perturbs the trafficking of several transmembrane proteins through alterations of the endocytic pathway. This leads to the modulation of cell surface expression of cellular receptors, including CD4 and major histocompatibility complex class I molecules (MHC-I), both in CD4-positive T cells and...
macrophages (20, 36). While the Nef-mediated modulation of CD4 requires the C-terminal AP-binding Leu-based motif, MHC-I down-modulation is determined by distinct motifs located in the N-terminal part of Nef: an acidic cluster (E62EEE65) and the poly-Pro SH3-binding motif (P2XXP75), indicating that the Nef-mediated cell surface down-regulation of either CD4 or MHC-I are related to different mechanisms (for review, 22).

In addition to T cells, macrophages represent important targets of HIV-1 during the initial steps of infection and contribute to the establishment of viral reservoirs, even in patients under highly active anti-retroviral therapy (for review, 10). Infected macrophages also participate in propagation of virus in non-lymphoid tissues such as lungs or brain. Furthermore, we have recently shown that Nef expression during HIV-1 infection disturbs specialized functions of macrophages: i) Nef induces the fusion of infected macrophages leading to the formation of multinucleated giant cells through activation of the Hck tyrosine kinase (40); ii) Nef also inhibits phagocytosis through alteration of the polarized exocytosis of recycling endosomes regulated by AP-1 (28).

Specifically expressed in phagocytes, the Nef-targeted Hck kinase is present in macrophages under two isoforms: p59Hck is found at the plasma membrane and induces the formation of F-actin rich-protrusions, whereas p61Hck is localized at the membrane of lysosomes where it can induce the formation of podosome-rosettes (6, 11). Nef is able to activate both Hck isoforms leading to the formation of actin-rich protrusions and podosome-rosettes (43). By activating p61Hck, Nef also triggers the fusion of individual infected macrophages leading to the formation of giant multinucleated cells that could contribute to the establishment of latent virus reservoirs in some tissues (40). In addition, we recently showed that Nef expression in HIV-1 infected macrophages also resulted in a significant diminution of their phagocytosis ability (28). This defect of phagocytosis did not involve
down-regulation of phagocytosis-involved receptors, but we observed a decrease of AP-1-positive endosome recruitment at the phagocyte cup (28).

While it is well established that Nef is essential for AIDS pathogenesis, it is not currently targeted by antiviral therapeutic strategies. The first Nef inhibitor was described in the literature was based on inhibition of the Nef interaction with an optimized derivative of the SH3 domain of Hck (16). More recently, small Nef interacting proteins composed of a Nef-targeted SH3 domain, fused to a sequence motif of the CD4 cytoplasmic tail and combined with a prenylation signal for membrane association have been also developed (4). SH3 domains are conserved structures composed of five \( \beta \) leaflets organized in two superposed surfaces and linked with unstructured loops (18, 27). The first loop, called RT loop, is critical for the recognition by HIV-1 Nef (23). To characterize Nef inhibitors, we previously generated a SH3 domain library in which the RT loop was replaced by a random hexapeptide (RRT, for random RT loop), and we isolated RRT-SH3 peptides that bound Nef with high affinity (\( kD=7 \) nM) (4, 15, 16).

We have recently described a single-domain antibody (sdAb) that binds Nef with high affinity (\( kD=2 \) nM) and inhibits most of the functions of Nef in CD4-positive T lymphocytes (3). This antibody fragment, called sdAb19, is able to counteract the Nef-mediated endocytosis of CD4 from the cell surface, and it also inhibits the positive effect of Nef on viral replication and infectivity (21). Finally, sdAb19 is also active \textit{in vivo} by rescuing Nef-mediated thymic CD4+ T-cell maturation defects and peripheral CD4+ T-cell activation in a CD4c/HIV-1\textsuperscript{Nef} transgenic mouse model (13). However, sdAb19 does not inhibit Nef-mediated down-regulation of MHC-I molecules (3), probably because it is not able to target the Nef residues involved in this function.

Here, we aimed to further improve the inhibitory activity of sdAb19 by expression as fusion with RRT-SH3 peptides (random RT-loop) that were previously selected for binding to
Nef with a high affinity (16). Two chimeric constructs, called Neffins, have been generated, and were able to potently inhibit all of the functions of Nef that we tested, including cell surface down-regulation of MHC-I. When expressed in T cells, Neffins specifically inhibited the Nef-mediated mislocalization of the Lck kinase involved in the Nef-induced impairment of the organization and function of the immunological synapse. In macrophages, Neffins inhibited the Nef-induced formation of multinucleated cells and rescued the inhibitory activity of Nef on phagocytosis through disruption of interactions of Nef with both AP complexes and Hck.
Materials and methods

Plasmids
Plasmids encoding wild-type or mutated Nef-hemagglutinin (Nef-HA) and Nef-green fluorescent protein (Nef-GFP), as well as plasmid for expression in bacteria of Nef fused to the glutathione-S-transferase (GST), have been described previously (20, 24). Plasmids for expression of sdAb19 (pET-sdAb19) in bacteria and in mammalian (pCDNA3.1-sdAb19) cells were also described (2). Plasmids for expression of Neffin-B6 and Neffin-C1 in mammalian cells were constructed by transferring a RRT-SH3 (B6 or C1) DNA fragment, amplified by PCR using specific primers, from the vector pEBB-myr-GST-RRT-SH3 (15, 16) into the NotI site of the sdAb19-pCDNA3 vector. For expression in bacteria, Neffins were amplified by PCR using specific primers, and then subcloned in pET15b as previously (2).

Cell culture and transfection
293T, HeLa, CEM, HPB-ALL, Jurkat, THP-1 and RAW264.7 cell lines were grown and transfected as previously described (2, 28, 36).

Immunofluorescence analysis
Immunofluorescence on HeLa cells was done as described previously (2), and cells were examined under an inverted Leica DMI6000 microscope equipped with a spinning Disk (Yokogawa CSU-X1M1), a 63x oil immersion objective and a cooled charge-coupled device camera (CoolSnap HQ2, Photometrics). Images were analyzed and processed with ImageJ (National Institutes of Health) and Photoshop (Adobe System Inc.). To represent the co-localization of the two proteins in white (see Figure 1B), the ImageJ co-localization plug-in was used. To quantitatively evaluate the level of co-localization of the two proteins, we
calculated the Pearson’s correlation coefficient (25) with the ImageJ JACoP plug-in (2) for at
least 10 independent cells.

Immunofluorescence on Jurkat T cells was done as described previously (37). Briefly, Jurkat
cells were permeabilized with 0.1% Triton X-100, then stained with anti-Lck (mAb 3A5,
Santa Cruz Biotech) and anti-c-Myc (71D10, Cell Signaling Technology) primary antibodies,
followed by CY3- and Alexa647-labelled secondary antibodies, respectively. Cells were
examined under a confocal Zeiss LSM700 microscope a using a 63x objective. To quantify
Lck mislocalisation, 3 independent experiments were carried out and 50 cells of each
condition were counted. Results are expressed as the percentage of transfected cells with Lck
relocated in an intracellular compartment, compared to total counted transfected cells.

Immunostaining in RAW264.7 cells was done as previously described (40), and fluorescent
cells were visualized using a Leica DM-RB fluorescence microscope. For analysis of cell
fusion, cells were examined 48 h after transfection in areas with similar cell density. All cells
were counted and cell fusion index was calculated as follows: total number of nuclei in
macrophage giant cells (cells with ≥2 nuclei) divided by total number of nuclei x 100. For
each condition, at least 200 cells were counted (40). For analysis of podosome-rosette
formation, slides were coated with vitronectin (Sigma) and 100 U/mL recombinant mouse
IFN-γ (Peprotech) were added 8 h after transfection as described (40). Cells were then
examined 16 h later. Statistically differences were determined using the Student’s t test, and
differences were considered significant if P < 0.01 (**), P < 0.001 (***)..

Phagocytosis assay
Phagocytosis of red blood cells (RBCs) or zymosan was performed as described previously
on transfected RAW264.7 macrophages (28). To quantify phagocytosis, the number of
internalized particles (RBCs or zymosan) was counted at 60 min in 50 cells randomly chosen
on the coverslips, and the phagocytic index (i.e., the mean number of phagocytosed particles per cell) was calculated. The index obtained for transfected cells was divided by the index obtained for control cells (negative cells of the same coverslip) and expressed as a percentage of control cells. We also counted the number of initial cell-associated particles (at 3 minutes), calculated the association index (mean number of associated particles per cell), and expressed it as a percentage of control cells (negative cells of the same coverslip).

**Viral production and infectivity assay**

Single-round HIV-1 carrying the GFP gene virus particles were produced in 293T cells as described (21). Infectivity of HIV-1 HXBc2 and VSV-G envelope glycoprotein-pseudotyped viruses was analyzed on HPB-ALL T cells as described (21).

**Immunoprecipitation and immunoblot analysis**

Immunoprecipitations were carried out on lysates of transfected 293T cells as described (2, 21) using anti-c-Myc (9E10 clone, Roche) antibodies. Immunoprecipitated proteins were then analyzed by Western Blot with anti-c-Myc and anti-HA (sc-8334, Santa Cruz) as described (2, 21).

**Nef-induced CD4 and MHC-I down-regulation**

Transfected HPB-ALL T cells were stained at 4°C with phycoerythrin(PE)-conjugated anti-HLA-A2, for MHC-I, or PE-CY5-conjugated anti-CD4 (clone RPA-T4, BD Biosciences), and cell surface expression was measured by flow cytometry as described previously (5). Results were expressed as the percentage of the mean fluorescence intensity determined in GFP-positive cells relative to that determined in GFP-negative cells.
GST pull-down assay

GST-Nef, sdAb19 and Neffins were produced in E. coli BL21 strain as described (19, 28). Briefly, 1 nmol of recombinant GST or GST-Nef was immobilized on 30 µL of glutathione-sepharose beads (GE Healthcare) and incubated for 1 h at 4°C with 1, 3 or 9 nmol of recombinant sdAb19, Neffin B6 or Neffin C1. Beads were washed twice in PBS, and then incubated with 1 mg of THP-1 cell lysate during 3 h at 4°C as described (28). Bound material was analyzed by Western blotting. Immunoblots against AP-1 and Hck were performed using mouse monoclonal anti-γ-adaptin (100/3, Sigma-Aldrich) and rabbit-polyclonal anti-Hck (SC-72, Santa Cruz Biotechnology), respectively.
Results

Interactions between Nef and Neffins in cells.

To improve the inhibitory activity of the anti-Nef sdAb19 and to expand its activity against the multiple functions of Nef, we generated two chimeric proteins in which the parental sdAb19 was fused to the N-terminal end of SH3 polypeptides previously selected for high affinity binding to HIV-1 Nef (16). These polypeptides were derived from the macrophage-specific Hck kinase but they carried substitutions within a hexapeptide region in the RT-loop of this SH3 domain (Neffins B6 and C1, see on Figure 1A). A more complete description of the design and biochemical properties of Neffins will be described elsewhere (Järviiluoma et al., submitted).

As described for the parental sdAb19 (2), we first examined the ability of sdAb19-SH3 Neffins to target Nef by immunofluorescence investigation in HeLa cells expressing Myc-tagged Neffins in combination with Nef-GFP (Figure 1B). When expressed alone (see supplementary Figure S1), both Neffins and sdAb19 were randomly distributed between the nucleus and the cytoplasm of transfected cells. In Nef-GFP expressing cells (Figure 1B), Neffin B6 or Neffin C1, as well as sdAb19, were redistributed to the cytoplasm and co-localized with the dotted Nef-positive structures concentrated in the perinuclear region, and corresponding to the endosomal compartments described previously (24). Calculation of the Pearson’s coefficient (r) to evaluate the level of co-localization between Nef-GFP and sdAb19, Neffin B6 or Neffin C1 gave rise to high r values (0.911, 0.944 and 0.914, respectively). Interestingly, the level of co-localization with Nef-GFP was significantly higher in cells co-expressing Neffin B6 than in cells expressing Neffin C1 (p=0.03) or sdAb19 (p=0.02) as evaluated by the Pearson’s coefficient.
To confirm that this co-localization was due to direct interactions between Nef and Neffins, co-immunoprecipitation analyses were performed on cells co-expressing c-Myc-Neffins and HA-tagged Nef (Figure 1C). Using anti-c-Myc antibody, Nef-HA was efficiently and specifically co-precipitated with either sdAb19, Neffin B6 or Neffin C1. These results indicate that Neffins are functional in the intracellular environment and can efficiently associate with co-expressed Nef protein. Under these conditions, the additional Nef-binding interface provided by the SH3 domain did not have an obvious impact on the amount of co-precipitated Nef.

Neffins do inhibit both CD4 and MHC-I cell surface down-regulation.

Having established that Nef was efficiently recognized by Neffins B6 and C1, they were subjected to a thorough analysis regarding their inhibitory potential on the best-characterized functions of Nef, including cell surface down-regulation of CD4 and MHC-I. Because sdAb19 was not able to inhibit MHC-I down-regulation (Ref 2, and Figure 2A), we first investigated whether the SH3 fusion could provide the Neffins with a capacity to inhibit this Nef function. As shown in Figure 2A, Nef-GFP efficiently down-regulated MHC-I from the surface of transfected T cells, and the poly-Pro motif of Nef was required for this Nef activity since MHC-I down-regulation was abrogated when the two proline residues were mutated to alanines (NefPxP/AxxA mutant). When cells were co-transfected with Nef-GFP and increasing amounts of Neffins B6 or C1 encoding plasmids (1:1 to 1:3 ratios), MHC-I down-regulation by Nef was inhibited in a dose-dependent manner leading to the restoration of cell surface levels of MHC-I molecules equivalent to that measured in the absence of Nef (Figure 2A and 2B).

Similar studies on Nef-induced down-regulation of cell surface expression of CD4 (Figure 2C) or CCR5 (supplementary Figure S2) showed that both Neffins B6 and C1 were
also able to inhibit these Nef effects in a dose-dependent manner. However, no obvious increase in their potency in blocking CD4 and CCR5 down-regulation could be seen compared to the parental sdAb19. As reported in the supplementary Figure S3A where cell-surface CD4 was plotted as a function of the GFP fluorescence, inhibition of the CD4 down-regulation activity by sdAb19 or Neffins was efficient even at the highest level of Nef expression. Similarly, CD4 down-regulation could be efficiently neutralized by sdAb19 and Neffins when expression of the native Nef protein was driven by the HIV-1 LTR promoter (supplementary Figure S3C). Finally, we checked that the inhibitory effects provoked by increasing amounts of sdAb19 or Neffin expression was not due to a decrease of Nef-GFP expression, as evidenced by Western blot analysis (supplementary Figure S3B).

Interestingly, Neffin B6 was able to inhibit the CD4 down-regulation activity of some Nef alleles, such as Nef proteins from the NA7 or YBF30 HIV-1 strains, that were not affected by co-expression of sdAb19 (Ref 2, and Figure 2D, left part). The MHC-I down-regulation activity of these Nef proteins was now also counteracted in cells co-expressing Neffin B6 (Figure 2D, left part). Together, these results show that Neffins are potent inhibitors of cell surface down-regulation of two major cellular targets of Nef. They also indicate that Neffins display a broader inhibitory activity than the parental sdAb19 against a very large panel of HIV-1 Nef proteins.

**Neffins specifically inhibit the Nef-induced mislocalization of the Lck kinase.**

While it has been shown that Nef expression impairs the ability of infected T lymphocytes to form immunological synapses with antigen-presenting cells, this effect was related, at least in part, to a mislocalization of the TCR proximal Lck kinase in endosomal compartments (34, 37). Since this mislocalization of Lck specifically depends on the poly-Pro motif of Nef (12), we investigated by immunofluorescence analysis whether Neffins were
able to counteract the intracellular redistribution of Lck observed in T cells expressing Nef-GFP. As shown in Figure 3A, Lck was mainly localized at the plasma membrane in control cells expressing GFP. In contrast, expression of Nef-GFP resulted in a net redistribution of Lck which accumulated in an intracellular compartment (Figure 3A), and the poly-Pro motif of Nef, but not the Leu-based motif, was required for this Nef activity (Figure 3B). When cells were co-transfected with Nef-GFP and Neffins B6 or C1 encoding plasmids (1:1 ratio), the mislocalization of Lck induced by Nef was largely inhibited leading to the restoration of a plasma membrane Lck staining almost equivalent to that observed in the absence of Nef (Figure 3A and 3B). In contrast, sdAb19 failed to counteract the Nef-induced mislocalization of Lck (Figure 3A), indicating that the additional binding interface provided by the SH3 domain in Neffins has an inhibitory impact on this Nef function involved in the impairment of the immunological synapse. Interestingly, whereas Nef-GFP was mainly localized at the cell cortex and in the perinuclear region both in control cells (Figure 3A, no sdAb) and in cells expressing sdAb19, co-expression of Neffins B6 or C1 resulted in a more diffuse cytoplasmic distribution of Nef-GFP (Figure 3A, left panels), suggesting that Neffins may affect cellular functions of Nef in T cells through alteration of its intracellular distribution.

Neffins and sdAb19 inhibit Nef-mediated enhancement of virus infectivity

Since the positive impact of Nef on virus infectivity depends on the integrity of both the Leu-based and poly-Pro motifs of the protein (24, 35), we also addressed if Neffins were able to affect Nef-mediated infectivity enhancement of new progeny virions. The ability of Neffins and sdAb19 to impact virus infectivity when expressed in virus-producing cells was investigated in a single-round infection assay. Nef-deleted reporter viruses carrying the gene encoding GFP were produced in the absence or presence of Nef expressed in trans, and in combination with a fixed concentration of Neffins or sdAb19 (1:3 ratio of the plasmids...
encoding Nef and Neffins or sdAb19, respectively). As expected, viruses provided with the HIV-1 envelope produced from Nef-expressing cells showed a net increase of virus infectivity, whereas Nef expression did not increase the infectivity of viruses pseudotyped with the VSV-G envelope (Figure 4). When HIV-1 env-containing viruses were produced in the presence of Neffins C1 or B6, an 80 and 95% decrease of the specific effect of Nef on virus infectivity was observed, respectively. A similar strong decrease of virus infectivity was observed when viruses were produced in cells transfected with the same ratio of the plasmid encoding the parental sdAb19. These results show that Neffins can abrogate Nef-induced enhancement of HIV-1 infectivity, but similar to CD4 modulation, do not show greater potency than sdAb19 in this function of Nef.

**Neffins and sdAb19 inhibit macrophage fusion and podosome-rosette formation induced by Nef in macrophages**

Since the poly-Pro-dependent binding of Nef to Hck, the Src tyrosine kinase that is specifically expressed in macrophages, is the best characterized interaction of Nef with cellular proteins containing SH3 domains (23), we decided to investigate the potential inhibitory effects of Neffins on the perturbations induced by Nef on specific functions of macrophages. For this, we expressed Nef in the RAW264.7 macrophage cells, in which expression of Nef was sufficient to recapitulate all the Nef-dependent alterations observed in HIV-1 infected primary human monocyte-derived macrophages (40). As previously reported (40), Nef expression triggered the formation of multinucleated macrophages with an increase by more than 2-fold in the fusion index compared to control cells, and this effect was dependent on the poly-Pro motif required for interaction with Hck (Figure 5A and 5C). Interestingly, the NefLL/AA variant with substitutions of the two leucine residues of the Leu-based motif required for Nef interaction with the clathrin-associated AP complexes was also
deficient for macrophage fusion (Figure 5A). This result demonstrates that the Nef-induced fusion of macrophages is also related, in addition to the Nef effects on the Hck-dependent signalling pathway, to the perturbations provoked by Nef expression on intracellular trafficking. When RAW264.7 cells were transfected with the Nef-GFP expression plasmid in combination with plasmids for expression of sdAb19 or Neffins, a significant decrease of the cell fusion index was measured (Figure 5A). These results show that both Neffins, as well as the parental sdAb19, are able to inhibit the Nef-triggered fusion of macrophages.

Next, we examined whether the Neffins and sdAb19 also inhibited the actin remodelling phenotypes characteristic of activation of endogenous Hck by Nef in RAW264.7 macrophages (43). We have previously shown that Nef-expressing macrophages re-arranged their podosomes (Figure 5C, arrowhead) into podosome-rosettes (arrow) by activation of the p61 isoform of Hck (6, 40). When cells expressed Nef-HA in combination with Neffins B6, C1 or sdAb19, a significant decrease of the number of cells with podosome rosettes was observed (Figure 5B and 5C). Altogether, these results show that both Neffins and sdAb19 are able to inhibit the Nef phenotypes induced, at least in part, by Hck activation in macrophages. They also reveal that the Nef-induced phenotype on macrophage fusion was dependent of both Leu-based and poly-Pro motifs, indicating that this effect is related to the functional perturbations induced by Nef on both intracellular trafficking and signaling pathways.

**Neffins and sdAb19 restore the phagocytosis function of macrophages expressing Nef**

Finally, the anti-Nef inhibitory activity of Neffins and sdAb19 was explored on the reduction of phagocytosis induced by Nef expression in macrophages (28). The efficiency of cell-association and phagocytosis of particles was evaluated in RAW264.7 cells expressing Nef by fluorescence microscopy after interactions with IgG- or complement-opsonized sheep red blood cells, or with fluorescent zymosan (heat-inactivated yeast). As reported in Figure
6B, a significant reduction of FcR- and CR3-mediated phagocytosis, as well as uptake of zymosan, was observed in Nef-expressing cells, whereas Nef had no impact on initial binding of the particles to cells (Figure 6A). In agreement with previous results showing that this Nef activity was related to a decrease of the recruitment of AP-1-positive endosomes at the phagocyte cup (28), the NefLL/AA mutant was deficient for inhibition of phagocytosis (Figure 6B). Interestingly, the NefPxxP/AxxA mutant, with substitutions of the proline residues of the poly-Pro motif, also failed to reduce macrophage phagocytosis (Figure 6B). These results indicate that the Nef-induced inhibition of phagocytosis is also related, in addition to the Nef effects on the endocytic pathway, to the perturbations provoked by Nef expression on signalling pathways in macrophages. Interestingly, an efficient reduction of phagocytosis, equivalent to that measured in cells expressing the wild-type Nef protein, was measured by co-expression of both NefLL/AA and NefPxxP/AxxA mutants (Figure 6B), confirming that Nef acts on different intracellular pathways for alterations of phagocytosis in macrophages.

To analyze the inhibitory activity of Neffins and sdAb19 on Nef-induced reduction of phagocytosis, RAW264.7 cells were co-transfected with Nef-GFP and sdAb19 or Neffins expression plasmids in a 1:1 ratio, and then submitted to phagocytosis of IgG-opsonized sheep red blood cells. As shown in Figure 6D, the rate of phagocytosis in cells co-expressing Neffins B6, C1 or sdAb19 was almost totally restored to a level equivalent to the control cells. We checked that the association of particles on macrophages was not affected by the expression of Neffins or sdAb19 (Figure 6C). These results indicate that sdAb19 and Neffins are both able to counteract the negative effect of Nef on phagocytosis by macrophages.

Neffins and sdAb19 disrupt interactions of Nef with AP-1 complex and Hck
Together, the results reported in Figures 5 and 6 demonstrated that the Nef-induced alterations provoked by Nef on macrophage functions were dependent of both the Leu-based and poly-Pro motifs, suggesting that the inhibitory activity of Neffins and sdAb19 on these functions could be related to modulation of the specific interactions of Nef with both AP complexes and Hck. Therefore, the impact of Neffins and sdAb19 on these interactions was challenged using an in vitro pull-down assay. Recombinant Nef fused to GST was expressed in E. coli (Figure 7A, lower panel), immobilized on GSH-sepharose beads, and then tested for interactions with native AP-1 complexes and Hck from a lysate of the human monocytic THP-1 cells (right panels) in the presence of increasing amounts of recombinant sdAb19 or Neffins. Bound proteins were analyzed by Western blot with anti-γ-adaptin and anti-Hck antibodies (Figure 7A, upper and middle panels, respectively).

As reported previously (28), both the full-length and a cleaved form of γ-adaptin, which plays a role in the regulation of recycling from endosomes to plasma membrane, were pulled-down by GST-Nef, but not by the GST control (upper panel). At the equimolar ratio with GST-Nef (1:1, Figure 7B), sdAb19 was not able to inhibit Nef/AP-1 interaction, whereas a significant inhibition (65%) of the Nef recruitment of γ-adaptin was measured with both Neffins. By increasing the amount of Neffins or sdAb19 to 1:3 or 1:9 ratios, we could totally block the Nef/AP-1 interaction. Even if the interaction between Nef and AP-1 does not depend of the poly-Pro motif, but rather required the Leu-based motif, these results indicate that the presence of the SH3 part in Neffins helps to the inhibition of the interaction of Nef with AP-1 complexes.

Like AP-1 complexes, Hck was specifically pulled-down by GST-Nef from a THP-1 cell lysate (Figure 7A, middle panels). An efficient dose-dependent inhibition of this interaction was evidenced with both Neffins, but also with sdAb19 (Figure 7C). This latter result indicates that the poly-Pro motif is not fully accessible for interaction with the SH3 domain of...
Hck after binding of sdAb19 to Nef. However, comparison of Hck binding to Nef in the presence of Neffins or sdAb19 revealed that Neffins were more effective for inhibition of this interaction (Figure 7A and 7C).

Together, the results reported in Figure 7 show that both Neffins and sdAb19 are able to impair, at least in vitro, the specific interactions of Nef with AP-1 complex and Hck. They also suggest that the fusion of the modified SH3 domains to sdAb19 results in a higher affinity and/or avidity of sdAb19 for Nef.
Discussion

In the present study, we report the characterization of the inhibitory activity of the anti-Nef single-domain antibody fragment sdAb19 and its derivate fused to modified SH3 domains, so-called Neffins, on distinct intracellular functions of HIV-1 Nef, including Nef-induced alterations of T lymphocyte and macrophage functions. These anti-Nef inhibitors were able to block several functions of Nef that mediate the effects of this viral protein in HIV-1 pathogenesis. Indeed, Neffins showed potent effects on Nef-induced down-regulation of the cell surface expression of both CD4 and MHC-I molecules, and they also inhibited the Nef-induced mislocalization of the T cell-specific Lck kinase. Neffins were also able to reduce drastically the Nef-mediated enhancement of HIV-1 infectivity. Finally, Neffins showed important inhibitory effects in macrophages. First, they inhibited macrophage fusion and formation of podosome-rosettes induced by Nef. Second, they restored the phagocytosis activity inhibited by Nef in macrophages. Neffins likely inhibited these Nef effects on macrophage functions through their ability to inhibit interactions of Nef with both AP-1 complex and Hck.

Some of the Nef functions examined in this study, such as MHC-I down-regulation, mislocalization of Lck, and association with Hck or AP-1, were more efficiently inhibited by Neffins than by the parental sdAb19. On the other hand, other Nef functions, such as CD4 down-regulation, were equally well suppressed by Neffins and sdAb19. These differences most likely reflect the capacity of Neffins to occupy a larger area on the surface of Nef and thereby mask additional functional determinants, especially the Nef poly-Pro motif. They could also reflect an increased overall strength of binding to Nef provided by the extended interaction interface, as evidenced by the ability of Neffins to counteract some Nef proteins that were not affected by sdAb19. Indeed, biochemical studies on the Neffin-Nef interaction
have revealed a more than 25-fold increase in the binding affinity compared to the sdAb19-Nef complex, which is mainly due to the significantly slower dissociation of the Neffin-Nef complex (Järveluoma et al., submitted). Furthermore, it has been suggested that Nef functions may involve transitions between alternative conformations (17), and thus a higher capacity to restrain the flexibility of the Nef protein might also contribute to the increased potency of Neffins in inhibiting some effects of Nef. Similarly, efficient Nef inhibitors engineered for targeting of the multiple interaction sites of Nef were recently reported (4).

Down-regulation of MHC-I molecules is a common function developed by several viruses, including herpesviruses and poxviruses (44), for escape from the immune system. For HIV-1, this important function is mediated by Nef, and the development of an anti-viral strategy targeting Nef needs to inhibit this function of Nef to help the immune system to target infected cells. The parental sdAb19 was not able to restore physiological MHC-I surface expression in the presence of Nef, probably because it was unable to mask the two main motifs (i.e., E_{62}EEE_{65} and P_{72}xxP_{75}) required for this Nef function (26, 29, 32). In contrast, fusion of sdAb19 to RRT-SH3 domains in Neffins led to the specific targeting of the poly-Pro motif of Nef involved in the recruitment of the Src family kinase required for induction of MHC-I endocytosis by Nef (8). Nef association with AP-1 complexes was also involved in the mechanism of Nef-induced MHC-I down-regulation (29, 33, 42). The N-terminal acidic cluster (E_{62}EEE_{65}) of Nef and a single residue in the poly-proline helix, P_{78}, which is not required for the SH3-binding activity of Nef, also participate in the down-regulation of MHC-I through direct or indirect interactions with AP-1 (29, 41). The close proximity of both E_{62}EEE_{65} and P_{78} with the P_{72}xxP_{75} SH3-binding motif could explain why Neffins inhibited AP-1 binding to Nef with a higher efficiency than sdAb19. Indeed, this effect of Neffins may be due to their ability to alter the folding of Nef, thus preventing the ability of E_{62}EEE_{65} and P_{78} motifs to recognize AP-1 leading to the inhibition of Nef-induced
MHC-I down-regulation (29). The fact that Neffins are still active for inhibition of the cell surface CD4 down-regulation, as efficiently as sdAb19, indicates that there is no competitive effect between the respective RRT-SH3 and sdAb19 parts of Neffins on Nef. Therefore, Neffins are probably able to target simultaneously the acidic cluster, the poly-proline rich region comprised between residues P72 and P78 and the conformational epitope recognized by sdAb19 on Nef. This targeting may also affect cellular functions of Nef in T cells through alteration of its intracellular distribution.

Similarly, the parental sdAb19 was not able to counteract the mislocalization of the TCR proximal Lck kinase in endosomal compartments induced by Nef in T cells, probably because it was unable to mask the poly-Pro motif required for this Nef function (23, 27, 30). In contrast, addition of the RRT-SH3 domains in Neffins led to the specific targeting of this motif of Nef and thus inhibition of the Lck mislocalization, a function responsible, at least in part, for the alterations of the organization and function of the immunological synapse formed between infected T cells and antigen-presenting cells (34, 37). Moreover, our preliminary results indicate that the decrease observed in the formation of conjugates between T cells expressing Nef and B cells used as antigen-presenting cells was also inhibited by co-expression of Neffins (data not shown).

In a recent study, we showed that Nef expression induced the fusion of infected macrophages leading to the formation of multinucleated giant cells through activation of the p61 isoform of Hck (40). The function of these giant cells in HIV-1 pathogenesis is not well established, but they have been identified in several tissues from AIDS patients such as the brain, and have been proposed as a marker of progression of HIV-1-induced dementia (30, 31). The formation of such giant cells, by an Hck-dependent mechanism, could also contribute to the establishment of latent virus reservoirs in some tissues (40). In addition, the Nef-induced activation of p61Hck at the level of lysosomes led to the formation of podosome-
rosettes (40). These structures are involved in proteolytic degradation of the extracellular matrix, and are required for macrophage migration in dense and compact 3-D environments (7, 38, 39). As expected, both fusion of macrophages and formation of podosome-rosettes are dependent on the poly-Pro motif of Nef required for Hck binding (40). More surprisingly, we show here that the NefLL/AA mutant was also impaired for the cell fusion phenotype, indicating that this function of Nef involves the recruitment and activation of p61Hck at the lysosomes, but also some other effectors of Nef that are dependent on the Leu-based motif required for interactions with endosomal AP complexes. We can speculate that Nef is able to disturb the intracellular trafficking and the cell surface expression of molecules regulating membrane fusion in macrophages. Therefore, the total inhibition of the effect of Nef on giant cells formation and podosome-rosette induction observed by co-expressing Nefsins or sdAb19 in macrophages is not surprising, since we show here that they are able to disrupt interactions of Nef with both AP complexes and Hck.

Similarly, we revealed here that the NefLL/AA and NefPxxP/AxxA mutants failed to inhibit macrophage phagocytosis. While we previously showed that this Nef-induced inhibition was dependent of the Leu-based motif (28), these data indicate that it is also dependent of the poly-Pro motif. The phagocytosis process and the fusion of macrophages are complex processes that show some similarities (for review, Ref 14). The two processes involve reorganization of the actin cytoskeleton and require Hck activation. During Fcγ receptor-mediated phagocytosis, p59Hck is initially recruited at the phagocytic cup, while p61Hck is recruited in phagosomes (11). The specific role of Hck in phagocytosis is still unclear but the sequential recruitment of p59 and p61Hck likely plays a major role in this process. By interacting with the two isoforms of Hck through its poly-Pro motif (40), Nef could have a detrimental effect on phagocytosis. Interestingly, by co-expressing the two NefLL/AA and PxxP/AxxA mutants, we could rescue the inhibitory effect of Nef on
phagocytosis. Two hypotheses could be suggested to explain this result. First, Nef could inhibit at least two steps of the phagocytosis process, and these two steps may involve the Leu-based and poly-Pro motifs of Nef. Second, Nef oligomerization could be required for the inhibition of phagocytosis, and the formation of oligomers between the two Nef mutants would lead to the reconstitution of fully active Nef proteins. Again, the impairment of phagocytosis induced by Nef was efficiently inhibited by sdA19 and Neffins, and likely results from inhibition of the interactions of Nef with AP-1 complexes and Hck.

While we previously showed that sdAb19 was able to interfere with the association of HIV-1 Nef with the cellular p21-activated kinase 2 (PAK2) involved in the resulting inhibitory effect of Nef on actin remodeling in T lymphocytes (2), we report here that Neffins, but also the parental sdAb19, are able to disrupt interactions of Nef with clathrin-associated AP complexes and the phagocyte-specific Hck tyrosine kinase from CD4-positive myeloid cells. This latter result indicates that the poly-Pro motif is not fully accessible for interaction with the SH3 domain of Hck after binding of sdAb19 to Nef. However, quantitative comparison of Hck binding to Nef in the presence of Neffins or sdAb19 revealed that Neffins were more effective for inhibition of this interaction. This observation suggests that the fusion of the modified SH3 domains to sdAb19 in Neffins results in a higher affinity and/or avidity of sdAb19 for Nef. Together, these results demonstrate that Neffins are highly active for inhibition of associations of HIV-1 Nef with its best-characterized cellular partners, responsible for most of the critical roles of Nef during the virus life cycle and in the progression of the natural infection (for review, Refs 1, 9).

In conclusion, Neffins, generated to improve the inhibitory activity of sdAb19, were able to inhibit all the functions of Nef, including the cell surface down-regulation of MHC-I, in both lymphoid and myeloid CD4-positive cells. When expressed in macrophages, Neffins inhibited the Nef-induced formation of multinucleated giant cells and podosome-rosettes, and
also rescued the inhibitory activity of Nef on phagocytosis through disruption of specific interactions of Nef with both AP complexes and Hck. Altogether, these results demonstrate that it is possible to inhibit all Nef functions, both in T lymphocytes and macrophages, with a single ligand that represents an efficient tool to develop new antiviral strategies targeting Nef.
Acknowledgments

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References


Figures legends

Figure 1: Association of sdAb19 and Neffins with Nef. A) Schematic representation of Neffins resulting from the fusion of sdAb19 to RRT-SH3 B6 or C1 domains (highlighted) using a flexible GGSGG loop. TAG, c-Myc-epitope tag. B) Intracellular distribution of Nef-GFP and sdAb19, Neffin B6 or C1. HeLa cells were transfected with the plasmid for expression of the c-Myc-tagged sdAb19 or Neffins in combination with the plasmid for expression of Nef-GFP. SdAb19 and Neffins were detected by indirect immunofluorescence with anti-c-Myc. The co-localization of Nef-GFP with sdAb19, Neffin B6 and C1 is shown in white (right panels), and the Pearson’s coefficient ($r$) for evaluation of the level of co-localization is indicated on the right. C) Co-immunoprecipitation of Nef-HA with sdAb19 or Neffins. 293T cells were transfected with the plasmid for expression of the c-Myc-tagged sdAb19 or Neffins in combination with the plasmid for expression of either Nef-HA or HA (mock). Control corresponds to cells that did not express sdAb19 or Neffins. Cell lysates (right part) were submitted to immunoprecipitation with anti-c-Myc (left part). Immunoprecipitates were analyzed by Western blotting (WB) using anti-c-Myc (bottom) or anti-HA (top). IP, immunoprecipitation.

Figure 2: Neffins inhibit Nef-induced MHC-I and CD4 down-regulation. A), B) and C) HPB-ALL T cells were transfected with plasmids for expression of either Nef-GFP (NL43 allele) or GFP in combination with increasing amounts of the plasmid for expression of sdAb19 or Neffins as indicated. Transfected cells were stained with PE-conjugated anti-HLA-A (A and B) or PE-conjugated anti-CD4 (C) at 4°C, and surface expression of MHC-I or CD4 in Nef-GFP- or GFP-expressing cells was measured by flow cytometry. The PxxP/AxxA or NefLL/AA mutants were used as controls. D) Cells were transfected (1:3 ratio) with the
plasmid for expression of sdAb19 or Neffin B6 in combination with the plasmid for expression of the indicated Nef protein (NL43, NA7 or YB30 alleles) expressed as GFP fusions. Surface expression of MHC-I (right part) or CD4 (left part) in Nef-GFP- or GFP-expressing cells was then assessed as indicated above. Results are expressed as the percentage of the mean fluorescence intensity (MFI) determined in GFP-positive cells relative to that determined in GFP-negative cells. Values are the means of at least 3 independent experiments. Error bars represent 1 standard deviation (SD) from the mean.

Figure 3: Neffins inhibit Nef-mediated mislocalization of Lck. Jurkat T cells were transfected with plasmids for expression of either wild-type or mutated Nef-GFP in combination with plasmids for expression of sdAb19, Neffin B6 or C1 (1:1 ratio). A) Subcellular distribution of Nef-GFP (in green) and Lck (in red) in GFP or Nef-GFP transfected Jurkat cells. Representative images of cells expressing GFP (used as a control) or Nef-GFP alone (no sdAb) or in combination with sdAb19, Neffin B6 or Neffin C1 (as indicated) are shown. B) Percentages of transfected cells showing Lck mislocalization were calculated by observation of 50 GFP-positive cells for each condition. Results are expressed as the percentage of total GFP-positive cells. Values are the means of 3 independent experiments. Error bars represent 1 SD from the mean.

Figure 4: Neffins and sdAb19 inhibit Nef-mediated enhancement of virus infectivity. Single-round GFP reporter viruses, pseudotyped with either HIV-1 Env or VSV-G, were produced in 293T cells in the absence or presence of the plasmids expressing sdAb19 or Neffins. Forty-eight hours later, viruses were pelleted from cell culture supernatants and were used to infect HeLa-CD4 cells. The percentages of GFP-positive infected cells were then measured by flow cytometry 60 h later. Viral infectivity was normalized to that of viruses
produced in the presence of Nef. Values are the means of 3 independent experiments. Error
bars represent 1 SD from the mean.

**Figure 5:** *sdAb19 and Neffins inhibit Nef-induced cell fusion and podosome-rosette formation in macrophages. A-C) RAW264.7 macrophages were transiently transfected with a pEGFP vector (control GFP) in combination with plasmids for expression of wild-type or mutated HA-tagged Nef and for expression of sdAb19, Neffin B6 or C1 (1:2 ratios). Then, cells were immunostained with anti-HA antibodies and with Alexa Texas Red-coupled phalloidin for F-Actin, and DAPI for nuclei. A) Quantification of cell fusion index evaluated 48 h after transfection (n=5). B) Percentage of cells with individual podosomes or podosome-rosettes 24 h after transfection (n=3). C) Immunofluorescence microscopy of RAW264.7 macrophages. F-Actin is shown in red, Nef-HA in green and nuclei in blue (DAPI). White arrowheads show a cluster of individual podosomes, arrows show a podosome-rosette. Bar, 10 μm.

**Figure 6:** *sdAb19 and Neffins restore efficient phagocytosis in macrophages expressing Nef. A and B) Motifs of Nef involved in phagocytosis inhibition. RAW264.7 macrophages transiently expressing GFP or wild-type or mutated Nef-GFP were processed for phagocytosis of IgG-RBCs, C3bi-RBCs or Zymosan for 60 min at 37°C. C and D) RAW264.7 macrophages transiently expressing GFP or Nef-GFP in combination with sdAb19, Neffin B6 or C1 were processed for phagocytosis of IgG-RBCs. The efficiencies of association (A and C) and phagocytosis (B and D) were calculated for 50 GFP-positive and 50 GFP-negative cells (GFP control). Results are expressed as the percentage of control GFP-negative cells. Values are the means of 3 independent experiments. Error bars represent 1 SD from the mean.
Figure 7: Neffins and sdAb19 inhibit Nef association with AP-1 and Hck. A) Purified recombinant GST or GST-Nef were immobilized on glutathione sepharose beads (lower panel, Coomassie blue), and were then incubated with THP-1 cell lysates in the presence of increasing amounts of sdAb19 or Neffins. In A) Bound material (left panels) and total lysates (right panels) were subjected to Western blotting with anti-AP-1 (upper panels) or anti-Hck (middle panels) antibodies. The percentages of association of AP-1 (B) or Hck (C) with GST-Nef were normalized to the association without inhibitor (100%).
**Figure 1**

**A**

sdAb19

GGSGG

RRT-SH3

TAG

Neffin B6

Neffin C1

sdAb19

Neffin B6

Neffin C1

control

**B**

Nef-GFP

merge

Nef-GFP

sdAb19

merge

Nef-GFP

Neffin B6

merge

Nef-GFP

Neffin C1

merge

r = 0.911 +/- 0.023

r = 0.944 +/- 0.020

r = 0.914 +/- 0.025

**C**

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**WB**

- anti-HA
- anti-c-Myc

**IP anti-c-Myc**

**Cell lysate**
Figure 3

A

B

Lck redistribution in intracellular compartment (% of transfected cells)

GFP  Nef-GFP  Nef-GFP Lck merge

Nef-GFP  Lck  merge

Nef-GFP  Lck  merge

Nef-GFP  Lck  merge

Nef-GFP  Lck  merge

Nef-GFP  Lck  merge

Figure 3

GFP  Nef-GFP  Nef-GFP Lck merge

Nef-GFP  Lck  merge

Nef-GFP  Lck  merge

Nef-GFP  Lck  merge

Nef-GFP  Lck  merge

Nef-GFP  Lck  merge
Figure 5

Fusion Index (%)

A

B

C

Nef-GFP

Nef-GFP + sdAb19 (1:2)

Nef-GFP + Neffin B6 (1:2)

Nef-GFP + Neffin C1 (1:2)

Control GFP

Nef-GFP

Nef-GFP, PxxP/AxxA

Nef-GFP, LL/AA

Nef-GFP + sdAb19 (1:2)

Nef-GFP + Neffin B6 (1:2)

Nef-GFP + Neffin C1 (1:2)

Podosome rosettes

Individual podosomes

(%)
Figure 7

A

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| GST-Nef     |     |     |     |     |     |     |     |     |     |     |       |
| GST         |     |     |     |     |     |     |     |     |     |     |       |

B

Coomassie
Blue

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Figure 7