HIV-1 Nef Induces the Release of Inflammatory Factors from Human Monocyte/Macrophages: Involvement of Nef Endocytotic Signals and NF-κB Activation

Eleonora Olivetta,* Zulema Percario,‡ Gianna Fiorucci,§ Gianfranco Mattia,¶ Ilaria Schiavoni,* Caitriona Dennis,¶ Joachim Jäger,¶ Mark Harris,¶ Gianna Romeo,§ Elisabetta Affabris,‡ and Maurizio Federico2*

It has been recently reported that the endogenous expression of HIV-1 Nef in human monocyte/macrophages induces the release of chemokines and other as yet unidentified soluble factors leading to multiple effects of pathogenic significance, such as the recruitment and activation of quiescent lymphocytes. However, the description of underlying molecular mechanisms remained elusive. We recently demonstrated that human monocyte-derived macrophages (MDM) efficiently internalize soluble rNef, thereby inducing effects largely resembling those observed in cells endogenously expressing Nef. By exploiting the rNef/MDM model, we sought to gain more insights on the molecular mechanisms underlying the response of MDM to Nef. Array analysis for the detection of transcripts from a large number of monokines, chemokines, cytokines, and receptors thereof showed that MDM promptly responded to rNef treatment by increasing the transcription of genes for several inflammatory factors. Analysis of supernatants revealed that rNef treatment induced the release of macrophage inflammatory proteins 1α and 1β, IL-1β, IL-6, and TNF-α. Conversely, rNefs mutated in domains critical for the interaction with the endocytotic machinery (i.e., EE155-156QQ, and DD174-175AA) were ineffective. Interestingly, we found that the Nef-dependent release of inflammatory factors correlated with the activation of the NF-κB transcription factor, mainly in its p50/p50 homodimeric form, and in a de novo protein synthesis-independent manner. Our data add new hints supporting the idea that the presence of Nef is per se heavily detrimental for monocyte/macrophages and relative cross-talking cell types.

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The progression of AIDS deeply depends on alterations in immunological functions, whose molecular mechanisms in most cases still await a clear description. On the basis of molecular epidemiology data in humans (1–3), and, more stringently, of results obtained in animal models, a key role in AIDS pathogenesis has been proposed for the HIV regulatory protein Nef. This is a 27/34 kDa multifunctional lentiviral protein, expressed exclusively by HIV-1/2 and simian immunodeficiency virus, lacking any enzymatic activity. Concerning the studies on animals, the conclusions drawn by former observations demonstrating the lack of disease in macaques inoculated with nef-deleted SIV strains (4) have been strongly enforced by results obtained in transgenic mice. In particular, Hanna et al. (5, 6) reported that Nef expression in cells of lymphomonocytic lineage induced severe AIDS-like pathologies, mainly correlating with the presence of the Nef Src homology 3 (SH3)3 motif binding domain (7).

A huge mass of data consistently indicates that Nef interferes with the signaling pathways in lymphocytic cells (8–13), but much less is known about the effects of Nef on monocyte/macrophages.

Monocyte/macrophages play a central role in the immunological homeostasis, both as APCs, and by secreting soluble factors involved in several immune response reactions. They replicate HIV quasispecies most represented in the early phases of the disease (i.e., M-tropic HIV) (14, 15), and resist the cytoidal effects typically induced by HIV in lymphocytes. Hence, behavioral alterations they met during the development of AIDS pathology deserve detailed investigations. Some aspects of the response of human monocyte-macrophages to HIV-1 Nef expression have been already characterized, e.g., the increased release in supernatants of macrophage inflammatory protein (MIP)-1α and MIP-1β, as well as of still unidentified soluble factor(s) able to activate lymphocytes (16, 17). By investigating the intracellular signaling, we recently demonstrated the activation of STAT-1 upon the infection of monocyte-derived macrophages (MDM) with a Δenv, but not with a Δenv/Δnef, HIV-1 strain (18). Moreover, the human myeloid TF-1 cells showed STAT-3 activation upon transduction with a Nef-expressing retroviral vector (19).

We further investigated the molecular mechanisms underlying the response of MDM to Nef by exploiting the ability of MDM to efficiently internalize rNef. Besides representing a useful tool for molecular analyses, such a system seems relevant as significant amounts (i.e., from 0.5 to 10 ng/ml) of extracellular Nef have been detected in supernatants from HIV-1-infected cell cultures, as well as in the serum of AIDS patients (20).

Laboratories of *Virology and ‡Clinical Biochemistry, Istituto Superiore di Sanità, †Department of Biology, University of Roma Tre, and ¶Institute of Biomedical Technology, Consiglio Nazionale delle Ricerche, Rome, Italy, and §School of Biochemistry and Molecular Biology, University of Leeds, Leeds, United Kingdom.

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1 This work was supported by grants from AIDS project of the Ministry of Health, Rome, Italy.

2 Address correspondence and reprint requests to Dr. Maurizio Federico, Laboratory of Virology, Istituto Superiore di Sanità, Viale Regina Elena, 299, 00161-Rome, Italy. E-mail address: federico@iss.it

3 Abbreviations used in this paper: SH3, Src homology 3; MIP, macrophage-inflammatory protein; MDM, monocyte-derived macrophage; NMT, N-myristoyl-trans-

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In this study, we report that the treatment of MDM with rNef induced augmented transcription and release of a set of soluble factors (i.e., MIP-1α, MIP-1β, IL-1β and -6, TNF-α) suggestive of an inflammatory response. Through the analysis of rNef mutants, we established that two C-terminal diacidic domains involved in the interactions with the endocytotic pathway are critical for the release of inflammatory factors. Moreover, we found that the gene activation induced by Nef correlated with an increase in the activation of the NF-κB transcription factor, mainly in its p50/p50 homodimeric form. These data allow us to propose a model where Nef induces an activation state in MDM appearing both potent and redundant.

Materials and Methods
Cell cultures
PBMC were isolated from buffy coats obtained from 20- to 40-year-old healthy male donors. Monocytes were isolated by 1-h adherence of PBMC followed by immunodepletion conducted by using anti-CD2, -CD3, and -CD19 Dynal beads (Dynal, Oslo, Norway). The purity of recovered cell populations was assayed by FACS analysis by means of PE-conjugated anti-CD14 mAb (Abbiotti, Abbott Park, Illinois). Ten nanograms of pseudotyped HIV-1 were used to infect 10⁶ 7-day-old MDM. The virus adsorption was conducted in 48-wells plates by incubating the cells for 1 h at 37°C with the viral inoculum diluted in 100 μl of complete medium. Afterward, the viral inoculum was removed, and the cells were washed and refed with 300 μl of complete medium.

Percentages of cells expressing intracytoplasmic HIV-1 Gag-related products were evaluated by FACs analysis after treatment with Permea (Ortho Diagnostic, Raritan, NJ) for 30 min at room temperature (r.t.), and labeling for 1 h at r.t. with a 1/50 dilution of KC57-RD1 conjugated anti-HIV-1 Gag mAb (Coulter, Hialeah, FL).

RNA isolation and cDNA array hybridization
Total RNA was isolated from MDM by lysing cells in RNA FAST reagent (Molecular Systems, San Diego, CA), treated with RNase-free DNase I for 10 min at r.t. to remove residual DNA, and cleaned through a RNasy mini column (Qiagen, Valencia, CA). To exclude genomic DNA contamination, recovered RNA was tested in a PCR by using primers for an housekeeping gene (i.e., human β-globin). 32P-labeled cDNA probes were generated by reverse transcription of 4 μg of total RNA following the manufacturer’s recommendations. To isolate the labeled cDNA from unincorporated 32P-labeled nucleotides and small cDNA fragments, the probes were purified with a MicroSpin Extraction Spin Column (Clontech Laboratories, Palo Alto, CA). Each resulting probe was then applied to an Atlas cytokine array (Clontech Laboratories) for an overnight hybridization with continuous agitation at 68°C. Hybridization and washing were performed according to the manufacturer’s recommendations. Signals from autoradiograms were quantified by using specific software (AtlasImage 2.01; Clontech Laboratories). The adjusted (i.e., subtracted from background) intensity ratio between RNA arrays from rNef treated vs untreated MDM was indicative of the modulation of gene transcripts.

Protein detection and confocal microscopic analysis
For Western blot analyses, MDM were washed twice with PBS, pH 7.4, and lysed in 1% Triton X-100 in the presence of 0.5 mM DTT, 20 mM sodium molybdate, 10 mM sodium orthovanadate, 100 mM sodium fluoride, 10 μM/ml leupeptin, 0.5 mM PMSF for 20 min in ice. Whole cell lysates were centrifuged at 16,000 × g for 10 min at 4°C and the supernatants were frozen at −80°C. The protein concentration of cell extracts was determined by the Bio-Rad ( Hercules, CA) protein assay. Aliquots of 30 μg of cell extracts were resolved on 7–10% SDSPAGE and transferred by electroblotting on polyvinylidene difluoride (Immobilon-P, Millipore, Bedford, MA) membranes for 60 min at 100 V with a Bio-Rad trans-blot. For the immunoblot, polyclonal anti-mouse antibodies to the indicated protein (e.g., MIP-1α, MIP-1β, IL-1α, IL-1β, TNF-α, TNF-β, IL-6, and IFN-α) were used. Membranes were blocked in 5% BSA fraction V (Sigma-Aldrich) in Tween 20 + TBS/EDTA (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.1% Tween 20) for 30 min at r.t., then incubated for 1 h at r.t. with specific Abs diluted in 1% BSA/Tween 20 + TBS/EDTA. Anti–IFN regulatory factor (IRF)-1 Abs were obtained from Santa Cruz Biotechnology (Santa Cruz), and monoclonal anti-β tubulin were obtained from ICN Biomedicals (Costa Mesa, CA).

Detection of soluble factors in MDM supernatants were performed through ELISA kits from R&D Systems (Minneapolis, MN) following the manufacturer’s recommendations.

For the confocal analyses, >98% pure monocytes were seeded in complete medium on eight chamber glass slides (BD Biosciences). After 7 days, the medium was replaced with fresh complete medium complemented with 100 ng/ml rNef-FITC, recovered as previously described (17). Sixteen hours after cells were washed twice with 1× PBS, and labeled for 1 h at 4°C with 100 μl of a 1/50 dilution in 1× PBS/2.5% FCS of PE-conjugated anti-CD14 mAb. Finally, cells were washed and fixed by adding 200 μl of 1× PBS, 2% (v/v) formaldehyde buffer. After an additional 30 min at r.t., glass coverslips were mounted, and cells observed. Images were analyzed by using a Leika CS4D confocal microscope (Deerfield, IL).

DNA EMSA
MDM were washed twice in ice-cold PBS and lysed for 20 min at +4°C in EMSA lysis buffer (20 mM HEPES, pH 7.9, 50 mM NaCl, 10 mM EDTA, 2 mM EGTA, 0.5% Igepal CA-630; Sigma-Aldrich; 0.5 mM PMSF, 10 mM sodium molybdate, 100 mM NaF, 10 μg/ml leupeptin, and 0.5 mM DTT). Three picomoles of the double-stranded oligonucleotide containing the NF-κB binding site of the IRF-1 promoter (5′-GGG CCG GCC AGG GCT GGG GAA TCC CGC TAA GTG TTT GGA T-3′; GCC AGG GCT GGG GAA TCC CGC TAA GTG TTT GGA T-3′) were end-labeled with γ-32P]ATP (0.74 MBq, 222 TBq/mmol; NEN, Boston, MA) by T4 polynucleotide kinase (New England Biolabs, Beverly, MA). Pounum-labelled products were incubated in the presence of the labeled oligonucleotide probe (37 mBq) at 4°C for 30 min and at r.t. for 40 min in 20 μl of binding buffer (20 mM Tris, pH 7.5, 75 mM KCl, 1% glycerol, 1 mM DTT, 1 μg of BSA, and 2 μg of poly(dI/dLc)). Cold competitor was added in a 100-fold molar excess of the radiolabeled probe. Supershift was performed by using 2 μg of the specific Abs (anti NF-κB p50 or anti NF-κB p65 polyclonal Abs; Santa Cruz Biotechnology) to the cell extracts. DNA-protein complexes were resolved on 5% polyacrylamide gels in 25
**Results**

**Analysis of gene transcription upon rNef treatment of MDM**

The internalization in human MDM of rNef leads to effects largely superimposable to those observed in cells endogenously expressing Nef, that are specifically abrogated by anti-Nef immunodepletion procedures (16–18). Hence, we exploited such an experimental model to further study the molecular events induced by Nef. As a first step, we analyzed the influence of Nef in the MDM transcriptional profile by evaluating the levels of transcripts from a large number of genes after rNef treatment. Purified monocytes from four donors were cultivated separately for 7 days and, thereafter, were treated with 100 ng/ml rNef for 2 h, a time lag selected on the basis of the kinetics of Nef-induced gene activation we previously described (18). Thereafter, control and treated MDM cultures were pooled, and the mRNA transcription extents of a broad panel of chemokines, cytokines, monokines, growth factors, and receptors were evaluated through an array detection system.

**Table I. Stronger constitutive activity in MDM genes whose expression was increased by rNef treatment**

| Gene Product | Folds of Increase Upon rNef Treatment | EMBL Gene Bank Accession Number |
|--------------|--------------------------------------|---------------------------------|
| IL-1α        | 23                                   | X02851                          |
| IL-1β        | 2.1                                  | X02532                          |
| IL-6         | 12.1                                 | X04602                          |
| TNF-α        | 3.2                                  | X01394                          |
| MIP-1α       | 34                                   | M23452                          |
| MIP-1β       | 70                                   | J04130                          |
| IRF-1        | 2.2                                  | X14454                          |
| TNF-stimulated gene 6 | 97     | M31165                          |
| CD40L        | 2.1                                  | X60592                          |
| Erb B-3      | 8.9                                  | M29366                          |
| GMF-β        | 4.1                                  | M86492                          |

**Table II. Lower constitutive activity in MDM genes whose expression was increased by rNef treatment**

| Gene Product | Folds of Increase Upon rNef Treatment | EMBL Gene Bank Accession Number |
|--------------|--------------------------------------|---------------------------------|
| IL-5         | 4.5                                  | X04688                          |
| IL-7         | 2.2                                  | J04156                          |
| IL-10R       | 4.6                                  | U00672                          |
| G protein-coupled receptor HM74 | 3.3   | D16431                          |
| Insulin-like growth factor binding protein 1 | 5.7 | M31145                          |

**Table III. Genes whose expression was reduced upon rNef treatment**

| Gene Product             | Folds of Decrease Upon rNef Treatment | EMBL Gene Bank Accession Number |
|--------------------------|--------------------------------------|---------------------------------|
| IFN-γ receptor β subunit | 4.2                                  | U05875                          |
| Macrophage inhibitory factor | 3.3  | M25639                          |
| TNF-α receptor           | 3.7                                  | M32315                          |
| Heparin-binding EGF-like growth factor | 7.2 | M60278                          |
| CXCR4                    | 3.7                                  | D10924                          |
| Platelet-activating factor receptor | 14.3 | D10202                          |
| Notch protein            | 2.7                                  | M99437                          |
| TNF C                    | 25                                   | L11015                          |
| Fibroblast growth factor receptor 1 | 9.1  | X66945                          |
| Vascular endothelial growth factor B | 11.1 | U48801                          |
| Vascular endothelial growth factor | 9    | M32977                          |
| Stem cell tyrosine kinase-1 | 2.5  | U02687                          |
| Tyrosine kinase receptor tie-1 | 3    | X69575                          |
| Endothelial monocyte-activating polypeptide 2 | 4.2 | U10117                          |

Products whose mRNA increased significantly upon rNef treatment are listed in Tables I–III, and grouped in genes showing stronger (Table I) or lower (Table II) constitutive activity. Genes whose expression was reduced upon rNef treatment are listed in Table III. It should be noticed that most of the genes positively modulated by Nef code for inflammatory chemokines and cytokines (i.e., MIP-1α, MIP-1β, IL-1α, -1β, and -6, TNF-α), indicating that Nef significantly altered the MDM gene expression program in a manner resembling a typical inflammatory response. Although we cannot formally exclude some variability among the gene profiles of the cells from the four donors tested, the results concerning the induction of genes for the inflammatory factors appeared consistent with data we accumulated over the time indicating that the MDM from only a small minority of donors (i.e., 3 of 82) did not respond to the rNef stimulation in terms of increase in the release of chemokines/cytokines (not shown).

**Nef induces the release of inflammatory chemokines and cytokines from MDM**

Data from the cytokine array indicated that the expression of genes for chemokines and cytokines typically involved in the inflammatory response were increased upon rNef treatment. To define whether the enhanced transcription levels correlate with an increase in the release of the respective soluble factors, MDM were treated for 16 h with 100 ng/ml rNef or left untreated, and supernatants were tested for the presence of the inflammatory chemokines and cytokines. As depicted in Table IV, the rNef treatment induced a strong enhancement in the release of MIP-1α, MIP-1β, IL-1β, IL-6, and TNF-α, reaching the concentrations of 4.3, 37, 0.35, 1.2, and 1.1 ng/ml, respectively.

Despite the apparently great increase in gene transcription, no detectable levels of IL-1α have been observed until 24-h posttreatment (not shown). Similarly, no detectable release of IL-12 or IL-18 has been observed (data not shown). Of note, qualitatively
superimposable results have been obtained by decreasing the amount of rNef added in MDM cultures up to 10-fold (not shown). Moreover, kinetic studies showed that the increase of soluble factors could be noticed as early as 2-h posttreatment, remaining clearly above background levels until 48 h after rNef addition (not shown).

Data from Table IV clearly show that MIP-1α and MIP-1β were the soluble factors whose release was more efficiently induced upon rNef treatment, thus representing a reliable hallmark of the MDM response to Nef. We exploited such a feature to test the dose-response curve was recovered by treating MDM with concentrations of rNef ranging from 0.1 to 100 ng/ml. As reported in Fig. 1, rNef was proved to efficiently stimulate MDM in releasing both MIP-1α and MIP-1β at a concentration of 1 ng/ml. Next, we asked whether Nef could lead to a prompt response in terms of the release of soluble factors also when expressed by HIV-1. For this purpose, we infected MDM with the (VSV-G) pseudotyped Δenvelope HIV-1, a protocol that we have recently demonstrated to be highly effective in challenging MDM (18). Cells were infected with 1 ng/10⁶ cells of (VSV-G) Δenvelope HIV-1 strains expressing or not expressing the nef gene, supernatants at 8- and 16-h postinfection were harvested, and the contents of both MIP-1α and -1β were measured by ELISA. Even if significantly reduced compared with rNef-treated MDM, higher levels of chemokines have been observed in supernatants from MDM infected with the Δenvelope vs the Δenvelope Δenvelope HIV-1 (Fig. 2A). To control the infection efficiencies, levels of intracytoplasmic HIV-1 Gag-related products were evaluated by intracytoplasmic immunofluorescence FACS analysis 16 h postinfection (Fig. 2B). Such data appear consistent with those obtained from rNef-treated MDM, at the same time establishing a stringent temporary correlation among HIV-1 infection, Nef expression, and an increase in the release of both MIP-1α and -1β.

**Analysis of Nef mutants. I. The N-terminal myristoylation of rNef boosts the increase in the release of soluble factors**

Data from gene activation profiling strongly suggest that Nef triggers an activation stimulus in MDM, consistently to that recently reported in Nef-expressing human lymphoblastoid cells (25). To investigate the underlying mechanisms, we sought to individuate the Nef domains involved in the MDM response. First, we investigated the role of membrane association of the internalized rNef, being a large part of the effects of endogenously expressed Nef dependent on the cell membrane interaction through its N-terminal myristoylation (for reviews, see Refs. 26 and 27). Although wild-type (wt) rNef lacked the N-terminal myristoylation, it is conceivable that, at least in part, it undergoes myristoylation upon cell internalization. To dissect the contribution of myristoyl-dependent membrane association, MDM were treated with either wt rNef, a rNef mutant lacking the Gly residue critical for the intracellular N-terminal myristoylation (G2A rNef), or a fully myristoylated wt rNef (myr⁺ rNef), expressed in E. coli cotransformed with a vector coding for a human NMT. The efficiency of myristoylation was estimated to be >99%, as calculated by electrospray mass spectrometry (not shown). The trace from the spectrometry analysis revealed a major peak of molecular mass of 24,346.7 Da that was in strong agreement with the predicted molecular mass of myristoylated rNef (24,343 Da). Two additional peaks were detected at 24,230 and 24,289.7 Da, respectively, likely the product of shorter chain fatty acyl groups added by the NMT, that, however, were clearly not nonmyristoylated rNef (whose predicted molecular mass is 24,133 Da) (data not shown).

We already proved that both wt and G2A rNefs are efficiently internalized by MDM, both displaying in a typical intracytoplasmic punctate pattern (17). Similarly, the intracellular disposition of myr⁺ rNef-FITC closely resembled those detectable after internalization of unmyristoylated wt rNef-FITC, as shown by the confocal analysis reported in Fig. 3A. FACS analyses failed to reveal significant differences in the internalization extents among diverse FITC-conjugated rNef isoforms (not shown).

By measuring the effectiveness of each rNef isoform in stimulating the release of inflammatory effectors, we observed an overall increase in the release of MIP-1α, MIP-1β, IL-1β, IL-6, and TNF-α whatever rNef isoform was used (Fig. 3B). Interestingly, the amounts of soluble factors released from MDM treated with wt and G2A rNef appeared comparable, and (except for IL-6) lower than those detected in supernatants from MDM treated with myr⁺ rNef. This indicates that full membrane targeting, even if not required, could improve in some extent the activation of effector genes. In addition, the evidence that wt and myristoylation-defective (i.e., G2A) rNefs had virtually identical effects suggested that the extent of myristoylation to which wt rNef may undergo upon cell internalization was ineffective in boosting the release of soluble factors.

Differently to that observed for the other inflammatory factors, it appeared that the Nef myristoylation had no effect in terms of increase of IL-6, at least at the concentration used (i.e., 100 ng/ml). To test whether this was the consequence of the rNef dose used, or, alternatively, a phenomenon of a general significance, we conducted dose-response experiments by treating MDM with increasing doses (i.e., from 1 to 100 ng/ml) of wt or myr⁺ rNefs, and measuring the release of IL-6 in the supernatants (Fig. 3C). We
proved that doses from 5 to 25 ng/ml myr+ rNef induced levels of IL-6 significantly higher than those detected upon treatment of wt rNef. Nevertheless, such a difference disappeared with the increase of rNef concentrations, suggesting that the apparent lack of influence of the rNef myristoylation on the release of IL-6 we previously observed was mainly the consequence of a saturation effect in terms of IL-6 production when MDM were treated with 100 ng/ml myr+ rNef. Importantly, the concomitant measurements of both MIP-1α and -1β revealed that MDM released higher amounts of both chemokines when treated with any dose of myr+ with respect to wt rNef (not shown).

FIGURE 2. Nef expressed by HIV-1 induces a prompt increase in the release of MIP-1α and MIP-1β from infected MDM. A. MDM cultures were infected with 1 ng/10^5 cells of VSV-G pseudotyped HIV-1-expressing or not expressing the nef gene. Two hours after the challenge, cell cultures were extensively washed, and refed. Supernatants were collected 6 and 16 h thereafter, and contents of chemokines were measured by ELISA, as compared with mock-infected control cultures (Ctrl). B. FACS analysis for the expression of HIV-1 Gag-related products in MDM 16 h after challenge. MDM treated as described in a (panels I and II) were labeled with a PE-conjugated anti-Gag HIV-1 mAb, and analyzed by FACS. Histograms from either uninfected or infected cells labeled with a PE-conjugated isotype-matched IgG overlapped that of uninfected MDM after labeling with anti-Gag HIV-1 mAb. Results from one representative experiment of five are reported.

Analysis of Nef mutants. II. Nef domains involved in the interactions with the endocytotic machinery regulate the release of chemokines and cytokines

We tested the effects of additional rNef mutants in terms of the release of MIP-1α, MIP-1β, IL-1β, IL-6, and TNF-α. In Fig. 4, data concerning rNef mutants defective for the interaction with the endocytotic machinery (EE-QQ and DD-AA rNefs) (28, 29), or for the interaction with SH3-binding domains (AxxA rNef) (30) are reported. In detail, whereas the phenotype of AxxA rNef resembled that of the wt isoform, the treatment with either the EE-QQ or...
DD-AA rNef mutants seemed largely ineffective, with a residual increase in MIP-1α release for the EE-QQ rNef mutant. Importantly, no differences in the rNef internalization extents were noticed by FACS analysis of MDM treated with different FITC-conjugated rNef mutants (not shown). By means of confocal microscope analysis, we proved that both EE-QQ and DD-AA rNefs mutants disposed in an intracytoplasmic pattern resembling that of wt rNef (Fig. 5A). Moreover, they proved to be biologically

FIGURE 3. The N-terminal myristoylation is not required for the Nef-induced increase in the release of inflammatory factors from MDM. A, Confocal microscope analysis of MDM after 16 h of treatment at 37°C with 100 ng/ml wt (I), or fully myristoylated (II) rNef-FITC, and 1 h labeling at 4°C with an anti-CD14 PE-conjugated mAb. In the inset of panel II, a cell internalizing the myr⁺ rNef-FITC was analyzed by means of FITC fluorescence alone. B, MIP-1α, MIP-1β, IL-1β, IL-6, and TNF-α contents in clarified supernatants of 10⁶ MDM that were treated for 16 h with 100 ng/ml wt, G2A, or myr⁻ rNefs, or were left untreated (Ctrl). Results are expressed in picograms per milliliter, and statistics were calculated from data of seven (for MIPs), and three (for IL-1β, IL-6, and TNF-α) independent experiments. C, Dose-response curve of IL-6 release from MDM treated with increasing concentrations of either wt or myr⁺ rNefs. MDM were treated with the indicated doses of rNef, and IL-6 contents were measured 16 h thereafter. Data reported are the mean values of duplicated cultures of 10⁶ MDM, and are representative of two independent experiments.
active, as both retained at least one already characterized Nef function in MDM, i.e., the induction of the IRF-1 after treatment for 5 h with rNef (18) (Fig. 5B), as the consequence of the STAT1 activation that both Nef mutants trigger as efficiently as the wt Nef does (Z. Percario, unpublished results).

The diacidic 155–156 EE domain acts as a lysosomal targeting signal, possibly through the interaction with the /H9252 subunit of the coat protein (COP)I complex (29) whereas the 174–175 DD Nef domain is involved in the interaction with the V1H regulatory subunit of the V-ATPase (28, 31), i.e., an enzyme localized in the most part to the single membrane-bound intracellular organelles that is required for the acidification of endosomes, lysosomes, and other intracellular vesicles. The fact that rNefs defective for either function resulted inactive, suggests that Nef-induced activation depends on the interaction with the V-ATPase in lysosomes. Conversely, the fact that the AxxA rNef, i.e., a Nef mutated in critical positions for the binding of the Nef polyproline region with the SH3 motif (30), induced effects similar to the wt form, suggests that SH3 Src protein kinases, known to interact specifically with Nef (i.e., Hck and Lyn in macrophages) (26, 27), are not involved in the observed phenotype.

The rNef treatment of MDM induces activation of NF-κB

Recently, it has been reported that murine peritoneal macrophages responded to the bafilomycin A1-induced inhibition of the V-ATPase with increased expression of TNF-α, mediated by the activation of the NF-κB transcription factor (32). In contrast, it has been proposed that the interaction of Nef with the V1H subunit of the V-ATPase leads to the inhibition of the proton pump (28). If Nef induces the activation of effector genes as a consequence of the V-ATPase inhibition, as suggested by the ineffectiveness of the DD-AA rNef mutant, then one would expect that such a gene activation correlates with increased NF-κB activation.

To test such a hypothesis, we treated MDM with rNef and, at different times, we evaluated the extent of NF-κB activation as the increase of binding activity to a specific target sequence (i.e., from the IRF-1 promoter) in an EMSA assay. Cell lysates were incubated also with Abs specifically recognizing the p50 or the p65 NF-κB subunits, or, as control, with a cold competitor. As shown in Fig. 6, the rNef treatment of MDM induced a strong and persistent increase in the binding activity of p50/p50 NF-κB homodimer, whose signals were efficiently shifted upon the incubation with anti-p50 Abs, and, in lesser extents, of the p65/p50 heterodimer. This result strongly supports the idea that the activation of the NF-κB transcription complex is part of the MDM response to Nef.

Nef-dependent NF-κB activation correlates with the stimulation of chemokine/cytokine release

In nonactivated conditions, NF-κB resides mostly in the cytoplasm bound to IκB, i.e., its specific inhibitor. Upon receipt of a stimulatory signal, IκB undergoes inactivation through phosphorylation by IκB kinase, and NF-κB translocates into the nucleus upon dimerization, leading to gene activation. The NF-κB transcription complex results in a homo- or heterodimer combination of correlated subunits (for review, see Ref. 33). More commonly, NF-κB acts in the form of either the p65/p50 heterodimer or the p50/p50 homodimer. Notably, such a transcription factor recognizes
sequences in promoters of several genes involved in the monocyte-macrophage activation, including MIP-1α, MIP-1β (34), IL-1β (35), IL-6 (36), and TNF-α (37).

To enforce the hypothesis that the Nef-dependent NF-κB activation plays a role in the induction of MDM effector genes, we reproduced the NF-κB activation assay by using cell lysates from MDM treated with the rNef mutants we already characterized in terms of induction of inflammatory cytokines and chemokines. Although the treatment for 2 h with activating rNefs led to a clear increase in NF-κB binding activity (Fig. 7A), appearing stronger in myr+ with respect to wt rNef-treated MDM (Fig. 7B), the signal from the p50/p50 NF-κB resulted in a clear reduction in MDM treated with rNef mutants (i.e., EE-QQ and DD-AA) unable to induce the effector genes (Fig. 7C), also extending the rNef treatment up to 8 h (data not shown). Hence, we found a good correlation between effector gene activation and the increase of NF-κB binding activity, strongly enforcing the hypothesis that the Nef-dependent activation of transcription is mediated by NF-κB activation.

The Nef-dependent NF-κB activation does not require de novo protein synthesis

To further investigate the mechanism of Nef-dependent NF-κB activation, we sought to establish whether the activation of the transcription factor depended on a de novo synthesis of interme-
mediate protein product(s). Thus, the 2 h rNef treatment of MDM was preceded by a 3 h incubation with 5 \mu g/ml cycloheximide. We observed that NF-\kappa B binding activity appeared even slightly enhanced in cells treated with both rNef and cycloheximide with respect to those incubated with rNef alone (Fig. 8). This was likely due to a slight NF-\kappa B activation induced by the cycloheximide treatment per se, as previously described (38). The effective block of protein synthesis was controlled by the inhibition of IRF-1 production (39) through Western blot analysis (not shown). The lack of an inhibitory effect of the cycloheximide treatment on the Nef-dependent NF-\kappa B increase of binding activity indicates that the observed Nef-dependent NF-\kappa B activation depended on intracellular signaling not requiring de novo protein synthesis.

**Discussion**

It has been recently reported that the expression of HIV-1 Nef in human lymphocytes triggers a transcriptional program overlapping that induced by a physiological activation by 97% (25). Results from the array analysis we performed on rNef-treated MDM support the idea that Nef induces an activation state also in human monocyte/macrophages. According to data from the cytokine array, the analysis on MDM supernatants demonstrated the increase in the release of MIP-1\alpha, MIP-1\beta, IL-1\beta, IL-6, and TNF-\alpha. Single cycle infection of MDM with (VSV-G)\_env HIV-1 pseudotypes proved that the expression of Nef induces an increase in the release of chemokines also when expressed in the viral context.

Indeed, genes not coding for inflammatory soluble factors were also induced by rNef treatment, namely TNF-stimulated gene 6, Erb-B3, CD40, IRF-1, and glia maturation factor (GMF)-\beta. Whereas TSG-6 is typically induced in the context of a negative feedback control of the inflammatory response (40), the lack of an inhibitory effect of the cycloheximide treatment on the Nef-dependent NF-\kappa B increase of binding activity indicates that the observed Nef-dependent NF-\kappa B activation depended on intracellular signaling not requiring de novo protein synthesis.

**FIGURE 7.** rNefs mutated in domains involved with the endocytotic machinery fail to activate NF-\kappa B. EMSA performed by incubating a \{\textsuperscript{32}P\}-labeled probe from the NF-\kappa B binding site of the IRF-1 promoter with total cell extracts from MDM untreated (Ctrl), or treated for 2 h with 100 ng/ml of wt, AxxA, or G2A rNef mutants (A), with 100 ng/ml wt or myr\^+ rNefs (B), or with 100 ng/ml wt, EE-QQ, or DD-AA rNef mutants (C). Supershifts were conducted with anti-p50 NF-\kappa B Abs, whereas anti-NF-\kappa B p65 and an IRF-1 cold competitor were used as controls. Electrophoretic mobility for p65/50 and/or p50/50 NF-\kappa B dimers are indicated on the left side.
It has been previously reported that human MDM transduced with a Nef-expressing adenoviral vector were stimulated in releasing both MIP-1α and MIP-1β, but not IL-1β or TNF-α (16). The discrepancies with data presented here could originate from the different cell culture conditions used, i.e., the treatment for 2 days with monocyte CSF described by Swingler et al. (16), could influence the maturation/differentiation processes typically occurring in monocyte cultures, in a way that MDM could modify the response to the same stimulus. In addition, we previously demonstrated that the treatment with 10–100 ng/ml rNef routinely leads to internalization in >95% of the cell population (17), while the transduction efficiency reported in the adenoviral vector system was at best 60–70%, as measured in cells transduced with a green fluorescence protein-expressing vector.

Nef induces similar effects in MDM whether endogenously expressed or upon internalization. In particular, lymphocyte activation mediated by the conditioned medium, STAT-1 activation, and increase of MIP-1α and -1β release have been proved in both models (16–18). However, the fate of rNef upon internalization conceivably resembles only in part that of endogenously expressed Nef which, through its N-terminal myristoylation, is targeted very efficiently to the cell membrane, where it can interact with a wide number of signaling host molecules. Afterward, Nef starts the retrograde intracellular path by associating with clathrin-coated pits, ultimately accumulating in the endosomal/lysosomal compartment (for review, see Refs. 26 and 27). Although mechanisms underlying the Nef internalization in MDM have not yet been elucidated, it is conceivable that MDM pick-up extracellular Nef through a pynocytotic/endocytotic process, leading to accumulation in vacuolar intracellular compartments, as suggested by the Nef intracytoplasmic punctated pattern we already described in rNef-FITC-treated MDM (17). Furthermore, the colocalization of rNef-FITC with lysosome-associated membrane protein-2 (i.e., a lysosomal subunit of the COPI coatomers in endosomes, a complex coating vesicle involved in the transport from early to late endosomes and lysosomes (29). Considering that binding of Nef to the β-COP should act as a lysosomal targeting signal, the strongly impaired activation we observed in MDM treated with the EE155–156QQ rNef may imply that crucial events occur in the lysosomal compartment. In summary, it is conceivable that Nef internalized by MDM interacts with endolysosomal V-ATPase, leading to the inhibition of luminal acidification, and, by consequence, of activation of lysosomal proteases. This in turn could favor the persistence of Nef in lysosomes, and thus a prolonged inhibition of lysosomal V-ATPase, with induction of effector genes.

We correlated the Nef-dependent induction of effector genes with an increased binding activity of the transcription factor NF-κB, mainly in its p50/p50 homodimeric form. It has been demonstrated that such NF-κB homodimer specifically stimulates the transcription from the Igκ-site sequences (46), that is equivalent to the HIV-1 enhancer site. Considering that Nef is the viral product first synthesized during the HIV life cycle, and is still the only viral product detectable in infected quiescent lymphocytes (47), it is tempting to speculate that, for the infecting virus, the Nef-dependent induction of p50/p50 NF-κB homodimers could represent a way of appropriation of the cellular machinery, leading to sustained long-terminal repeat activation.

The effects of Nef we described in macrophages should be considered as an additional contribution to the idea that Nef plays an important role in the development of AIDS pathogenesis. In particular, both present and already published data could help to delineate more clearly the effects of Nef in human monocyte-
macrophages (Fig. 9). The interaction of Nef (either upon internalization or endogenously expressed) with the endocytotic machinery leads to prompt activation of NF-κB, and, by consequence, of the transcription of HIV genome, in the case of infected cells, and/or of effector genes coding for inflammatory cytokines, chemokines, and additional, yet unidentified factor(s) efficiently inducing the activation of STAT-1. In turn, activated STAT-1 leads to the transcription of an additional set of genes, including STAT-1 itself, and other transcription factors, such as IRF-1, ultimately enforcing the stimulatory effects. It is noteworthy that the impressive induction of IRF-1 5 h after treatment with either EE-QQ or DD-AA rNef mutants proved that it was not per se sufficient to induce the increased release of inflammatory factors, being rather the consequence of the Nef-dependent STAT-1 activation we previously described (18), and that we detected also in MDM-expressing either Nef mutant (Z. Percario, unpublished results).

In sum, it appears that MDM react to the presence of Nef through a redundant mechanism of cell activation, and the consequent release of diverse activating factors likely extends the impact of Nef-dependent macrophage activation also to neighboring cells.

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