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Gene expression profiling reveals Nef induced deregulation of lipid metabolism in HIV-1 infected T cells

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ABSTRACT

Human Immunodeficiency Virus-1 (HIV-1) encodes a 27 kDa Negative Factor or Nef protein, which is increasingly proving to be a misnomer. Nef seems to be crucial for AIDS progression as individuals infected with *nef*-deleted strain of HIV were reported to become Long Term Non Progressors (LTNP). These findings necessitate tracing of Nef's footprint on landscape of cellular transcriptome favoring HIV-1 pathogenesis. We have tried to explore effect of Nef on cellular gene expression profile in conjunction with rest of HIV-1 proteins. Our results show that 237 genes are differentially regulated due to the presence of Nef during infection, which belong to several broad categories like "signaling", "apoptosis", "transcription" and "lipid metabolism" in gene ontology analysis. Furthermore, our results show that Nef causes disruption of lipid content in HIV-1 infected T cells. Molecular inhibitors of lipid metabolism like Atorvastatin and Ranolazine were found to have profound effect on wild type virus as compared to *nef*-deleted HIV-1. Thus our results suggest that interference in lipid metabolism is a potential mechanism through which Nef contributes in enhancing HIV-1 pathogenesis.

1. Introduction

According to the latest global UNAIDS report, there are nearly 35 million people infected with HIV living today, even after three decades of intense research on this virus. This necessitates further studies to unravel the unsolved mysteries in existing literature about deleterious effects of the virus. HIV-1, in addition to regular structural and regulatory proteins expresses several accessory proteins namely Nef, Vpr, Vpu and Vif. HIV-1 Nef has been credited as one of the most important proteins for viral life cycle and pathogenesis. It is a ~27 kDa, multifunctional adaptor protein lacking enzymatic activity [1,2]. Nef is produced early and abundantly in the viral life cycle right after proviral transcriptional activation [1,3]. Nef participates in an array of functions including down-regulation of CD4, and class I and II Major Histocompatibility Complex, stimulation of viral replication and enhancement of virion infectivity [1–3]. Presence of non-functional, truncated or mutant Nef during infection by HIV-1 has been shown to decrease viral infectivity and pathogenicity [4]. *Nef*-deleted HIV was also found to exist in humans having barely detectable viral load [4,5]. Long Term Non Progressors (LTNP)/Slow progressors (SP) of HIV infection were detected to be carrying mutations in *nef* region [6,7]. So it is evident that lack of Nef or its partial functionality contributes to non-progressive HIV infection, projecting Nef as a subject of intense studies.

Numerous studies have therefore, been directed towards Nef's effects on transcription of viral and cellular genes. While few reports point towards direct effect of Nef on enhancing transcription from HIV-1 promoter [8,9], there are also reports revealing Nef induced modulation of cellular transcriptome. Exogenous expression of Nef followed by gene expression studies have been done on a variety of cells like Jurkat [10], HeLa [11], astrocytes [12] and U937 [13]. All such studies seem to have neglected the complex molecular interaction between Nef and other viral proteins [14] having simultaneous effect on HIV infected host cells. Thus it is important to study Nef's effect on host cell in presence of rest of the viral proteins. In the present study, we have compared the gene expression profile between wild type and *nef*-deleted virus infected T-cells in order to identify genes modulated due to presence Nef during HIV-1 infection. Interestingly, our results indicate changes in a number of genes including that of lipid metabolism related genes due to HIV-1 infection in presence of Nef. Consequently we also found lowering of lipid content of T cells due to HIV-1 infection suggesting a role of nef in regulating lipid metabolism. Moreover,

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molecules like Atorvastatin [15] and Ranolazine [16] that inhibit lipid metabolism at different stages, inhibited WT HIV-1 replication more than *nef*-deleted HIV-1. Thus, our results implicate Nef in perturbation of lipid content of $CD4^+$ T cells during HIV-1 infection.

2. Materials and methods

2.1. Plasmids, cell lines and transfections

pNL4-3, a molecular clone of HIV-1 was received from NIH AIDS reagent program, USA [17]. *Nef*-deleted molecular clone of HIV-1, pNL4-3Δ*nef* was a kind gift from Dr J C Guatelli [18]. NL4-3 Nef cloned in pCDNA (pCDNA-Nef) was a kind gift from Dr. M. Federico [19]. Cell lines HEK-293T and U937 were obtained from NCCS cell repository, India. Human $CD4^+$ T reporter cell line CEM-GFP [20] and HeLa based $CD4^+$ reporter cell-line TZM-bl was received from NIH AIDS reagent program, USA [21]. Cells were transfected with pCDNA-Nef in increasing concentrations using XtremeGene HP (Roche Applied Sciences, Germany) according to the manufacturer's protocol. Cells were harvested 60 h post-transfection for quantitation of GFP expression.

2.2. HIV-1 infection and quantitation

CEM-GFP, U937 and TZM-bl were given 0.5 multiplicity of infection (MOI) of Δnef or WT HIV-1 NL4-3 as described previously [22]. HIV-1 replication was quantitated using $p24^{gag}$antigen capture ELISA (Advanced Biosciences Laboratories, USA) according to manufacturer's instructions. HIV-1 LTR promoter activity after infection was monitored in TZM-bl by luciferase assay as described earlier [23]. In CEM-GFP cells, GFP expression as a measure of gene expression from LTR promoter due to HIV-1 infection was monitored by quantitating GFP expression using M5 Microplate multimode reader from Molecular Devices, USA. Zidovudine (AZT), Atorvastatin and Ranolazine (Sigma–Aldrich, USA) were used at already reported concentration of 5 μM, 10 μM and 50 μM respectively [24–26] during HIV-1 infection.

2.3. Gene expression profiling

Total RNA was isolated from uninfected, 0.5 MOI of Δnef or WT HIV-1 infected CEM-GFP at 72 h in triplicates, using RNeasy Mini kit (Qiagen, Germany). RNA integrity was evaluated using a Lab-on-Chip-System Bioanalyzer 2100 (Agilent, USA) and was quantified using NanoDrop ND-1000 (Thermo, USA). For gene expression profiling, oligonucleotide microarray analysis was performed using 4X44 K Agilent Human Genome microarray (Agilent Technologies, USA) according to the manufacturer's protocol along with Feature extraction software, version 9.5.3 – Agilent Technologies, USA. The data analysis was done using GeneSpring 13 software (Agilent, USA). This microarray data has been deposited to the GEO repository and is available via the accession number GSE76793 at http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE76793. RNA extraction and qRT-PCR for selected genes was done as reported [23].

2.4. Lipid quantitation using flow cytometry and spectrophotometry

Uninfected CEM-GFP cells along with 0.5 MOI Δnef or WT HIV-1 infected cells were harvested 72 h post infection, fixed with 1% Paraformaldehyde and stained with 0.3% Oil Red O (ORO) (Sigma, USA) in 60% Isopropanol as described earlier [27]. Acquisition and analysis of GFP expression and ORO staining along with controls were quantified on FACS Canto II flow cytometer using FACS Diva software (Becton Dickinson, USA). Histograms were plotted using FlowJo Software (Tree Star Inc., USA). To quantitate ORO stain using spectrophotometer, stained or unstained control cells were lysed using lysis buffer (50 mM Tris–HCl pH 7.4, 5 mM EDTA, 0.12 M NaCl, 0.5% NP40, 0.5 mM NaF, 1 mM DTT, 0.5 mM PMSF) on ice for 45 min. 100 μl of lysate was read for optical density at 518 nm in M5 microplate multimode reader.

2.5. Antibodies and immunoblotting

Cells were lysed in lysis buffer as mentioned above. Equal amounts of protein were taken from cell lysates and were resolved by SDS-PAGE followed by immunoblotting for Nef and GAPDH. A polyclonal anti-Nef serum was obtained from Dr S. Jameel [28] as a kind gift whereas antibody specific for GAPDH was obtained from Santa Cruz Biotechnology, USA.

2.6. Statistical analysis

Each individual experiment was repeated at least three times. The error bars in the figures represent the mean ± S.D. of three independent experiments. Statistical analysis of the experimental data was performed using Student's t test with the levels of significance denoted by *p-value*.

3. Results

3.1. Nef induces HIV-1 replication and transcription in infected cells

Although Nef has been reported in enhancement of virus replication and infectivity [1,2], we wanted to reconfirm its role in HIV-1 replication and gene expression in different microenvironments exemplified by different cell lines infected with HIV-1. Supernatant from *nef*-deleted or wild-type HIV-1 NL4-3 virus infected cells were collected and virus production was assessed by measuring p24 antigen. In both the cell lines *nef*-deleted virus production seems to lag behind WT HIV-1 production (Fig. 1A, B). Being a positive strand RNA virus, transcription from HIV-1 LTR promoter has a direct bearing on its replication. Thus, we also measured transcriptional activity from HIV-1 LTR promoter in reporter cell lines CEM-GFP and TZM-bl. Analysis of GFP expression and luciferase activity in these infected cell lines exhibit enhanced transcriptional activity due to presence of Nef in WT HIV-1 infection as compared to *nef*-deleted virus infection (Fig. 1C and D). The difference between Δnef HIV-1 and WT HIV-1 is reflected maximally at 72 h post infection in CEM-GFP (Fig. 1A and C). So this time point was chosen to explore how Nef can affect cellular gene expression profile during HIV-1 infection progression.

3.2. Gene expression profiling of nef-deleted and wild type HIV-1 infected CEM-GFP cells

Microarray technology used as described above enabled us to visualize gene expression profile of *nef*-deleted and wild type virus infected cells thereby identifying genes specifically affected due to presence of Nef amongst all other viral proteins. On comparison of WT HIV-1 infected CEM-GFP (WTHI) with uninfected cells (UI); we obtained a gene list (DEG-1) of 231 significantly deregulated genes [Supplementary Data Table 1]. We also obtained another gene list (DEG-2) of 30 significantly deregulated genes due to *nef*-deleted HIV-1 infection of CEM-GFP cells (DNHI) as compared to uninfected cells (UI) [Supplementary Data Table 2]. Then we used Δnef HIV-1 infected cell gene expression profile as control and compared it with WT HIV-1 infected cell gene expression profile. We obtained a 3rd gene list (DEG-3) of 237 significantly deregulated genes

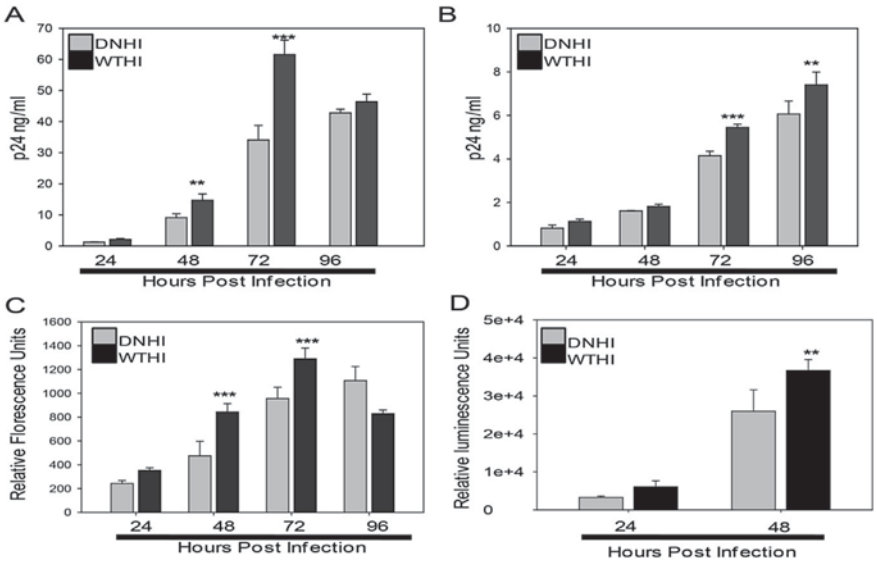

Fig. 1. ***nef*-deleted HIV-1 lags behind WT HIV-1 in terms of peak and kinetics of infection progression**. HIV-1 replication was quantitated using supernatants of 0.5 MOI Δnef HIV-1 (DNHI) or WT HIV-1 infected cells (WTHI) at different hours post infection by p24 antigen capture ELISA in (A) CEM-GFP cells (B) U937 cells. Quantitation of transcriptional activity from HIV-1 LTR promoter due to ΔnefHIV-1 or WT HIV-1 infection by measuring (C) GFP expression in CEM-GFP cells, (D) luciferase activityin TZM-bl cells. $^{**}p \leq 0.05$ $^{***}p \leq 0.01$.

[Supplementary Data Table 3]. These three gene lists were then analyzed for number of common and unique genes in each of them by plotting a Venn diagram (Fig. 2A). We found 98 genes exclusively deregulated due to presence of Nef during HIV-1 infection progression. There are also 123 genes which are common to WTHI vs DNHI and WTHI vs UI indicating that these genes are deregulated due to HIV-1 infection and probably also due to presence of Nef. To study the precise effect of Nef on gene expression profile of HIV-1 infected cells we further analyzed the third gene list i.e., DEG-3. Normalized intensity of expression of these genes in all the samples is depicted in a heat map (Fig. 2B). We manually then re-sorted the differentially expressed genes in categories like "Apoptosis", "Transcription", "Signaling" (Supplementary Fig. 1) and "Lipid metabolism" according to their associated GO term. Mean normalized intensities of lipid metabolism genes are represented in heat map (Fig. 2C) as we wanted to study them further. All these genes belonging to these four broad categories are also represented through cytoscape (Supplementary Fig. 2) which allows depiction of direction of expression change and corresponding interaction readily.

3.3. Validation of microarray results for selected lipid metabolism related genes

Amongst the 33 genes of DEGs belonging to category "lipid metabolism", we selected randomly 10 genes for validation showing a wide range of modulation pattern in microarray data. Validation of results obtained by microarray analysis was performed by qRT-PCR of selected deregulated genes. The transcript levels of all 10 genes were measured by qRT-PCR using GAPDH as an internal control. We found strong correlation between the deregulation trend observed in microarray and the qRT-PCR data (Fig. 3). Also to be noted here is that, a number of genes like formin binding protein 1 (FNBP1), sterol O-acyltransferase 2 (SOAT2), carnitine O-octanoyl transferase (CROT), fatty acid binding protein 3 (FABP3), peroxisome proliferator-activated receptor alpha (PPARα), 1-acylglycerol-3-phosphate O-acyltransferase 3 (AGPAT3), delta(4)-desaturase sphingolipid 1 (DEGS1) etc, are directly involved in lipid binding and oxidation.

3.4. Nef alters lipid constitution of HIV-1 infected cells

After confirming deregulation of several lipid metabolism related genes in microarray data, we have then looked at the fate of this deregulation on cellular physiology. Significant decline in Oil Red O (ORO) staining of Wild type infected CEM-GFP cells as compared to *nef*-deleted HIV-1 infected or uninfected cells was observed in flow cytometry based analysis (Fig. 4A). However, there was significant GFP expression in CEM-GFP due to *nef*-deleted HIV-1 or Wild-type HIV-1 infection (Fig. 4B) as compared to uninfected cells. Same data was plotted using Mean Fluorescent Intensities (M.F.I.) of ORO staining and GFP expression. MFI of ORO staining indicate that lipid content of WT HIV-1 infected cells is significantly lower than uninfected or Δnef HIV-1 infected cells (Fig. 4C) although MFI of GFP expression indicated high levels of infection in both Δnef HIV-1 and WT HIV-1 infected cells (Fig. 4D).

To further verify the interplay of Nef, we did the quantitation of ORO staining using spectrophotometry. Expression of increasing dose of Nef in *trans* with Δnef HIV-1 revealed gradual decline in ORO staining as compared to Δnef HIV-1 infected (Fig. 4E). Western blot for Nef in the lower panel confirmed dose-dependent increase in Nef expression where GAPDH was used as control. This experiment further proved that Nef induces decline in lipid content during HIV-1 infection progression. As visualized by GFP expression, infection levels also increased due to increase in Nef expression during *nef*-deleted HIV-1 Infection (Fig. 4F). We then used well characterized pharmacological inhibitors of lipid metabolism namely Atorvastatin [15] and Ranolazine [29]. We examined their ability to affect HIV-1 infection in presence or absence of Nef. Addition of Atorvastatin (10 μM) and Ranolazine (50 μM) respectively led to significant decline in wild type HIV-1 viral particle production as quantified by p24 antigen capture ELISA (Fig. 4G upper panel) as well as in GFP expression (Fig. 4G lower panel). However, inhibition of virus production or suppression of GFP expression was not visible to the same extent in Δnef HIV-1 infection (Fig. 4H). The concentrations used for the study did not induce any visible cytotoxicity in CEM-GFP. Zidovudine (AZT, 5 μM) was used as a positive control inhibitor.

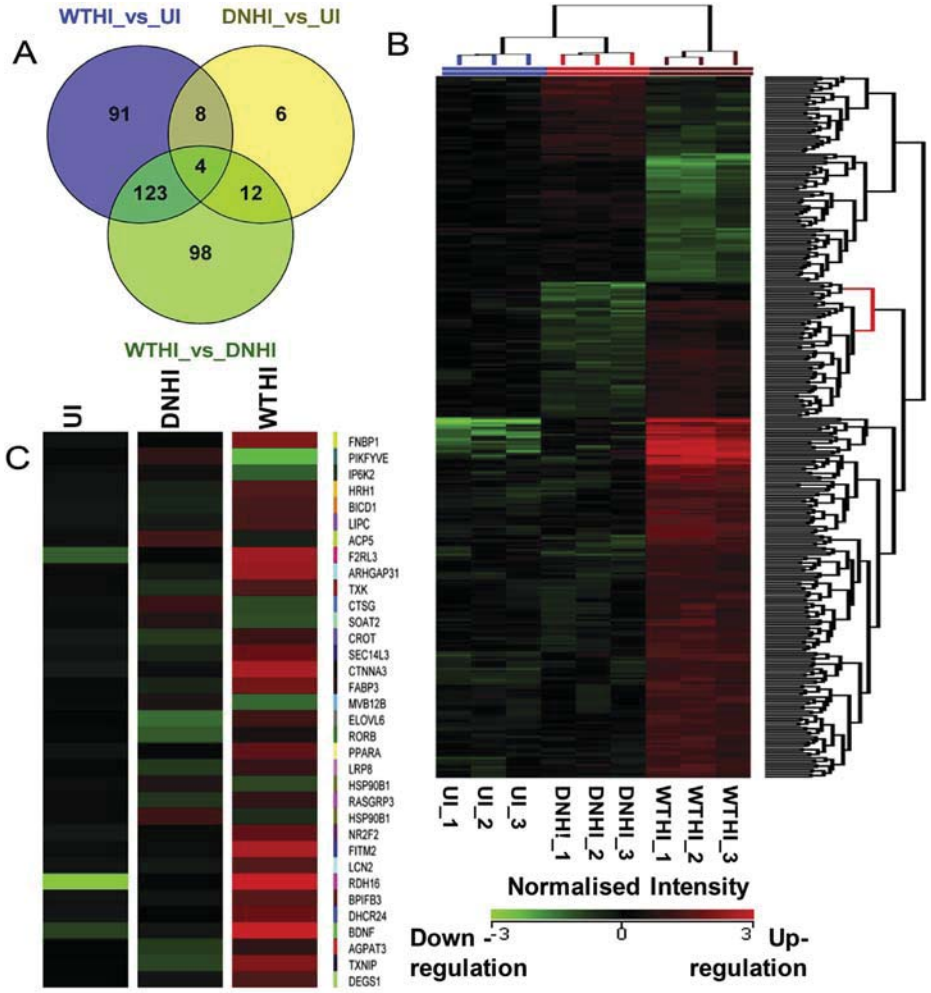

Fig. 2. Representation of differentially expressed genes in wild type and *nef*-deleted HIV-1 infected CEM-GFP as identified from microarray analysis. (A) Venn diagram showing number of genes common or unique to three different gene lists DEG.1, 2, 3 generated by gene expression profiling of uninfected, DNHI or WTHI. (B) Heat map representation of genes differentially expressed due to presence of Nef during infection. Red and green color show up-regulation and down-regulation respectively from baseline (black color). (C) Heat map illustration of mean normalized intensity of genes related to Lipid metabolism. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

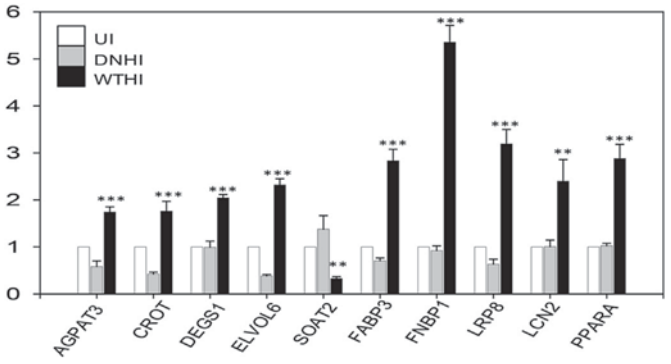

Fig. 3. Validation of microarray data for selected lipid metabolism related genes using qRT-PCR. qRT-PCR analysis of selected lipid metabolism genes from DEG3 was performed using RNA extracted from uninfected, DNHI, and WTHI CEM-GFP cells. The fold change values in the bar graphs depict the changes in gene expression in infected cells over the expression levels in uninfected cells normalized to GAPDH. The data is representative of three biological replicates. $^{**}p \leq 0.05$, $^{***}p \leq 0.01$.

4. Discussion

Antiretroviral therapy currently available to AIDS patients include inhibitors that block fusion of viral and host membrane, inhibitors of viral reverse transcriptase, protease and integrase activity, thereby impeding important steps in viral life cycle. Meanwhile the focus on identifying an alternative target for anti-HIV drug discovery has been inclined towards Nef due to increasing literature about its importance in viral life cycle [30]. Various other reports [2] and our preliminary results confirms Nef's integral role in viral replication and transcription irrespective of different microenvironments posed by different cell lines like CEM-GFP, U937 or TZM-bl.

There have been several reports on Nef's interaction with other viral proteins. These interactions can alter activities of Nef and rest of the viral proteins [14]. For instance protease mediated cleavage of Nef can alter its protein binding ability [31]. Nef–Tat interaction can cause enhanced gene expression from HIV-1 LTR promoter [8,9]. Either way, the interactions between Nef and other viral proteins can have an effect on host cellular machinery. Thus to explore the sole effect of Nef on viral and cellular transcription in

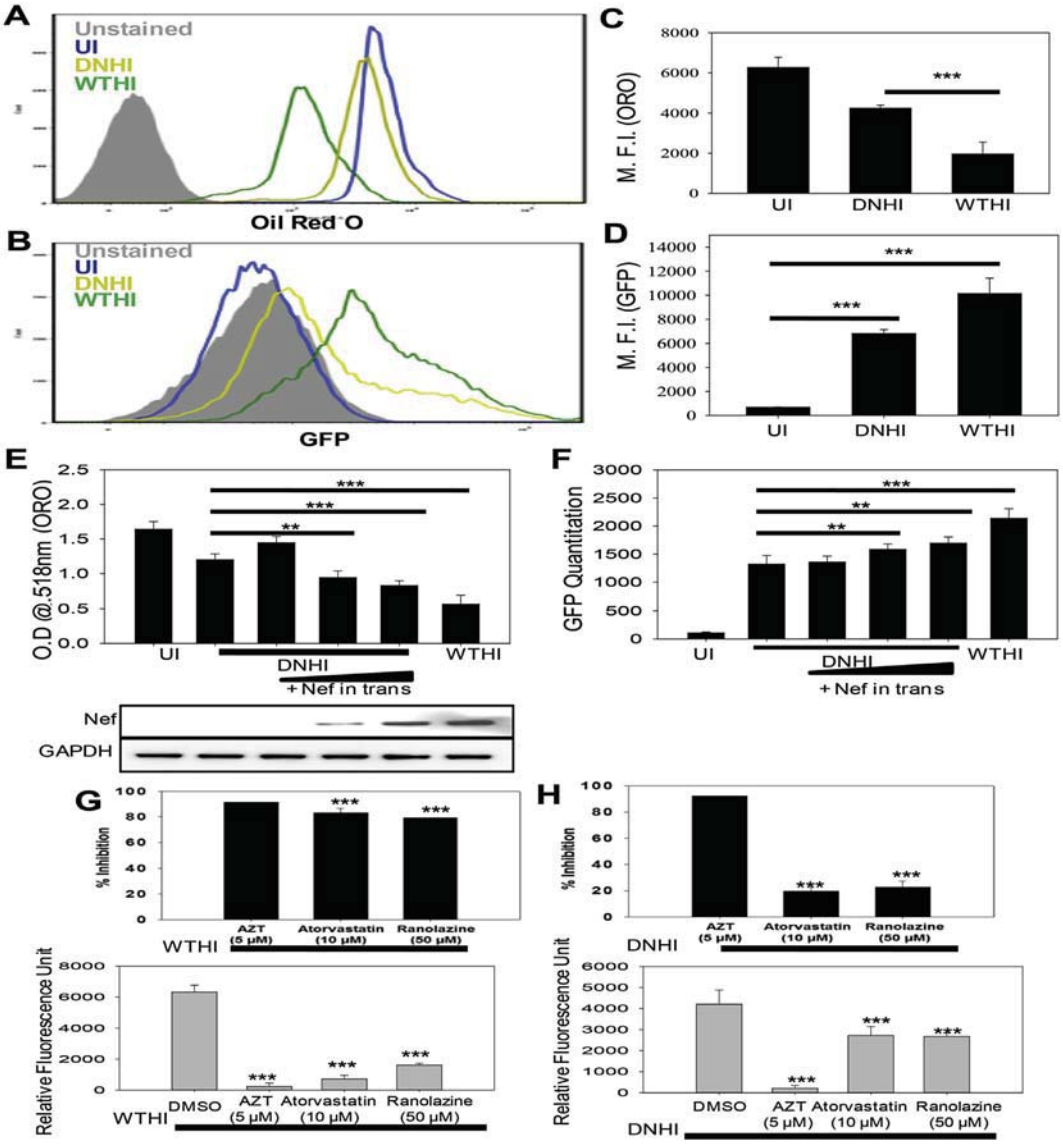

Fig. 4. Nef is responsible for decrease in cellular lipid content observed during HIV-1 infection, and its effect can be abrogated by pharmacological inhibitors of lipid metabolism. (A) Histogram of FACS based quantitation of Oil Red O staining of uninfected, ΔNef HIV-1 (DNHI) or WT HIV-1 infected (WTHI) CEM-GFP cells showing decline in ORO staining of WT HIV-1 infected cells as compared to ΔNef HIV-1 infected or uninfected cells. (B) Histogram of FACS based quantitation of GFP expression of uninfected, ΔNef HIV-1 or WT HIV-1 infected CEM-GFP showing enhanced WT HIV-1 infection and ΔNef HIV-1 infection. The results of one representative experiment out of three are shown (C) MFI of ORO staining indicates statistically significant decline in lipid content of WT HIV-1 infected CEM-GFP cells. (D) MFI of GFP expression indicates infection progression in ΔNef HIV-1 or WT HIV-1 infected CEM-GFP cells. (E) Increasing dose of Nef in trans with DNHI indicated gradual decrease in lipid content of CEM-GFP cells. (F) Gradual enhancement of GFP seen in nef-deleted HIV-1 infected cells due to expression of increasing dose of Nef in Trans. (G) WTHI CEM-GFP and (H) DNHI CEM-GFP treated with Atorvastatin (10 μM) and Ranolazine (50 μM). Zidovudine (AZT) (5 μM) was taken as positive control. 72 h post-infection, supernatant was checked for viral particle production inhibition (upper panel) and cell lysate was analyzed for GFP expression (lower panel).***$p \leq 0.01$, **$p \leq 0.05$.

absence of rest of the viral proteins during infection would be an imperfect biological microenvironment and physiologically extraneous. Hence we compared expression profiles of *nef*-deleted HIV-1 and WT HIV-1 infected cells which yielded information on a set of genes which are significantly deregulated due to presence of Nef during infection progression. Our model also corroborates with the methodology adopted to study the effect of Nef on infectivity during HIV-1 infection very recently [32,33]. Surprisingly on comparison of DNHI with uninfected cells we found only 31 genes deregulated, indicating the lower potency of *nef*-deleted HIV-1. It also reinstates our results that Nef can exercise transcriptional control and thus affect HIV-1 replication. While an array of reports point to Nef's role in signaling and apoptosis as described in previous reviews [1,34,35], there are only a few studies which points to Nef's interference in cellular transcription or lipid metabolism disruption. We found genes belonging to lipid metabolism as a subset of genes differentially expressed due to presence of Nef during HIV-1 infection. We validated our microarray results by checking the mRNA expression levels of a few of them through qRT-PCR. This implicates Nef in directing disruption of cellular lipid content. Oil Red O staining of *nef*-deleted and WT HIV-1 infected cells confirmed our hypothesis that Nef indeed disrupts lipid constitution of HIV-1 infected cells. Our observation falls in accordance with a prior study where over expression of Nef in HeLa lead to disruption of cellular lipid constitution [11]. However additional experiments are necessary to identify precise chemical nature of lipids and impact of their deregulation on HIV-1 infection progression. Another concurrent study done on plasma lipid levels of

nef-deficient HIV-1 infected patients recently revealed differences in abundance of High Density Lipoproteins (HDL), phosphatidylserine and sphingolipids [36]. While our study differs from these in terms of not only reporting the deregulation of lipid metabolism genes at transcription level but also showing down regulation of cellular lipid content. Atorvastatin is classically known to inhibit cholesterol synthesis and can therefore interfere in lipid metabolism in more than one ways and is also reported to inhibit HIV-1 [37]. Ranolazine on the other hand inhibits fatty acid oxidation and is widely used as a drug against angina [29]. As wild type HIV-1 can utilize lipid metabolism for its own replication, inhibitors of lipid metabolism can potentially suppress HIV-1 replication. Our results highlight the association of lipid metabolism disruption with presence of Nef which is why Δnef HIV-1 replication is inefficiently inhibited in presence of Atorvastatin and Ranolazine.

In view of the number of pathways in which Nef aids HIV infection progression, lipid metabolism perturbation can potentially contribute to some of the common clinical features seen in AIDS patients including weight loss, wasting, and immunodeficiency. This study implicates Nef in lipid metabolism perturbation to enhance HIV-1 replication. Our study thus reinstates Nef as a pivotal therapeutic target to enhance the quality of life of AIDS patients.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2016.02.089.

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