Inhibition of HIV-1 replication in primary human monocytes by the IκB-αS32/36A repressor of NF-κB

Camillo Palmieri1, Francesca Trimboli1, Antimina Puca2, Giuseppe Fiume2, Giuseppe Scala1,2 and Ileana Quinto*1,2

Address: 1Department of Clinical and Experimental Medicine, University of Catanzaro "Magna Graecia", Via T. Campanella 115, 88100 Catanzaro, Italy and 2Department of Biochemistry and Medical Biotechnology, University of Naples "Federico II", Via S. Pansini 5, 80131 Naples, Italy

Email: Camillo Palmieri - cpalmieri@unicz.it; Francesca Trimboli - trimboli@unicz.it; Antimina Puca - puca@dbbm.unina.it; Giuseppe Fiume - fiume@dbbm.unina.it; Giuseppe Scala - scala@unicz.it; Ileana Quinto* - quinto@unicz.it

* Corresponding author

Abstract

Background: The identification of the molecular mechanisms of human immunodeficiency virus type 1, HIV-1, transcriptional regulation is required to develop novel inhibitors of viral replication. NF-κB transacting factors strongly enhance the HIV/SIV expression in both epithelial and lymphoid cells. Controversial results have been reported on the requirement of NF-κB factors in distinct cell reservoirs, such as CD4-positive T lymphocytes and monocytes. We have previously shown that IκB-αS32/36A, a proteolysis-resistant inhibitor of NF-κB, potently inhibits the growth of HIV-1 and SIVmac239 in cell cultures and in the SIV macaque model of AIDS. To further extend these observations, we have generated NL(AD8)IκB-αS32/36A, a macrophage-tropic HIV-1 recombinant strain endowed to express IκB-αS32/36A.

Results: In this work, we show that infection with NL(AD8)IκB-αS32/36A down-regulated the NF-κB DNA binding activity in cells. NL(AD8)IκB-αS32/36A was also highly attenuated for replication in cultures of human primary monocytes.

Conclusions: These results point to a major requirement of NF-κB activation for the optimal replication of HIV-1 in monocytes and suggest that agents which interfere with NF-κB activity could counteract HIV-1 infection of monocytes-macrophages in vivo.

Background

HIV-1 infection is characterized by a long period of clinical latency followed by the development of acquired immunodeficiency syndrome, AIDS. During latency and when viral replication is being controlled in patients treated with antiretroviral therapy, HIV-1 is present in cellular reservoirs and continues to replicate, with each ensuing round of replication giving rise to escape mutants, which further replenish viral reservoirs [1,2]. This grim picture calls for novel targeted therapies for eradicating virus-infected cells and for preventing new infections.

Initial infection in vivo by HIV-1 is thought to occur in CD4-positive, CCR5-positive lymphocytes and monocytes. Accordingly, when HIV-1 envelope protein in its oligomerized g160 form contacts the cell surface receptor a signalling cascade is triggered that results in transcriptional activation of specific gene arrays, such as the
inflammatory cytokines IL-1β, IL-6, IL-8, TNF-α, TGF-β; these cytokines, in turn, function to enhance the transcriptional activity of the proviral long terminal repeat (LTR) promoter [3, 4]. This cytokine-driven inflammatory-like setting is mediated molecularly by the NF-κB family of transcription factors [5, 6]; thus, it serves to reason that preventing NF-κB activation would attenuate HIV-1 replication. Indeed, the LTR of HIV-1 does contain two tandem NF-κB sites [7] and three repeated Sp1 sites [8] upstream of the TATAA box with an additional NF-κB site located in the 5' untranslated region of viral genome [9]. Both sets of NF-κB sequences enhance HIV-1 transcription in response to various signals [9]. However, the Sp1 sites and TATAA box can redundantly sustain the Tat-mediated transactivation of the HIV-1 LTR in the absence of NF-κB sites [10]. It is controversial whether NF-κB cellular factors are required for the HIV-1 replication. Mutant HIV-1 carrying deletions or base-pair substitutions in the NF-κB enhancer in the LTR have been shown to be either competent or incompetent for replication [11-13]. These divergent observations are likely explained by differing cellular contexts, such as primary cells versus immortalized cell lines, and varying levels of cellular activation.

IKB inhibitors regulate NF-κB activity [14]. In response to activating stimuli, IκB proteins become phosphorylated, ubiquitinated and degraded by proteasomes. This releases cytoplasmic-sequestered NF-κB to enter the nucleus to activate the transcription of responsive genes [14]. The mutant IκB-αS32/36A is defective for serine 32- and serine 36-phosphorylation and is resistant to proteolysis. IκB-αS32/36A acts as a potent inhibitor of the NF-κB-dependent gene transcription, including those from the HIV-1 genome [15]. To verify the requirement of NF-κB in the replication of HIV-1 in primary cells, we previously designed HIV-1 and SIV molecular clones containing the IκB-αS32/36A cDNA positioned into the nef region of the respective viral genome [16, 17]. We found that these recombinant viruses were highly attenuated for replication in T cell lines as well as in human and simian PHA-activated peripheral blood mononuclear cells, PBMCs [16, 17]. These findings supported an interpretation that in these cellular contexts NF-κB is required for efficient viral replication. We also showed that a recombinant SIV which expressed IκB-αS32/36A inhibitor was also highly replication attenuated in vivo in rhesus macaque [17]. Here, we have extended our analysis of IκB-αS32/36A function in HIV-1 replication to primary monocytes. We report that a macrophage-tropic derivative of NL4-3 strain that expresses the proteolysis-resistant IκB-αS32/36A inhibitor of NF-κB replicated poorly in cultured primary human monocytes.

Results

Construction of pNL(AD8)IκB-αS32/36A

To generate a macrophage-tropic HIV-1 expressing the IκB-αS32/36A cDNA fused to the FLAG epitope, the CXCRI4-tropic envelope of pNLIkB-αS32/36A [16] was replaced with the CCR5-tropic envelope from pNL(AD8) [18]. Briefly, the 2.7 Kb EcoRI-BamH1 fragment of pNL(AD8) was religated to the 13.1 Kb EcoRI-BamH1 fragment of pNLIkB-αS32/36A or pNLIkB-antisense, thus generating pNL(AD8)IkB-αS32/36A and pNL(AD8)IkB-antisense, respectively (Fig. 1A). Both molecular clones are Nef-minus because our cloning strategy deleted the first 39 amino acids from the N terminus of Nef and engineered a translational frameshift into the remaining Nef encoding codons [16]. The respective molecular clones were transfected into 293T cells to analyse for the expression of HIV-1 proteins and IκB-αS32/36A polypeptide by immunoblotting (Fig. 1B, C). As expected the IκB-αS32/36A-FLAG protein was expressed by pNL(AD8)IkB-αS32/36A (Fig. 1C, lane 4).

Inhibition of NF-κB activity by pNL(AD8)IκB-αS32/36A

To assess the functional impact of IκB-αS32/36A expressed from the recombinant NL(AD8) genome, 293T cells were transfected individually with pNL(AD8), pNL(AD8)IkB-αS32/36A or pNL(AD8)IkB-antisense, and the respective nuclear extracts were evaluated for NF-κB (Fig. 2A) and Sp1 DNA binding activity (Fig. 2B). A significant reduction in NF-κB DNA binding activity was observed upon transfection of pNL(AD8)IkB-αS32/36A (Fig. 2A, lane 5) as compared to the other viral transfections (Fig. 2A, lanes 3, 4). The specificity of the IκBαS32/36A-mediated inhibition of NF-κB was verified by the demonstration that Sp1 binding to DNA was unaffected (Fig. 2B). These results support the interpretation that IκBαS32/36A expressed from the recombinant viral genome functionally inhibited NF-κB activity.

Attenuation of pNL(AD8)IκB-αS32/36A in primary monocytes

We next analyzed the replication properties of the recombinant HIV-1 genomes in cultured human monocytes from different individuals. Based on normalized amounts of input virus, we found that NL(AD8)IkB-αS32/36A was highly attenuated for replication when compared to NL(AD8) and NL(AD8)IkB-antisense (Fig. 3A-B). Accordingly, virus-induced syncitium formation was also strongly inhibited in monocytes infected with NL(AD8)IkB-αS32/36A (Fig. 4A, B). Taken together, our results underscore a critical contribution of NF-κB to HIV-1 growth in monocytes.

Discussion

Substantial numbers of monocytes are preserved in infected individuals even at later clinical stages of AIDS,
when T cell numbers are dramatically reduced. Consistently, in animal models of HIV-1 infection, monocytes are the major reservoir after acute depletion of CD4-positive T cells [19,20]. This indicates that these cells are long lasting infected moieties that shuttle from mucosal sites to lymph nodes and could function as a major HIV-1 reservoir in vivo. In addition, monocytes are programmed to produce a large amount of inflammatory cytokine, includ-
ing IL1-β, IL-6, TNF-α, which are strong inducers of HIV-1 replication [5]. Indeed, HIV-1 envelope binding to CCR5 receptor activates an intracellular signalling cascade that promotes high levels of transcription factors, including NF-κB, which sustain the initial rounds of viral replication and induce the production of inflammatory cytokines which activate surrounding cells to become more susceptible to virus infection [3,4].

Based on the published literature, the role of NF-κB in HIV-1 replication has been controversial [13,16,21]. For instance, the deletion of NF-κB binding sites from HIV-1
and SIV LTRs [22] has suggested that NF-κB activity may not be required for HIV-1 LTR-directed transcription. Moreover, deletion of NF-κB sequences in the LTR has also been reported not to affect HIV-1 replication in defined cellular settings [11,12]. These latter studies relied on short-term infections of immortalized cells that may not express a physiologic concentration of transcription factors. To address this issue, we have developed a novel HIV-1 strain, NL(AD8)IκB-αS32/36A, which was engineered to express a proteolysis-resistant IκBαS32/36A, and is a strong inhibitor of NF-κB activity. This recombinant virus expresses the envelope of the AD8 strain, a macrophage-tropic virus. Our findings show that NL(AD8)IκB-αS32/36A replication profile is different from that of the NL(AD8)IκB-antisense control. NL(AD8)IκB-αS32/36A failed to produce a productive infection in primary monocytic cells over a thirty-days acute infection (Fig. 3). These results were correlated with a strong inhibition NF-κB activity in NL(AD8)IκB-αS32/36A-infected cells (Fig. 2), indicating that in the setting of HIV infection of primary monocytes NF-κB plays a non-redundant role. These results are in agreement with the evidence that IκB-αS32/36A negatively affected the replication of HIV and SIV in PBMC cultures and in monkeys [16,17].

Because IκB-αS32/36A constitutively inhibits NF-κB [15], the potent inhibition of HIV/SIV replication could be due to repression of the NF-κB-dependent activation of HIV/SIV transcription. However, additional mechanisms might explain the potent inhibition of HIV/SIV replication by IκB-αS32/36A. In this regard, IκB-α regulates the transcriptional activity of NF-κB-independent genes by interacting with nuclear co-repressors, histone acetyltransferases and deacetylases [23,24]. Further studies are required to clarify novel activities of IκB-α in the modulation of the transcriptional machinery. Our results underscore a central role for IκB-α as a potent inhibitor of the replication of HIV-1 in both T cells [16] and monocytes (this study), and point to the NF-κB/IκB network as a suitable target for therapeutic intervention of AIDS.

**Conclusions**

In this study we have addressed the role of NF-κB/IκB proteins in the replication of HIV-1 in primary human monocytes. We show a strong attenuation in the replication of a macrophage-tropic HIV-1 strain expressing the IκB-αS32/36A repressor of NF-κB in primary cultures of human monocytes. These results are consistent with previous evidence of HIV/SIV inhibition by IκB-αS32/36A in PBMCs and in macaques [16,17]. In addition, these findings...
Reduced syncitia formation by NL(AD8)IkB-αS32/36A in infection of primary human monocytes. Panel A shows the kinetics of syncitia generation upon infection of primary human monocytes with $10^5$ cpm RT activity of the indicated viral stocks. The average of syncitia observed per optical field is reported. Panel B shows the picture of primary human monocytes at 14 days post-infection with $10^5$ cpm RT activity of the indicated viral stocks (original magnification × 430).
further support a role of NF-κB inhibitors in blocking HIV-1 replication and suggest novel strategies for the development of anti-viral therapy that targets NF-κB factors.

**Methods**

**Transfections and Viral stocks**

293T cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% v/v heat-inactivated fetal bovine serum and 3 mM glutamine. Viral stocks were produced by transfecting 293T cells (10⁶) with viral plasmids (10 µg) using calcium phosphate. Forty hours later, the cell culture supernatant was passed through a 0.45-µm filter and measured for RT activity as previously described [16].

**Immunoblotting analysis**

293T cells were transfected with viral plasmids (10 µg) and lysed in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris-HCl pH 8.0) 24 hours later. Proteins (10µg) were separated by electrophoresis in 10% SDS-polyacrylamide gel and transferred to Immobilon-P (Millipore). Filters were blotted with an AIDS patient serum or with anti-FLAG monoclonal antibody by using Western-Light Chemiluminescent Detection System (Tropix, Bedford, MA).

**Electrophoretic Mobility Shift Assays**

Nuclear extracts and gel retardation assays were performed as described previously [9]. Briefly, cells were harvested, washed twice in cold phosphate-buffered saline, and resuspended in lysing buffer (10 mM Hepes, pH 7.9, 1 mM EDTA, 60 mM KCl, 1 mM DTT, 1 mM phenylmethysulfonyl fluoride, 0.2% v/v Nonidet P-40) for 5 min. Nuclei were collected by centrifugation (500 × g, 5 min), rinsed with Nonidet P-40-free lysing buffer, and resuspended in 150 µl of buffer containing 250 mM Tris-HCl, pH 7.8, 20% glycerol, 60 mM KCl, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride. Nuclei were then subjected to three cycles of freezing and thawing. The suspension was cleared by centrifugation (7000 × g, 15 min), and aliquots were immediately tested in gel retardation assay or stored in liquid phase N2 until use. The HIV-1 NF-κB oligonucleotide probe was 5’-CAAGGGACTTTCCGCTGGGGACTTTCCAG-3’; the Sp1 oligonucleotide probe was 5’-GGGAGGTGTGGCCTGGGCGGGACTGGGGAGTGGCG-3’. The probes were end-labelled with [γ-³²P]ATP (Amerham Int., Buckinghamshire, UK) using polynucleotide kinase (New England Biolabs, Beverly, MA). Equal amounts (5 µg) of cell extracts were incubated in a 20 µl reaction mixture containing 10% glycerol, 60 mM KCl, 1 mM EDTA, 1 mM DTT, and 2 µg of poly [d(G-C)] (Boehringer Mannheim, Germany) for 5 min on ice. One µl of [γ³²P]-labelled double-stranded probe (0.2 ng, 5 × 10⁴ cpm) was then added with or without a 100-fold molar excess of competitor oligonucleotide. The reactions were incubated at room temperature for 15 min and run on a 6% acrylamide:bisacrylamide (30:1) gel in 22.5 mM Tris borate, 0.5 mM EDTA. Gels were dried and autoradiographed.

**Monocytes cultures and infections**

Human monocytes were isolated from PBMC by elutriation, cultured in RPMI, 10% FCS and GMCSF (20 ng/ml) for 48 hours. Infections were performed with viral stocks measured by reverse-transcriptase (RT) activity [16]. Usually, cell cultures (10⁵ cells) were infected with 10⁵ - 10⁶ cpm of RT activity. The cell culture supernatants were collected every two days and replaced with fresh medium. The viral production was measured as RT activity in the culture supernatants as previously described [16]. The syncitia formation in cell cultures was evaluated by calculating the average number of syncitia in at least six optical fields.

**List of abbreviations used**

NF-κB, nuclear factor kappa B

IκB, inhibitor of nuclear factor kappa B

IL-1, interleukin-1

IL-6, interleukin-6

IL-8, interleukin-8

TNF-α, tumor necrosis factor alpha

TGF-β, transforming growth factor-beta

cpm, counts per minute

FCS, fetal calf serum

GMCSF, granulocyte-macrophage colony-stimulating factor

**Competing interests**

The author(s) declare that they have no competing interests.

**Authors’ contributions**

CP carried out the analysis of viral growth and DNA bandshift assays. FT was responsible for cell cultures. AP performed the immunoblotting analysis. GF produced the viral plasmids and viral stocks, and performed the artwork of the paper. GS participated in the design of the study and discussion of results. IQ designed this study and edited the manuscript.
Acknowledgements

We thank K. T. Jeang for helpful discussions, and E. Freed for providing pNL(AD8). This work was supported by Ministero della Sanità-Istituto Superiore della Sanità-Programma Nazionale di Ricerca sull’AIDS, and Ministero dell’Istruzione, dell’Università e della Ricerca. C.P and A.P were recipients of FIRC fellowships.

References

1. Chun TW, Davey RT, Ostrowski M, Justement JS, Engel D, Mullins JI, Fauci AS: Relationship between pre-existing viral reservoirs and the re-emergence of plasma viremia after discontinuation of highly active anti-retroviral therapy. Nat Med 2000, 6:757-761.

2. Chun TW, Stuyver L, Mizel SB, Ehler LA, Mican JA, Baseler M, Lloyd AL, Novak M, Fauci AS: Presence of an inducible HIV-1 latent reservoir during highly active antiretroviral therapy. Proc Natl Acad Sci USA 1997, 94:13193-13197.

3. Devadas K, Hardegen NJ, Whal LM, Hewlett IK, Clouse KA, Yamada KM, Dhawan S: Mechanism for macrophage-mediated HIV-1 infection. J Immunol 2004, 173:6735-6744.

4. Orenstein JM, Fox C, Wahl SM: Inducible expression of IkappaBalpha repressor mutants interferes with NF-kappaB activity and HIV-1 replication in Jurkat T cells. J Biol Chem 1998, 273:743-7440.

5. Viatour P, Legrand-Poels S, van Lint C, Warnier M, Merville MP, Gie len J, Piette J, Bours V, Chariot A: Cytoplasmic IkappaBalpha increases NF-kappaB-independent transcription through binding to histone deacetylase (HDAC) I and HDAC3. J Biol Chem 2003, 278:6404-6410.

6. Yedavall Vi, Benkiranee M, Jeang KT: Tat and trans-activation-responsive (TAR) RNA-independent induction of HIV-1 long terminal repeat by human and murine cyclin T1 requires Sp1. J Biol Chem 2003, 278:46541-46548.

7. Aguilera C, Hoya-Arias R, Haegeman G, Espinosa L, Bigas A: Recruitment of IkappaBalpha to the hes1 promoter is associated with transcriptional repression. Proc Natl Acad Sci U S A 2004, 101:16537-16542.

8. Recruit-