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HIV Impairs Alveolar Macrophage Function Via MicroRNA-144-Induced Suppression of Nrf2

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Abstract

Rationale: Despite anti-retroviral therapy, HIV-1 infection increases the risk of pneumonia and causes oxidative stress and defective alveolar macrophage (AM) immune function. We have previously determined that HIV-1 proteins inhibit antioxidant defenses and impair AM phagocytosis by suppressing nuclear factor (erythroid-derived 2)-like 2 (Nrf2). Given its known effects on Nrf2, we hypothesize miR-144 mediates the HIV-1 induced suppression of Nrf2.

Methods: Primary AMs isolated from HIV-1 transgenic (HIV-1 Tg) rats and wild type littermates (WT) as well as human monocyte-derived macrophages (MDMs) infected *ex vivo* with HIV-1 were used. We modulated miR-144 expression using a miR-144 mimic or an inhibitor to assay its effects on Nrf2/ARE activity and AM functions *in vitro* and *in vivo*.

Results: MiR-144 expression was increased in AMs from HIV-1 Tg rats and in HIV-1-infected human MDMs compared to cells from WT rats and non-infected human MDMs, respectively. Increasing miR-144 with a miR-144 mimic inhibited the expression of Nrf2 and its downstream effectors in WT rat macrophages and consequently impaired their bacterial phagocytic capacity and H_2O_2 scavenging ability. These effects on Nrf2 expression and AM function were reversed by antagonizing miR-144 *ex vivo* or in the airways of HIV-1 Tg rats *in vivo*, but this protection was abrogated by silencing Nrf2 expression.

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Conflicts of interest: The authors declare no conflicts of interests.

Conclusions: Our results suggest that inhibiting miR-144 or interfering with its deleterious effects on Nrf2 attenuates HIV-1-mediated AM immune dysfunction and improves lung health in individuals with HIV.

Keywords

miR-144; Nrf2; alveolar macrophage; HIV-1 transgenic rat

Introduction

Infection by the human immunodeficiency virus (HIV) remains a major global threat with ~38 million individuals living with HIV worldwide.[1–3] Although combination antiretroviral therapy (ART) is effective at decreasing viral load (often to undetectable levels) and slowing disease progression, individuals living with HIV remain remarkably susceptible (5-10-fold or greater) to serious pneumonias and lung injury, and pulmonary diseases remain the dominant causes of death in this vulnerable population.[4, 5] Importantly, we determined that even otherwise healthy-appearing individuals living with HIV and adherent to ART have profound oxidative stress and macrophage dysfunction within their alveolar compartment,[6] and recent evidence from our group and others demonstrates that HIV-1 integrates into alveolar macrophages[7] and airway epithelial cells.[8] Such integration in other tissues is known to lead to the chronic expression of HIV-1 viral proteins, including gp120 and Tat, which can directly induce alveolar macrophage and epithelial dysfunction.[8, 9] As the alveolar macrophage is the primary innate immune cell within the alveolar space, impairments of its function by HIV-1 and/or HIV-1 viral proteins within this space likely contributes to the extraordinarily high risk of pneumonia in these individuals even when circulating virus is effectively suppressed and peripheral CD4 cell counts are in the normal range. However, the molecular mechanisms by which HIV viral proteins impair alveolar macrophage function remain only partially understood.

Using clinically relevant HIV-1 transgenic rodent models that express HIV-1 viral proteins in the alveolar space, we discovered that these proteins induce oxidative stress by inhibiting Nrf2 (Nuclear factor (erythroid-derived 2)-like 2), the master transcription factor that activates anti-oxidant and immune defenses.[10, 11] Because of the important role of Nrf2 in key alveolar macrophage functions, it is critical that we understand how HIV-1 inhibits Nrf2 activity and thereby disrupts alveolar macrophage and epithelial cell function. In this study we focused on a novel mechanism by which HIV-1 infection and/or HIV-1 viral proteins within the alveolar space could inhibit Nrf2 expression and innate immune function within the alveolar macrophage. Specifically, microRNAs regulate myriad physiological mechanisms[12] as they bind to the 3’ untranslated region (UTR) of their target messenger RNAs and inactivate them. Importantly, microRNA-144 (miR-144) has been identified as having protean effects that are consistent with the aforementioned effects of HIV-1 in the alveolar space. In particular, miR-144 targets the 3’-UTR of Nrf2 and inhibits Nrf2 signaling[13–15] and HIV-1-induced increases in miR-144 impair alveolar epithelial barrier function.[16] Therefore, we focused on miR-144 in this study and present new evidence for its role in mediating the suppression of Nrf2 and immune function in the alveolar macrophages of HIV-1 transgenic rats and possibly in human macrophages infected *ex vivo* with HIV.

Taken together, our new experimental findings suggest that HIV-1 infection and the consequent expression of HIV-1 viral proteins in the lung induces the expression of miR-144, which inhibits Nrf2-dependent macrophage functions in the alveolar space and thereby creates a stressed microenvironment that renders these individuals susceptible to pneumonia and lung injury even when they are on effective ART.

Methods

Cell culture:

Primary alveolar macrophages (AMs) were isolated from 7-12 month old wild type Fischer 344 (WT) or HIV-1 transgenic (HIV-1 Tg) rats (Harlan Laboratories, Indianapolis, IN) and cultured in DMEM/F12 (Cellgro, Manassas, VA) with 10% fetal bovine serum (FBS, Atlanta Biologicals, Lawrenceville, GA) and antibiotic-antimycotic solution (Sigma-Aldrich, St. Louis, MO) at 37°C, 5% CO_2. After culturing for two hours at 37°C and 5% CO_2, the macrophage population was enriched by removing non-adherent cells prior to use in further experiments. A rat alveolar macrophage cell line (NR8383) was cultured in F12K with 10% FBS and antibiotic-antimycotic solution (Sigma-Aldrich).

Human MDMs:

Cells were isolated from the buffy coat of healthy human peripheral blood by Ficoll centrifugation, according to a protocol approved by Cincinnati Children's Hospital institutional review board.[10] In brief, isolated and enriched cells were cultured in RPMI-1640 with 10% FBS, 1% nonessential amino acid and 1% sodium pyruvate. After 7 days culture, cells were infected with or without HIV-1 viral particles for 4 h and then replaced with fresh growth medium for 8 days.

RNA extraction and real-time RT-PCR:

Total RNA was extracted from primary AMs using the QuickRNA kit (Zymo Research, Irvine, CA). Real-time RT-PCR was performed as previously described[11] and target genes were normalized to 9S from the same RT samples.

MicroRNA isolation and analysis:

miRNA was extracted according to the mirVana miRNA Isolation Kit manufacturer's protocol (Thermo Fisher Scientific, Waltham, MA). miRNA cDNA was synthesized using the miScript II RT Kit (Qiagen, Germantown, MD). Real-time PCR for miR-144-3p expression was performed using the Qiagen QuantiTect SYBR Green PCR Kit as previously described.[16] Primers for miR-144-3p and SNORD25 were obtained from Qiagen.

Transfection of miR-144 mimic:

Primary AMs from WT rats were transfected with either miR-144-3p mimic from Qiagen (5'-UACAGUAUAGAUGAUGUACU-3') or miRNA mimic negative control (miRNA Ng CTL, Qiagen) using Lipofectamine 3000 (Thermo Fisher Scientific). Expression of Nrf2 and its downstream effectors were assessed at 72 hours post-transfection.

miR-144 silencing *in vivo*:

Sixteen HIV-1 Tg rats (8 male and 8 female rats) were randomly selected to treat with 50 nM of either miRNA inhibitor negative control or miR-144-3p inhibitor (Qiagen, 5'-UACAGUAUAGAUGAUGUACU-3') via intra-tracheal delivery three times over the course of one week as previously described.[16] Phagocytic function was assessed in freshly-isolated primary AMs, as were RNA and protein expression of Nrf2 and its effectors.

Nrf2 siRNA transfection:

Primary AMs from HIV-1 Tg rats were isolated and plated at 100,000/well in 16-well chamber slides or 200,000/well in 24-well plates. The next day, AMs were transfected with 10 nM of Nrf2 stealth select RNAi (Thermo Fisher Scientific, oligo ID: RSS343557) with or without 50 nM miR-144 inhibitor using the Lipofectamine 3000 transfection reagent (Thermo Fisher Scientific). Twenty-four hours later, Nrf2 mRNA expression and phagocytic function was assessed as described below.

Nrf2 luciferase assay:

A rat macrophage cell line (NR8383, 20,000 cells/well) was cultured in 96-well plates and transfected with the Qiagen Cigna ARE reporter (which includes an inducible, ARE-responsive firefly luciferase construct and a constitutively expressed *Renilla* luciferase construct) and 10 nM of either miR-144 mimic or miRNA mimic control (Qiagen) using the Lipofectamine 3000 transfection reagent. Forty-eight hours later, cells were lysed and luciferase activity was quantified using a dual-luciferase reporter assay per the manufacturer's instructions (Promega, Madison, WI). Nrf2/ARE promoter activity was expressed as a ratio of arbitrary units of firefly luciferase/*Renilla* luciferase activity.

Western immunoblotting:

Total proteins were isolated from the primary AMs using 2x Laemmli sample buffer (Bio-Rad Lab, Hercules, CA), electrophoresed in 4-20% polyacrylamide gels (NuSep, Germantown, MD), and transferred to a PVDF membrane. The membrane was incubated with primary antibodies against Nrf2 and NQO1 (SC-722x and SC-16464, respectively; Santa Cruz biotechnology, Heidelberg, Germany), GCLC (ab55435, Abeam, Cambridge, MA), and GAPDH for housekeeping (G9545, Sigma-Aldrich, St. Louis, MO) prior to incubation with a secondary antibody. Immuno-reactive bands were captured with the ChemiDoc XRS system (Bio-Rad).

Phagocytic assay:

Primary AMs (100,000 cells/well) were plated on 16-well chamber slides and cultured in DMEM/F12 plus 2% FBS with 10 nM of either miR-144 mimic or miRNA mimic negative control (Qiagen) for 3 days. 1×10^6 units of pHrodo Rad *S. aureus* BioParticle Conjugates (Thermo Fisher Scientific) were added during the final two hours of culturing. Images were captured by Olympus Fluorescent microscope and analyzed by ImageJ (NIH) as previously described.[10]

Hydrogen peroxide (H_2O_2) scavenging assay:

Primary rat AMs were cultured in 24-well plates and transfected with 10 nM of either miR-144 mimic or miRNA mimic negative control (Qiagen) for 48 hours. As previously described,[10] cells were cultured with assay buffer plus 5 mU/ml of glucose oxidase (Sigma-Aldrich) at 37 °C, *5%* $CO_2$for 2 hours in the dark. The Amplex red assay kit (Thermo Fisher Scientific) was used for quantifying H_2O_2concentration in the culture media by measuring fluorescence at excitation 540 nm and emission 590 nm. The H_2O_2concentration was normalized with cells measured by MTT. The results were expressed as the percentage of H_2O_2 scavenging in treatment group compared to cells in control group.

Statistical analyses:

One-way ANOVA with Newman-Keuls post-test was performed for multiple comparisons and Student's t-test was used for single comparisons using Prism (GraphPad, San Diego, CA). All data are presented as mean ± SEM. Significance was accepted at $P \leq 0.05$.

Study approval:

All animal studies were reviewed and approved by the Institutional Animal Care and Use Committee at Emory University. The isolation of human peripheral blood monocytes was performed at a core laboratory at Cincinnati Children's Hospital with the approval of the Institutional Review Board at that institution. Informed consent was obtained from all participants for blood collection. All methods were performed in accordance with the relevant guidelines and regulations.

Results

HIV-1 viral proteins increased miR-144 expression in primary rat alveolar microphages and human monocyte-derived macrophages (MDMs).

We previously reported that HIV-1 transgene expression and HIV-1-infected human MDMs significantly decreased Nrf2 expression and impaired alveolar macrophage function[10] and that Keap1 mRNA expression levels were stable in primary alveolar macrophages from HIV-1 transgenic rats (87.9 ± 21.7 %, n=16) compared to the cells from WT rats (100.0 ± 6.7 %, n=18). According to analysis *in silico*, two miR-144 binding sites are present in the Nrf2 3'-UTR. We hypothesized that HIV-1 transgene expression inhibited Nrf2 expression via alterations in miR-144 expression. As shown in Figure 1A, miR-144 expression was significantly increased 4-fold in primary alveolar microphages derived from HIV-1 Tg rats compared to cells from WT rats as well as in human MDMs infected with HIV-1 Figure 1B.

miR-144 mimic suppressed Nrf2 and its effectors and impaired macrophage phagocytic function.

We next introduced miR-144 mimic to primary alveolar macrophages from WT rats to determine its effects on Nrf2 expression and activity. As shown in Figure 2A, increasing miR-144 significantly decreased gene expression of Nrf2 and its effectors GCLC and NQO1 ($p<0.05$). We confirmed these findings using a dual-luciferase assay in NR8383 cells, a rat AM cell line, and found that miR-144 mimic significantly decreased Nrf2/ARE promoter

activity to around 50% of control levels (Figure 2B). Further, we determined that phagocytic function and H_2O_2 scavenging ability were also significantly impaired by miR-144 mimic (Figure 3A–B)

Silencing miR-144 *in vivo* restored both Nrf2 expression and macrophage phagocytic function.

Because miR-144 suppressed Nrf2/ARE activity and impaired macrophage functions, we hypothesized that administration of a miR-144 inhibitor *in vivo* would improve viral protein-induced dysfunction in alveolar macrophages. HIV-1 Tg rats were treated with 50 nM of either miRNA inhibitor negative control or a miR-144 inhibitor intratracheally three times over the course of one week. We found that gene and protein expression of Nrf2 and its effectors NQO1 and GCLC were significantly increased in the alveolar macrophages of rats treated with the inhibitor (Figure 4A–B). Most importantly, phagocytic function in macrophages from inhibitor-treated rats was restored to WT levels (Figure 5).

Macrophage phagocytosis is impaired by HIV-1-induced miR-144 expression.

After determining that miR-144 manipulation caused significant effects in Nrf2/ARE activity and phagocytic function, we next sought to connect the two findings through the use of Nrf2 silencing RNA. Primary alveolar macrophages from HIV-1 Tg rats were transfected *ex vivo* with Nrf2 silencing RNA (Nrf2 siRNA) ± miR-144 inhibitor. After 24 hours in culture, Nrf2 mRNA expression was assessed. In parallel, pHrodo-labelled *S. aureus* BioParticles Conjugates were added and phagocytic function was determined as described above. As expected, Nrf2 mRNA expression was significantly suppressed in cells treated with Nrf2 siRNA regardless of the presence or absence of the miR-144 inhibitor (Figure 6A). As shown in Figure 6B and consistent with the results shown in Figure 5, antagonizing miR-144 expression restored phagocytic function in alveolar macrophages from HIV-1 transgenic rats, but those effects were abrogated in cells transfected with Nrf2 siRNA. Taken together, these results are consistent with a direct mechanistic link between miR-144 and Nrf2 in the functional impairment of alveolar, macrophages during chronic exposure to HIV-1 viral proteins *in vivo*.

Discussion

In this study we determined that either HIV-1 transgene expression *in vivo* in rats, which causes chronic exposure of the alveolar macrophages to HIV-1 viral proteins, or direct HIV-1 infection of human monocyte-derived macrophages *ex vivo*, increased macrophage-specific expression of miR-144. Further, increasing miR-144 by transfecting a miR-144 mimic in primary alveolar macrophages from littermate control rats without HIV-1 transgene expression decreased Nrf2 protein levels, activity, and downstream antioxidant effector function. In parallel, miR-144 mimic decreased bacterial phagocytic capacity and hydrogen peroxide scavenging in primary alveolar macrophages. Direct increase of miR-144 in naïve macrophages recapitulated the effects of HIV-1 on these cells. In contradistinction, antagonizing miR-144 expression *ex vivo* in primary alveolar macrophages freshly isolated from HIV-1 transgenic rats increased the expression of Nrf2 and of downstream Nrf2-dependent antioxidants. Further, antagonizing miR-144 expression in the airways of HIV-1

transgenic rats *in vivo* restored alveolar macrophage phagocytic capacity. Antagonizing miR-144 expression in primary alveolar macrophages from HIV-1 transgenic rats *ex vivo* likewise restored their phagocytic function, but this salutary effect was abrogated by simultaneously silencing Nrf2 expression. Taken together, these experimental findings implicate the induction of miR-144 as a proximal mechanism by which HIV-1 inhibits Nrf2 expression and activity which, in turn, causes oxidative stress and immune dysfunction within the alveolar space thereby rendering individuals living with HIV-1 vulnerable to pneumonia and lung injury.

Despite the dramatic improvements in overall health and survival of people with HIV-1, even when they adhere to ART, they are at greater risk for pneumonias from diverse pathogens,[17–24] and lung function is irreversibly damaged following episodes of pneumonia.[25] Our group has been investigating the importance of Nrf2 suppression in HIV-1-mediated lung disease for years now. Our recent study on the effects of Nrf2 suppression in the macrophage is particularly germane here, as it highlights two key points relevant to the current investigation.[10] First, it ties the effects of Nrf2 suppression directly to innate immunity. Although Nrf2 is primarily known as an antioxidant effector, its importance to macrophage innate immunity cannot be overstated.[26] Second, we showed that direct infection of human monocyte-derived macrophages by HIV-1 affected Nrf2 in a similar fashion to the effects of HIV-1 viral proteins, which is important in establishing the credibility of the HIV-1 transgenic rat model we use throughout this study. Although that model is not infectious, it generates the same HIV-1 viral proteins that have been found in the alveolar space of human subjects with HIV-1. After our group noted that relatively few alveolar macrophages were infected with the virus despite the presence of global macrophage dysfunction,[7] we hypothesized that the effects of HIV-1 on alveolar macrophages and epithelium may be due to viral proteins, a hypothesis that our work to date has supported.[10, 11] The new findings presented in this study build on our recent manuscript in which we determined that the induction of miR-144 by HIV-1-viral proteins decreases Nrf2 expression and barrier function within the alveolar epithelium.[16] Therefore, the chronic expression of HIV-1 viral proteins within the alveolar space that occurs in individuals living with HIV-1 perturbs both alveolar epithelial barrier integrity and alveolar macrophage immune function. This common mechanism, namely miR-144-mediated inhibition of Nrf2, may explain at least in part why multiple pulmonary diseases such as emphysema, pulmonary fibrosis, lung cancer and related disorders develop earlier and far more often in individuals living with HIV.[27–30]

In recent years, the relevance of microRNAs to human disease has made them an important topic of study.[31–34] In HIV, the focus has primarily been on the effects of microRNAs in the process of HIV replication, and the manner in which microRNAs may be harnessed to control the virus.[35,36] In this study, as in our recent manuscript detailing the effects of miR-144 on alveolar epithelium,[16] we highlight the effects of HIV-1 on the myriad microRNAs present in the alveolar space. Because of the complex processing they undergo, microRNAs add layers of complexity to any interaction between host and pathogen. In the case of the lung, our two studies argue that miR-144 in particular is not only enhanced by HIV-1, but also represents a proximal defect in the milieu of the alveolar space that leads directly to lung dysfunction. Further, in the case of both the macrophage (in this study) and the alveolar epithelium,[16] we were able to mitigate the deleterious effects of miR-144 with

an antagomir *in vivo*, thereby offering a compelling possibility for therapy in human subjects.

Upregulation of miR144 associated with decrease in Nrf2 expression has also been seen in other diseases such as sickle cell disease,[15,37] thalassemia[38] and acute myeloid leukemia.[39] We focused on miR-144 based on our prior analysis *in silico* and our prior work on the alveolar epithelium, but our selection carries two important caveats worth mentioning here: first, several miRNAs are known to target Nrf2 based on an analysis of the miRDB (http://mirdb.org) and of the TargetScanHuman directory (http://www.targetscan.org/vert_72/). Yang et al[40] reported that miR28 also targeted the Nrf2 3'UTR and reduced the Nrf2 expression in the breast cancer cell line although the complex interaction between the Nrf2 activity and cancer development and progression. Therefore, we cannot exclude the possibility that other miRNAs contribute to the pathophysiological effects of HIV-1 viral proteins on Nrf2 and its effectors. However, at least in the experimental HIV-1 transgenic rat model employed in this study, specifically increasing miR-144 by transfecting a mimic recapitulates the deleterious effects of HIV-1 viral proteins on Nrf2 expression and phagocytic function in the alveolar macrophage and antagonizing miR-144 reverses these effects. Importantly, although we could not perform such parallel mechanistic studies in human cells, we determined that direct infection of human macrophages with HIV-1 induced the expression of miR-144, providing important albeit circumstantial evidence that this pathophysiological sequence is relevant in individuals living with chronic HIV. Interestingly, miR-144 appears to mediate influenza-induced lung injury as ablating miR-144 *in vivo* attenuates influenza replication and lung injury in mice.[41] However, to date, studies connecting HIV-1 and miR-144 have been few and far between, including one recent clinical investigation[42] and our own recent paper on HIV-1-induced epithelial dysfunction.[16]

In summary, we determined that HIV-1 infection of human macrophages, or the chronic expression of HIV-1 viral proteins within the alveolar space in a transgenic animal model, induces miR-144. In the experimental model we identified that miR-144 in turn inhibits the expression and functions of Nrf2 and thereby dampens bacterial phagocytic capacity. Further, antagonizing miR-144 in the airways of HIV-1 transgenic animals *in vivo* restores Nrf2 expression and function within the alveolar macrophage and reverses their phagocytic dysfunction. These findings provide new insights into why individuals living with HIV are at increased risk for pneumonia and other lung diseases despite effective viral suppression and apparent immune reconstitution with ART. Further, they suggest that targeting miR-144 and/or Nrf2 within the airway could enhance lung health in this vulnerable population.

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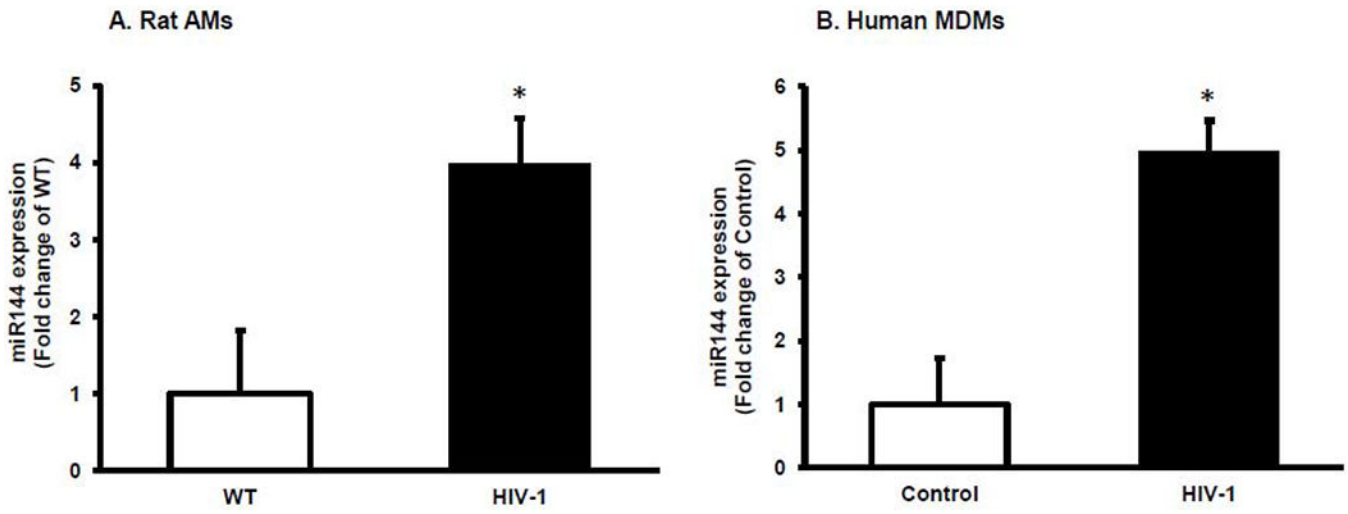

Figure 1. HIV-1 viral proteins induced miR-144 expression in macrophages.

(A) miR-144 was measured by RT-qPCR in the primary alveolar macrophages derived from WT or HIV-1 Tg rats, n=7 or **(B)** in human MDMs infected with HIV-1 for 8 days, n=4. Student's t-test was used for the single comparisons shown in each panel using Prism (GraphPad, San Diego, CA). In each panel, the data are shown as mean ± SEM; *$p<0.05$ compared to WT (panel A) and to Control (panel B).

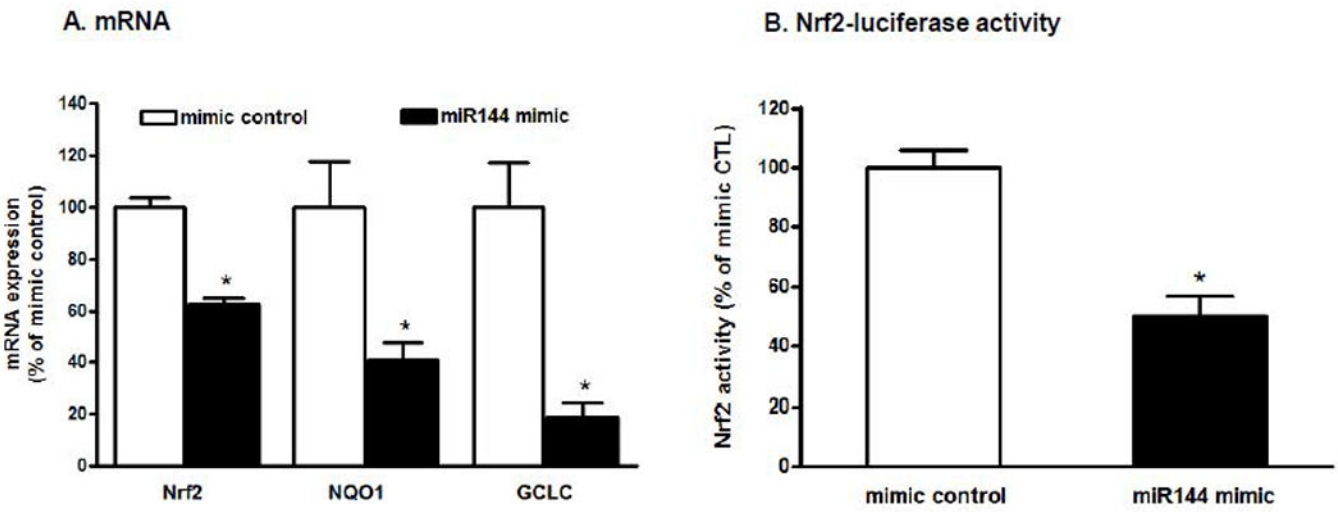

Figure 2. miR-144 mimic decreased Nrf2 activity in the alveolar macrophages *in vitro*.

A. Primary alveolar macrophages (AM) were isolated from WT rats and transfected with 5 nM of either the miRNA mimic negative control or the miR-144 mimic for 3 days; n=3-4 wells. *P<0.05 compared to mimic negative control (p<0.05). **B.** NR8383 cells were transfected with 10 nM either the miRNA mimic negative control or the miR-144 mimic with the Cignal Reporter was also added. Forty-eight hours later the cells (n=12 wells in each condition) were lysed and Nrf2/ARE activity was quantified by luciferase activity. Student's t-test was used for single comparisons using Prism (GraphPad, San Diego, CA). In each panel the data shown are the means ± SEM and *P<0.05 compared to cells treated with miRNA the mimic negative control.

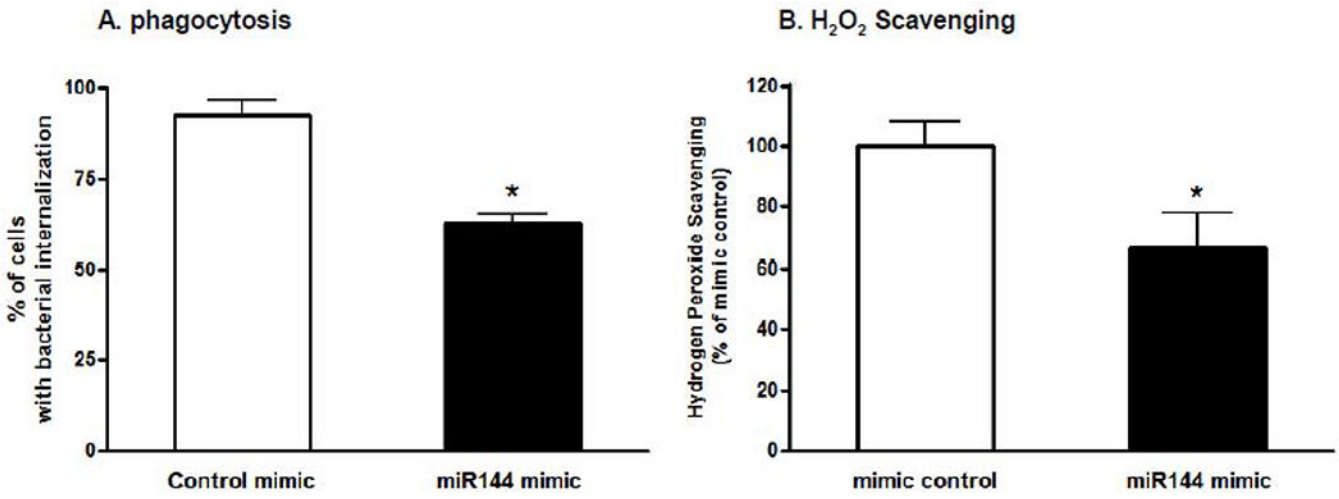

Figure 3. Increasing miR-144 impairs the alveolar macrophages function in the *in vitro*.

Primary alveolar macrophages (AM) were isolated from WT rats and treated with either miRNA mimic negative control or miR-144 mimic (10 nM). **A.** During the final 2 hours of culture, pHrodo Rad *S. aureus* BioParticles conjugates were added and images were captured by Olympus Fluorescent microscope and analyzed by Image J. Five images were taken from each well (150 – 160 cells per treatment); n=4-5 wells in each condition. **B.** H_2O_2 scavenging as quantified by the Amplex Red assay; n=4-5 wells in each condition. Student's t-test was used for the single comparisons shown in each panel using Prism (GraphPad, San Diego, CA). Data shown as mean ± SEM; *P<0.05 compared to cells treated with miRNA mimic negative control.

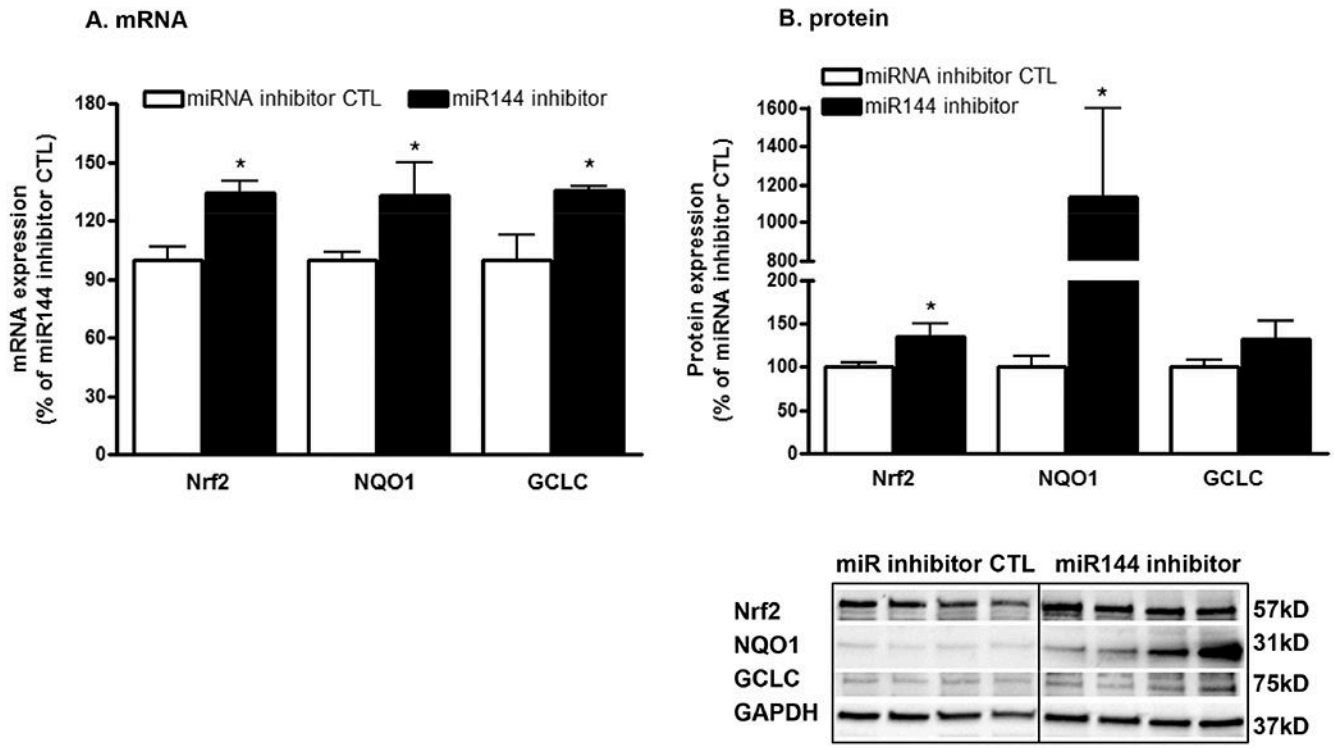

Figure 4. Delivering a miR-144 inhibitor into the airway of HIV-1 transgenic rats *in vivo* increases the expression of Nrf2 and Nrf2-dependent antioxidants in their alveolar macrophages.

Primary alveolar macrophages (AM) were isolated from HIV-1 Tg rats treated with either the miRNA inhibitor control (CTL) or the miR-144 inhibitor (50 nM ×3) for 1 week (2 female and 2 male rats in each group). AM were then analyzed for gene expression by qRT-PCR **(panel A)** and protein expression by western immunoblotting **(panel B).** Student's t-test was performed for single comparisons. Data are presented as mean ± SEM (n=4 rats); *P<0.05 increased compared to AM from rats treated with the miRNA inhibitor control.

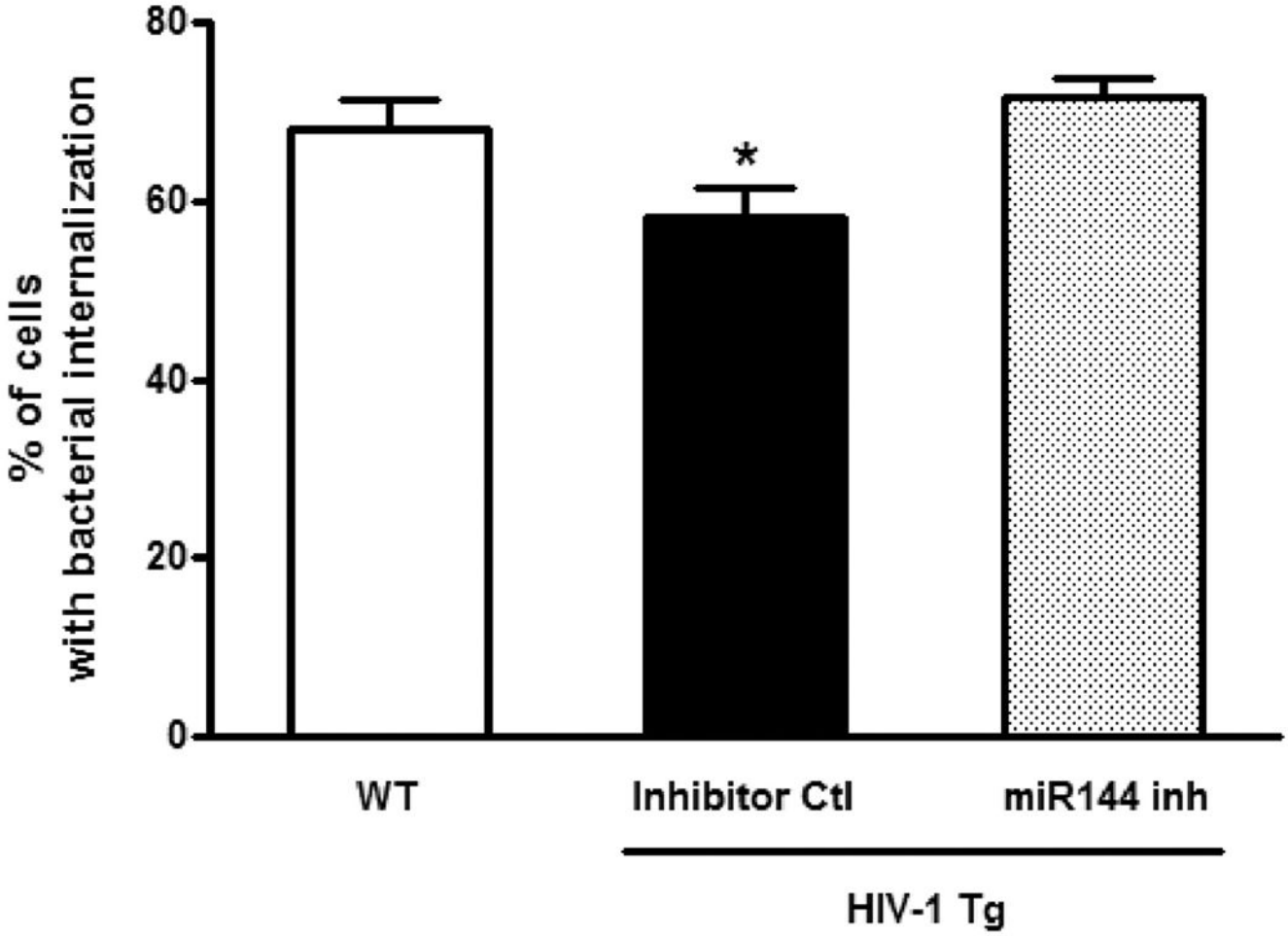

Figure 5. Delivering a miR-144 inhibitor into the airway of HIV-1 transgenic rats *in vivo* restores the phagocytic function in their alveolar macrophages.

Primary alveolar macrophages (AM) were isolated from wild type (WT) rats and HIV-1 Tg rats treated with either miRNA inhibitor control or miR-144 inhibitor *in vivo* as in Figure 4. The AMs were then cultured for 2 hours with pHrodo Rad *S. aureus* BioParticle Conjugates and phagocytosis was assessed using an Olympus Fluorescent microscope and quantifying the percentage of AMs with visible bacterial internalization using ImageJ software. 10-15 images/4 rats with 30-50 cells/field were analyzed. One-way ANOVA with Newman-Keuls post-test was performed for multiple comparisons and the data are presented as mean ± SEM; *P<0.05 decreased compared to AM from WT rats and from HIV-1 Tg rats treated with the miR-144 inhibitor.

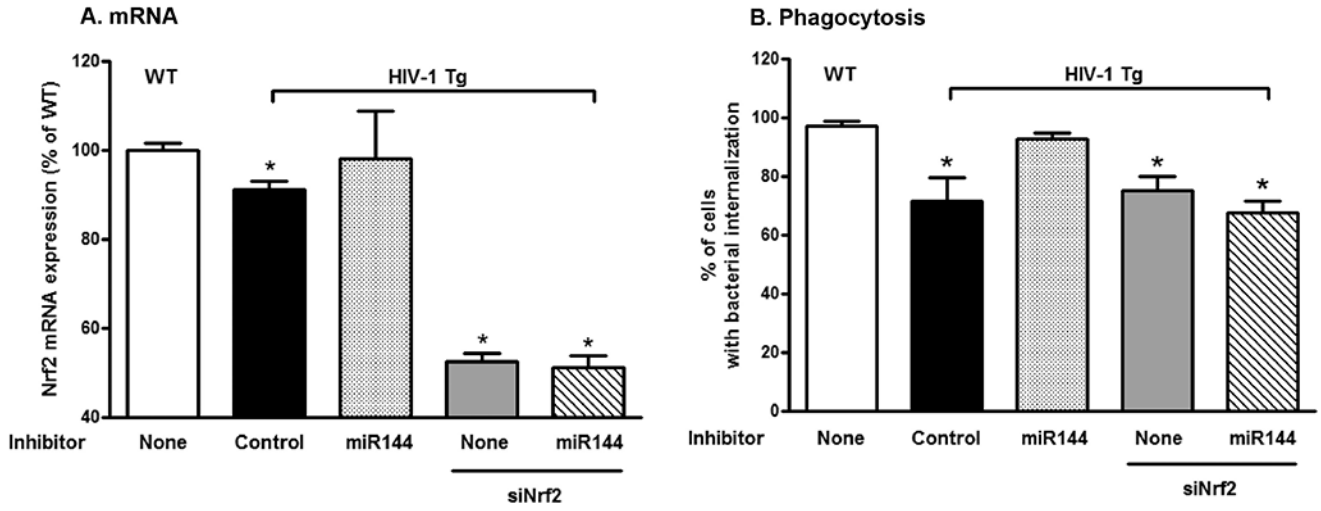

Figure 6. miR-144 inhibition restored phagocytic function in alveolar macrophages from HIV-1 Tg rats, and this protection was blocked by silencing Nrf2 expression.

Primary alveolar macrophages (AMs) were isolated from WT and HIV-1 Tg rats. AMs from HIV-1 Tg rats were transfected *ex vivo* with either a control inhibitor or a miR-144 inhibitor ± Nrf2 RNA silencing (siNrf2) and then incubated with pHrodo Rad *S. aureus* BioParticles Conjugates as before. Twenty-four hours later, cells were extracted for analysis of Nrf2 mRNA expression (n=6 wells; **panel A**) and phagocytosis images (3-4 wells/treatment) were captured using an Olympus Fluorescent microscope and analyzed by Image J (150 – 200 cells per treatment; **panel B**). One-way ANOVA with Newman-Keuls post-tests was performed for these multiple comparisons. Data are presented as mean ± SEM; *P<0.05 decreased compared to AM from WT rats.