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Nef-Induced Differential Gene Expression in Primary CD4+ T Cells Following Infection with HIV-1 Isolates.

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Abstract

Almost 80% of viral transcripts during early HIV-1 infection encode the Nef protein, which has been implicated in altering expression of a number of genes. In this study, we infected primary human $CD4^+$ T cells with pseudotyped Nef-containing or Nef-deleted (Δ-*nef*) NL4–3 virus and used RNA-Sequencing (RNA-Seq) for transcriptome analysis. Our results showed that the interferon response, IL-15 & JAK/STAT signaling, as well as genes involved in metabolism, apoptosis, cell cycle regulation, and ribosome biogenesis were all altered in the presence of Nef. These early Nef-mediated transcriptional alterations may play a role in priming the host cell for cellular activation and viral replication.

Keywords

HIV-1 Nef; RNA-Seq; HIV transcriptomics; delta-Nef

HIV-1 Nef is an early and highly expressed viral protein that has been implicated in setting the cellular stage for optimal viral replication [1]. Its replication bolstering effects have been

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AUTHORS' CONTRIBUTIONS

RLF, AA, OOY, and DFN conceived and designed the experiments; AA and RLF performed the experiments; RLF, AA, OOY, and DFN analyzed the data; AA and OOY contributed reagents/materials/analysis tools; RLF and DFN wrote the paper.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable

CONSENT FOR PUBLICATION

Not applicable

AVAILABILITY OF DATA AND MATERIAL

Sequencing data can be found under BioSample Accession Numbers SAMN07676177 and SAMN07676178.

COMPETING INTERESTS

The authors declare that they have no competing interests.

linked to alterations in cell signaling, vesicle trafficking, metabolic alterations, and transcriptional responses [2,3]. Previous studies have investigated the effects of Nef expression on transcriptional responses but vary in the context in which Nef was expressed, transcriptomic analysis technique, and cell type used including Jurkat [4], CEM-GFP [5], U937 [6], HeLa [7], and the astrocytoma cell line U251MG [8]. Some studies expressed *nef* alone without infection or the presence of other early genes *tat* or *rev* [7,8,4,6], while others compared cells infected with Wild-Type (WT) and *nef*-deleted HIV-1 viruses [5]. All of these studies used cDNA microarrays for transcriptomic analysis, and unlike RNA-Sequencing (RNA-Seq), this methodis limited by the number of genes that are screened. A more recent used updated transcriptomic methods for sequencing total and messenger RNA to investigate early transcriptional alterations following HIV-1 infection; however, the cell line SupT1 was used to look at total transcriptional effects by HIV-1 infection and did not specifically look at Nef-mediated transcriptional alterations [9]. In this study, we infected primary human $CD4^+$ T cells with HIV-1 Wild-Type (WT) NL4–3 or *nef*-deleted (Δ*nef*) NL4–3 for 24 hours prior to RNA-sequencing and analysis. RNA-Seq analysis identified Nef-mediated regulation of a different set of genes in primary $CD4^+$ T cells compared to the previous studies in cell lines.

Primary human $CD4^+$ T cells were isolated from anonymously collected PBMC through the UCLA AIDS Institute Virology Core by Ficoll separation and cultured in the presence of 0.5 μg/ml CD3:CD8 bispecific monoclonal antibody (NIH Reagent program #12277) and 50 Units/mL of human IL-2 for 6 days at 37°C and 5% CO_2. The T cells, which were 93% $CD4^+$ (Figure 1A), were then infected with tagged VSV-G pseudotyped HIV-1 containing a wild-type *nef* gene (NL4–3 *vpr* m*CD24* Δ*env*) or a deleted *nef* gene (NL4–3 Δ*nef vpr* m*CD24* Δ*env*) as previously described [10]. Five million $CD4^+$ T cells were incubated with 100 ng equivalent of p24 antigen for four hours with intermittent shaking followed by washing and resuspending in fresh RPMI media containing 10% FBS and 50 Units/mL of human IL-2 (R10–50) at a concentration of 5×10^5 cells/ml and cultured for 2 days. Both pseudoviruses expressed murine CD24 or heat stable antigen (HSAg) in place of Vpr. Flow analysis of the reporter marker mCD24 indicated that the cell population was more than 87% infected following positive selection using anti-PE Miltenyi microbeads and magnetic sorting (Figure 1C). Separated cells were washed two times with PBS and then used for total RNA isolation using a ThermoFisher Scientific Trizol kit. After air drying, RNA pellets were resuspended in 100 μl of Dnase/RNase free water containing 100 units of RNase inhibitor. The purity and concentration of RNA was measured by a NanoDrop 1000 and RNA integrity measurements were done using a bioanalyzer. Each of the two experimental conditions was performed in duplicate. Transcriptomic paired-end reads were sequenced using an Illumina HiSeq 2500 instrument (sequencing data can be found under BioSample Accession Numbers SAMN07676177 and SAMN07676178). Differential gene expression analysis was done using the Tuxedo protocol developed by Trapnell *et.al.* which is a component of the Green Line RNA-Seq Analysis pipeline of DNA Subway (https://dnasubway.cyverse.org/) [11]. The Tuxedo protocol is composed of TopHat, CuffLinks, CuffDiff and cummeRbund software packages. R programming was used to create a heat map of the significant differentially expressed genes between the two conditions ($p<0.05$, Figure 1D and 1E). After analyzing all of the genes in the WT-Nef infected $CD4^+$ T cells

and the ΔNef infected $CD4^+$ T cells, we identified19 differentially expressed genes altered by HIV-1 Nef . DAVID GO-term analysis indicates that the majority of these differentially expressed genes may have nuclear localization and are involved in transcriptional regulation and alternative splicing. Other identified pathways from GO-term analysis include the type I interferon signaling pathway, redox reactions, and apoptosis.

HIV-1 Nef inhibited expression of PCGF1, FHIT, RN7SK, MTO1, PGM3, EFTUD1, SCNN1A, CTD-2245F17.3, some of which are involved in translation and modification of proteins. EFTUD1 is involved in ribosome biogenesis as well as translational activation. PGM3 is involved with glycosylation of proteins trafficking through the trans Golgi network. Mutations in PGM3 are also linked to immunodeficiencies, suggesting a critical role in immune function [12].

Nef increased expression of MYO1B, ZNF573, ALDH1A2, KDM6B, PDSS2, PHLDA1, MDC1, NOC2L, ISG20, EGR1, and IL15. Several of these proteins act at the nuclear level and are involved in transcriptional signaling and chromatin organization (ZNF573, ALDH1A2, KDM6B, and NOC2L) or DNA damage response and cell cycle regulation pathways (PHLDA1 and MDC1), which are pathways previously reported to be altered by Nef [7,9,6]. *NOC2L* inhibits histone acetyltransferases and was shown in cDNA microarray analysis by Morou *et. al.* to be upregulated 70-fold in $CD4^+$ T cells treated with HIV-1 Env [13]. MDC1 is involved in regulating cell cycle checkpoints by recruiting proteins to site of DNA damage. Interestingly the *mdc1* locus is one reported hotspot for HIV-1 integration genome-wide [14].

Several of the previous studies reported alteration of immune related pathways including responses to viral infections [7], the interferon response [9,8,4], and the JAK/STAT pathway [8]. In our study, we saw a significant increase in transcripts of two proteins involved in the interferon response, ISG20 and EGR1. The 20 kD Interferon Stimulated Gene 20 protein (ISG20) is thought to increase cellular resistance to some RNA viruses by acting as a 3’ to 5’ exoribonuclease. Espert *et. al.* previously reported that ISG20 was highly and quickly upregulated following HIV-1 infection in the CEM cell line [15]. When they infected the cells with NL4–3 Δ-*nef* they still saw rapid upregulation of ISG20, suggesting other viral products also elevate the expression of this protein. Our results also indicate that Nef elevates ISG20 mRNA expression. Despite the reported antiviral activity of ISG20, HIV-1 is still able to thwart this intrinsic host restriction factor. The Early Growth Response protein 1 (EGR1) is a member of a group of early response genes that stimulate several environmental signals including cell growth, development, stress responses, hormones, and neurotransmitters. EGR1 was previously shown to be increased following Nef expression in transcriptomic studies [9,8] and its expression and activity has been shown to be altered by other HIV-1 proteins including Tat [16]. EGR1 has been found to play a role in autophagy and apoptosis and its upregulation by early HIV-1 proteins like Nef and Tat may emphasize its action HIV-mediated cytotoxicity [16]. Peng *et. al.* found a significant increase in HIV-induced differential expression of 888 and 941 genes downstream of IRF1 and EGR1 promoters respectively in SUP-T1 cells, further supporting HIV-mediated upregulation of the interferon response in HIV-1 infected cells [9].

JAK/STAT pathways are commonly activated following HIV-1 infection and Nef has been shown to upregulate STAT3 activation [8]. In our study, we saw an upregulation of the STAT3 activator IL-15. This cytokine has been reported to be upregulated in HIV-1 infection, with higher levels in untreated individuals than in those on antiretroviral treatment, yet its benefit is under debate [17]. IL-15 supports antiviral $CD8^+$ T cell and natural killer cell expansion and function, although it may also increase susceptibility of memory $CD4^+$ T cells to infection. HIV-1 Nef may play a role in elevating IL-15 during acute infection.

In this brief study, we utilized RNA-Seq to compare Nef-induced transcriptional regulation in primary human $CD4^+$ T cells infected with HIV-1 isolates . Although significant overlap was not found between specific genes identified in our study in primary CD4+ T cells with those using microarrays and cell lines like CEM-GFP [5]or RNA-Seq and SUP-T1 [9], HIV-1 Nef does appear affect early transcriptional events in all methods tested with an enhancement in interferon and metabolic pathway alterations. We provide further evidence that HIV-1 Nef alters host cell transcription early in infection and affects pathways involved in chromatin organization, the DNA damage response, the interferon response, and JAK/STAT signaling.

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ABBREVIATION LIST:

EGR 1	Early Growth Response protein 1
HIV-1	Human Immunodeficiency Virus-1
ISG20	Interferon Stimulated Gene 20 protein
RNA-Seq	RNA-Sequencing
VSV-G	Vesicular Stomatitus Virus G protein

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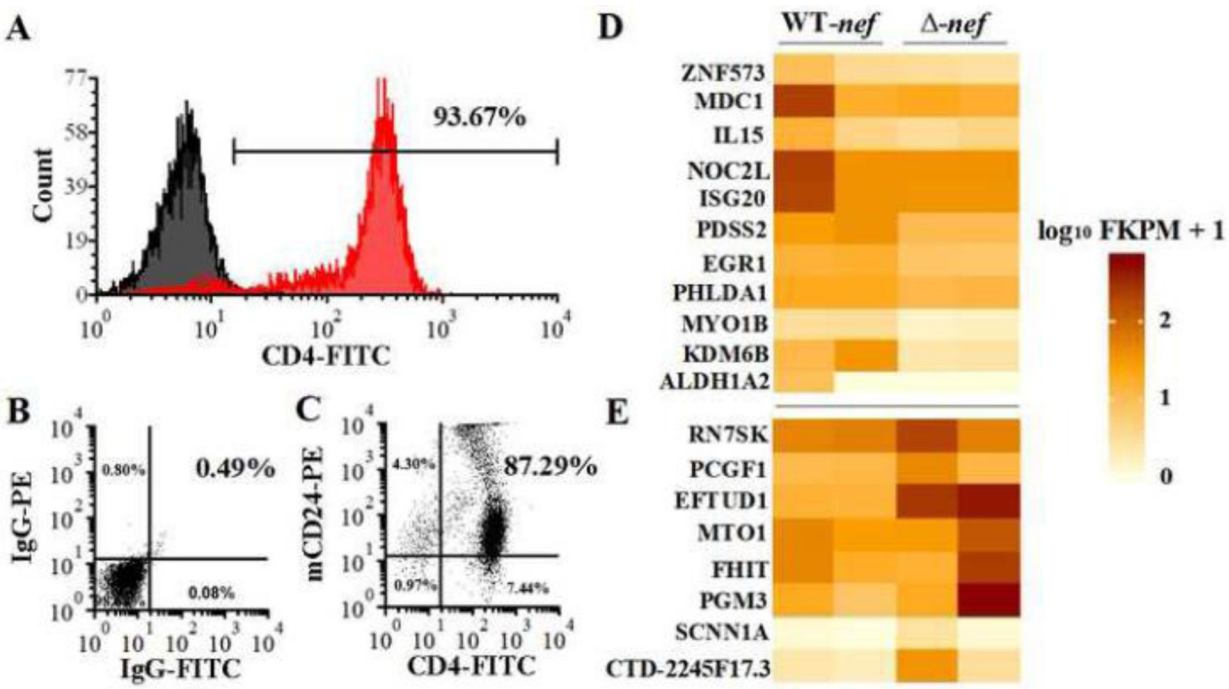

Figure 1:

HIV-1 Nef Regulation of Gene Transcription in Primary Human CD4$^+$ T Cells: (A) Primary human CD4$^+$ T cells were purified from PBMC using a bispecific antibody to CD3 and CD8. The majority of isolated cells post-selection were CD4$^+$ T cells as measure by flow cytometry. Following infection with pseudovirus, percentage of infected cells was measured by flow cytometry (B) antibody isotype control (C) 87.29% of the total cells were infected CD4$^+$ T cells indicated by coexpression of a murine CD24 reporter antigen. Transcriptomic analysis of primary human CD4$^+$ T cells infected with nef-containing (WT-nef) or nef-deleted (Δ-nef) pseudotyped HIV-1 NL4–3 was done 24 hours post-infection in duplicate. RNA-Seq reads were analyzed using the Tuxedo protocol on the green line of DNA Subway and subsequent graphs were created in R. Differentially expressed genes (DEGs) were determined by the log-fold change in FKPM +1 values and the significance was established using the p-value of the fold change between the ΔNef/Wildtype ratio ($p<0.05$). (D) DEGs increased in the presence of Nef or (E) DEGs increased in the absence of Nef are listed in duplicate runs.