Transcription Factor FOXO3a Mediates Apoptosis in HIV-1-Infected Macrophages

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Macrophages serve as a major reservoir for HIV-1 because a large number of macrophages in the brain and lung are infected with HIV-1 during late stage disease. Recent evidence suggests that those HIV-1-infected macrophages play a key role in contributing to tissue damage in AIDS pathogenesis. Macrophages undergo apoptosis upon HIV-1 infection; however, the mechanisms of this process are not well-defined. Previously, we demonstrated that HIV-1 infection inhibits Akt-1, a critical protein for cell survival of macrophages. In the present study, we investigated the involvement of transcription factor FOXO3a in the regulation of HIV-1-mediated apoptosis in macrophages. HIV-1 infection significantly decreased phosphorylation of FOXO3a and promoted FOXO3a translocation to the nucleus in human monocyte-derived macrophages (MDM). Overexpression of constitutively active FOXO3a increased DNA fragmentation with decreased cell viability in MDM, whereas a dominant-negative mutant of FOXO3a or small interfering RNA for FOXO3a to knockdown the function of FOXO3a in HIV-1-infected MDM decreased DNA fragmentation and protected macrophages from death in HIV-1-infected MDM. Overexpression of constitutively active Akt-1 increased FOXO3a phosphorylation, suggesting that FOXO3a phosphorylation in human MDM is dependent on Akt-1. We therefore conclude that FOXO3a plays an important role in HIV-1-induced cell death of human macrophage. Understanding the PI3K/Akt-1/FOXO3a pathway and its associated death mechanism in macrophages during HIV-1 infection would lead to identification of potential therapeutic avenues for the treatment of HIV-1 infection. The Journal of Immunology, 2008, 180: 898–906.
found HIV-1 infection decreased phosphorylation of FOXO3a and promoted nuclear translocation in infected monocyte-derived macrophages (MDM).3 Azidothymidine (AZT), an inhibitor for HIV-1 reverse transcriptase, blocked these effects. Overexpression of constitutively active FOXO3a decreased cell viability and increased DNA fragmentation in MDM. Moreover, both a dominant-negative (DN) mutant of FOXO3a and FOXO3a small interfering RNA (siRNA) knockdown partially reduced cell death in HIV-infected MDM. These studies provide insight into the apoptotic signaling events of HIV-1-induced cell death and identify FOXO3a as an important apoptotic factor in HIV-1-infected macrophages.

Materials and Methods

Isolation and culturing of primary human monocytes

Human monocytes were recovered from PBMC of HIV-1, HIV-2, and hepatitis B virus-seronegative donors after leukopheresis and purified by counter-current centrifugal elutriation (24). Monocytes were cultured in DMEM (Sigma-Aldrich) supplemented with 10% heat-inactivated human serum, gentamicin (50 μg/ml), ciprofloxacin (10 μg/ml), and M-CSF (1000 U/ml, a gift from Wyeth, Cambridge, MA). Monocytes were allowed to differentiate for 7 days at which time they were considered MDM. All human subject studies were performed in full compliance with the University of Nebraska Medical Center and National Institutes of Health ethical guidelines.

HIV-1 infection of MDM

Seven days after plating, MDM were infected with HIV-1 virus at a multiplicity of infection (MOI) of 0.1 virus/target cell (24). Briefly, viral stocks were diluted 1/15 in medium for overnight incubation with MDM. On the second day, medium was removed and substituted with MDM culture medium that was half-exchanged every 2 days (24). Stock virus was screened for mycoplasma and endotoxin using hybridization and Limulus amoebocyte lysate assays, respectively.

Measurements of reverse transcriptase (RT) activity

RT activity was determined in triplicate samples of cell culture fluids. For this assay, 10 μl of supernatant was incubated in a reaction mixture of 0.05% Nonidet P-40, 10 μl of poly(A)/ml, 0.25 μg of oligo(dT)/ml, 5 mM DTT, 150 mM KCl, 15 mM MgCl2, and [3H]TTP in Tris-HCl buffer (pH 7.9) for 24 h at 37°C. Radiolabeled nucleotides were precipitated with cold 10% trichloroacetic acid on paper filters in an automatic cell harvester and washed with 95% ethanol. Radioactivity was determined by liquid scintillation spectroscopy (13).

Immunofluorescence and confocal microscopy

Cells on coverslips were cultured 3 days following HIV-1 infected infection, fixed for 15 min in 4% paraformaldehyde in PBS, dried in methanol for 5 min, permeabilized, and blocked 30 – 60 min with 0.1% Triton X-100 with 2% BSA in PBS. Cells were incubated overnight with mouse anti-P24 mAb (DakoCytomation) and rabbit anti-FOXO3a Ab (Cell Signaling Technology), then conjugated with anti-mouse Alexa Fluor 488 nm (green; Invitrogen Life Technologies) and anti-rabbit Alexa Fluor 647 nm (red; Invitrogen Life Technologies) secondary Abs. Hoechst 33342 (Sigma-Aldrich) was used for nuclear staining. Triple immunostaining was examined by a Bio-Rad MRC1024ES laser scanning confocal microscope, using a triple laser line and simultaneous triple display mode of the Bio-Rad LaserSharp imaging program.

Western blot analysis

Cell lysates from MDM were prepared with M-PER Mammalian Protein Extraction Buffer (Pierce). Nuclear and cytoplasmic fractions were prepared from MDM with the Nucleus Protein Extraction kit according to the manufacturer’s instruction (Pierce). Protein concentration was determined by a BCA Protein Assay kit (Pierce). Protein (30 μg) was electrophoresed on 10% SDS-PAGE (Bio-Rad) and transferred to an Immune-Blot polyvinylidene difluoride membrane (Bio-Rad). Total FOXO3a, phospho-FOXO3a, Puma, Akt-1, β-actin, and TATA box binding protein (TBP) proteins were detected using anti-FOXO3a (Cell Signaling Technology), anti-phospho-FOXO3a at Ser318/321 (Cell Signaling Technology), anti-Puma (Cell Signaling Technology), anti-Akt-1 (Cell Signaling Technology), anti-β-actin (Sigma-Aldrich), and anti-TBP (Abcam) Abs. Membranes were treated overnight with primary Ab at 4°C followed by HRP-labeled anti-rabbit (Cell Signaling Technology) or anti-mouse (Cell Signaling Technology) secondary Ab for 1 h at room temperature. Ag-Ab complexes were visualized by ECL (Amersham Biosciences) and captured with CL-Xpose Film (Pierce). For data quantification, the films were scanned with a CanoScan 9950F scanner; the acquired images were then analyzed on a Macintosh computer using the public domain NIH image program (developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/).

Adenoviral infection

Replication-defective adenoviral vectors expressing dominant negative FOXO3a (Ad-DN-FOX3a) and constitutively active FOXO3a (Ad-AAA-FOX3a) were purchased from Vector Biolabs. The constitutively active FOXO3a was prepared by substituting alanine for amino acids threonine 32, serine 253, and serine 315. Both Ad-DN-FOX3a and Ad-AAA-FOX3a have a hemagglutinin tag at the N terminus and express GFP. Constitutively active Akt-1 (Ad-myrAkt-1)

![Figure 1](http://rsb.info.nih.gov/nih-image/). Phosphorylation of FOXO3a decreases in different HIV-1 strains infecting human MDM. A, Human MDM were infected by HIV-lADA, HIV-lJRFL, and HIV-lBAL strains. Cell lysates were collected 5 days after infection and subjected to Western blotting for p-FOXO3a (phosphorylated FOXO3a) and FOXO3a (total FOXO3a). Control denotes uninfected cells. B, Axial actin was used as a loading control. C, Viral strain infections were treated with AZT (5 μM). C and D, Results of A and B were densimetrically quantified as a ratio of p-FOXO3a to FOXO3a and presented as percentage of control in C and D, respectively. Average ± SD of three independent experiments from three donors were shown. **, p < 0.01 compared with control.
was generated as described (25) and provided by K. Walsh (Boston University School of Medicine, Boston, MA). The constitutively active Akt-1 construct has the c-src myristoylation sequence fused in-frame to the N terminus of the HA-Akt-1 (wild-type) coding sequence. Adenoviral constructs were amplified in HEK 293 cells and purified by ultracentrifugation through a CsCl gradient. MDM were infected with recombinant adenovirus at \( \frac{90}{900} \) confluency in serum-free DMEM for 2 h and then incubated for 48 h in a growth medium. Infection efficiency was close to 70% as determined by the GFP expression. For adenovirus and HIV-1 double infection, MDM were infected with HIV-1 for 24 h followed by infection with adenovirus. Apoptosis assays were performed 3 days after adenovirus infection.

**siRNA transfection**

Predesigned siRNA duplexes targeting FOXO3a (ID: 144672) and a control siRNA were synthesized by Ambion and Dharmacon, respectively. Control siRNA is the number one siCONTROL nontargeting siRNA from Dharmacon that has been tested to have minimal off-target effects. At 24 h after HIV-1 infection, cells were transfected with 100 nM siRNA duplexes for 4 h in DMEM culture medium without serum, in the presence of siIMPORTER reagent (Upstate Cell Signaling Solutions) according to the manufacturer’s instructions. To evaluate transfection efficiency, cells were transfected with siGLO (red fluorescence tagged nonspecific siRNA; Qiagen). At 48 h posttransfection, cells were incubated with Hoechst 33342 (Sigma-Aldrich) for nuclear staining. Transfection efficiency was measured by counting siGLO-positive cells and total cells in fluorescence microscopy.

**Assessment of cell viability and apoptosis**

To assess apoptosis, MDM were plated in 24-well plates with \( 1.1 \times 10^6 \) cells/well. After 48 h of adenoviral infection with or without HIV-1 infection, cells were lysed and assayed by Cell Death Detection ELISA (Roche), as previously described (26). After 72 h of adenoviral infection, a colorimetric MTT assay was performed to measure the cell viability as previously described (26).

**FACS analysis**

After 3 days of adenoviral infection, MDM were dissociated with Accutase (Chemicon International) for 15 min. Cells were then washed with 3% FBS/PBS and incubated with 1 \( \mu \)g/ml propidium iodide (PI; catalog no. 537059; Calbiochem) for 15 min at 4°C. Cells were washed twice with 3% FBS/PBS and resuspended in PBS containing 4% paraformaldehyde. Cell death was determined by flow cytometry and analyzed with CellQuest software. At least 10,000 cells were analyzed per sample.

**Statistical tests**

Data were analyzed as means \( \pm \) SD of the mean (SD) unless specified. The data were evaluated statistically by the ANOVA, followed by the Tukey test for paired observations. Significance was considered to be \(<0.05\) unless specified. To account for any donor-specific differences, all experiments were performed with at least three donors. All assays were performed at least three times with triplicate or quadruplicate determinations for each time.

**Results**

**HIV-1 infection significantly decreased phosphorylation of FOXO3a in human macrophages**

Phosphorylation regulates the function of FOXO3a. When in an unphosphorylated form, FOXO3a resides inside the nucleus. Phosphorylation of FOXO3a in the forkhead domain leads to disruption of DNA binding, cytosol exportation, abrogating its transcriptional
activity (27–29). To assess the regulation of FOXO3a in macrophages during HIV-1 infection, we used three macrophage-tropic HIV-1 strains (HIV-1ADA, HIV-1JRFL, and HIV-1BAL) to infect cultured human MDM. The phosphorylated FOXO3a, total FOXO3a, and β-actin were determined through Western blotting (Fig. 1, A and B). After densimetric quantification of the Western blotting, we found HIV-1ADA, HIV-1JRFL, and HIV-1BAL infection decreased phosphorylation of FOXO3a to 28.7, 34, and 45.7% of control MDM, respectively (Fig. 1C), when normalized with total FOXO3a. HIV-1 infection did not change total FOXO3a and β-actin levels. AZT, a reverse transcriptase analog, was used to inhibit HIV-1 replication. AZT treatment blocked the observed decrease of FOXO3a phosphorylation (Fig. 1D). Infection levels of MDM by HIV-1 were monitored by RT activity assay to confirm infection of

FIGURE 3. HIV-1 infection induces FOXO3a translocation from the cytoplasm to the nucleus (immunofluorescence staining). Human MDM were infected with HIV-1 for 3 days with or without AZT treatment and then stained with Abs to p24 (HIV-1 infection marker, green) and FOXO3a (red), conjugated with anti-mouse Alexa Fluor 488 nm and anti-rabbit Alexa Fluor 647 nm secondary Abs, respectively. 4′,6′-Di amidino-2-phenylindole (DAPI; blue) was used for DNA staining. A–D, Control uninfected MDM. E–H, HIV-1-infected MDM. I–L, AZT-treated control MDM. M–P, AZT-treated HIV-1-infected MDM. D, H, L, and P are merged pictures of A–C, E–G, I–K, and M–O, respectively. Images were acquired from a BioRad MRC1024ES laser scanning confocal microscope. Magnifications: A–P, ×600. Panels are representative of four separate donors.

FIGURE 4. HIV-1 infection induces FOXO3a translocation from the cytoplasm to the nucleus (immunoblotting). A, After 3 days of infection by HIV-1, with or without AZT treatment (5 μM), proteins from whole cell lysates, cytoplasmic fractions, and nuclear fractions were examined for phosphorylated FOXO3a, total FOXO3a, and β-actin were determined through Western blotting (Fig. 1A and B). After densimetric quantification of the Western blotting, we found HIV-1ADA, HIV-1JRFL, and HIV-1BAL infection decreased phosphorylation of FOXO3a to 28.7, 34, and 45.7% of control MDM, respectively (Fig. 1C), when normalized with total FOXO3a. HIV-1 infection did not change total FOXO3a and β-actin levels. AZT, a reverse transcriptase analog, was used to inhibit HIV-1 replication. AZT treatment blocked the observed decrease of FOXO3a phosphorylation (Fig. 1D). Infection levels of MDM by HIV-1 were monitored by RT activity assay to confirm infection of
FIGURE 5. Overexpression of constitutively active FOXO3a induces macrophage cell death. A, Human MDM were infected with adenovirus vector containing a GFP reporter system. Infection efficiencies were monitored by taking fluorescence images 48 h after adenovirus infection. Ad-GFP is GFP control vector adenovirus; Ad-AAA-O3a is adenovirus expressing constitutively active FOXO3a; Ad-DN-FOXO3a is adenovirus expressing DN FOXO3a. B, After 72 h of adenoviral infection at 2000 MOI, FOXO3a overexpression was determined by immunoblotting for total FOXO3a. Constitutively active FOXO3a and endogenous FOXO3a both appeared at 98 kDa, while DN FOXO3a appeared at 40 kDa. In these samples, induction of Puma by FOXO3a overexpression was also determined by Western blotting. β-actin was used as a loading control. C, MDM were infected with various MOI of viral particles for Ad-GFP, Ad-AAA-O3a, and Ad-DN-FOXO3a. Seventy-two hours after infection, cell viability was determined by MTT assay; results shown were performed in triplicate and normalized as a percentage of GFP vector control. **, p < 0.05, ***, p < 0.001 compared with GFP vector control. D, At 2000 MOI of adenovirus, after 48 h of infection, apoptosis was determined by DNA fragmentation apoptosis ELISA. Results were representative of experiments performed in triplicate and normalized as percentage of control. **, p < 0.01 compared with GFP control vector. NS denotes no statistical difference from GFP control. E, Hoechst staining was performed to detect nuclear condensation after delivery of FOXO3a to MDM. F, Positive cells with nuclear condensation were counted every 200 cells per well with triplicate. **, p < 0.01 compared with GFP vector control. NS denotes no statistical difference from GFP control. G, After 72 h of adenoviral infection, human MDM were harvested and apoptosis was determined by PI staining and subsequent flow cytometry analysis. Numbers appearing in the upper right corner are percentage of PI-positive cells in GFP-positive cells. Results are representative of three independent experiments.

MDM by all the viral strains tested (data not shown). To assess whether the inhibition of FOXO3a phosphorylation is in transcription level, we examined the mRNA expression of FOXO3a after HIV-1 infection. There were no significant changes in the mRNA levels of FOXO3a following HIV-1 infection (data not shown), suggesting the decrease of FOXO3a phosphorylation occurred posttranscriptionally. Among all HIV-1 strains tested, HIV-1ADA represented typical HIV-1 infection and was used thereafter.

Next, we examined phosphorylation of FOXO3a over time during HIV-1 infection. Phosphorylated FOXO3a decreased in a time-dependent manner following HIV-1 infection (Fig. 2A). Densimetric quantification determined that phosphorylation of FOXO3a was reduced to 60% of control MDM 3 days after infection and to 23% 5 days after infection (Fig. 2B). Furthermore, we infected MDM at different MOIs of HIV-1, and quantitated FOXO3a phosphorylation by Western blotting (Fig. 2C). The infection levels by different MOIs of HIV-1 were monitored by RT activity assay, which showed a dose-dependent increase of RT activities as the MOI increased (Fig. 2D). Densimetric quantification showed a dose-dependent decrease of FOXO3a phosphorylation as HIV-1 infection levels increased (Fig. 2D). We observed a significant negative correlation between RT value and the ratio of p-FOXO3a to FOXO3a in HIV-1-infected MDM (Fig. 2F, $r^2 = 0.7171$), suggesting the inhibition of FOXO3a phosphorylation is dependent on the levels of HIV-1 infection. Together, these results indicate HIV infection decreases the phosphorylation of FOXO3a, an effect that is dependent on HIV-1 replication.

HIV-1 infection causes nuclear translocation of FOXO3a

Phosphorylation of amino acids tyrosine 32, serine 253, and serine 315 of FOXO3a retains FOXO3a in the cytosol; loss of phosphorylation at those sites causes nuclear translocation of FOXO3a (28). To confirm decreased FOXO3a phosphorylation promotes nuclear translocation of FOXO3a, we immunostained MDM with or without HIV-1 infection and determined the subcellular localization of FOXO3a by confocal microscopy. p24 Ab against HIV-1 viral Ag was used to differentiate infected cells from noninfected cells. In control and AZT-treated cells, HIV-1 was not present or unable to replicate thus p24 staining was absent (Fig. 3, A, I, and M). In these cells, the majority of FOXO3a staining was localized in the cytoplasm (Fig. 3, B, J, and N). In HIV-1-infected MDM, there was staining of FOXO3a in the nucleus with minimal FOXO3a detected in the cytoplasm (Fig. 3, E–H). These results indicate HIV infection increases FOXO3a nuclear translocation. Furthermore, when treated with AZT, the nuclear translocation was blocked, confirming the translocation of FOXO3a was reliant upon HIV-1 replication (Fig. 3, M–P).
We also used subcellular fractionation as another approach to study the nuclear translocation of FOXO3a. Whole cells, cytoplasm, and nuclear protein lysates were prepared 72 h postinfection. Levels of phospho-FOXO3a and total FOXO3a, along with their corresponding loading control, were determined by Western blotting. The phosphorylation of FOXO3a decreased in a manner consistent with Fig. 1 in HIV-1-infected MDM in the whole cell lysates. In the subcellular fractions, the phosphorylation of FOXO3a decreased in the cytoplasm, whereas nuclear FOXO3a increased in HIV-infected MDM (Fig. 4A). After densitometric quantification of the Western blotting, we found HIV-1 infection significantly decreased phosphorylation of FOXO3a in the cytosolic fractions, while it increased the amount of total FOXO3a in nuclear fractions (Fig. 4, C and D). Decreased phosphorylation of FOXO3a in the cytosol and increased total FOXO3a in the nucleus was blocked by AZT treatment, suggesting this effect is reliant on HIV-1 replication (Fig. 4B). Together, these data further confirmed HIV-infection inhibited phosphorylation of FOXO3a, leading to FOXO3a translocation to the nucleus.

Constitutively active FOXO3a induces cell death in MDM

To investigate the function of FOXO3a in MDM, we used an adenoviral gene-delivery system to manipulate the activity of FOXO3a in MDM. Constitutively active FOXO3a (AAA-FOXO3a) delivered by adenovirus (Ad-AAA-FOXO3a) was used to increase activity of FOXO3a. The constitutively active FOXO3a was modified from native FOXO3a by mutating all three phosphorylation sites to alanine. Ad-DN-Foxo3a (DN-FOXO3a) was constructed by deletion of the transactivation domain from the C terminus of FOXO3a. Thus, DN-FOXO3a can competitively inhibit the regulatory function of the endogenous FOXO3a. GFP adenovirus was used as a vector control in all adenoviral infections. After 48 h of adenoviral infection, the infection efficiency was monitored by fluorescence microscopy (Fig. 5A). At 2000 MOI, the infection efficiency was ~70% for each adenovirus. FOXO3a overexpression in Ad-AAA-03a and Ad-DN-03a was confirmed by Western blotting (Fig. 5B), which also showed the endogenous FOXO3a of Ad-GFP group, 98 kDa of AAA-03a, and 40 kDa of DN-03a. We also examined Puma, one of possible downstream factors of FOXO3a. After infection with constitutively active FOXO3a for 72 h, the expression of Puma is increased as compared with the GFP vector control, whereas there are no significant changes in FOXO3a in the nucleus infected with Ad-DN-Foxo3a (Fig. 5B). This result indicated an induction of downstream factor Puma by FOXO3a overexpression in human MDM.

Next, cell viability and apoptosis were assessed by MTT, DNA fragmentation ELISA, nuclear condensation, and flow cytometry followed by adenoviral infection. Cell viability in AAA-FOXO3a cells was significantly decreased in a dose-dependent manner as compared with Ad-GFP and Ad-DN-FOXO3a (Fig. 5C). DNA fragmentation in Ad-AAA-FOXO3a dramatically increased after 48 h (Fig. 5D) and, consequently, nuclear condensation and nuclear fragmentation also increased (Fig. 5, E and F). Flow cytometric analysis showed PI-positive cells also dramatically increased following Ad-AAA-FOXO3a infection (Fig. 5G). These
First, we assessed FOXO3a expression 72 h after siRNA transfection, and found the FOXO3a protein was reduced ~70% by FOXO3a siRNA. Compared with nonspecific siRNA (Fig. 6, C and D), knockdown of FOXO3a by siRNA decreased DNA fragmentation by 50% in HIV-infected MDM (Fig. 6E). FOXO3a knockdown partially prevented the decreased cell viability in HIV-1-infected MDM (Fig. 6F). These results demonstrated that DN FOXO3a and FOXO3a siRNA knockdown prevented HIV-induced macrophage death, suggesting HIV-1-induced macrophage death is at least in part via FOXO3a transcription factor.

**Reduced phosphorylation of FOXO3a in HIV-1-infected macrophages is mediated by inhibition of Akt-1**

FOXO3a has been reported to be an important transcription factor downstream of PI3K/Akt-1 in various cell types (27). To test whether FOXO3a is a downstream factor of PI3K/Akt-1 pathway in MDM, PI3K inhibitor LY294002 was used at 0.5 and 5 μM to treat MDM (Fig. 7A). Phosphorylation of FOXO3a dramatically decreased after 2 h of both 0.5 or 5 μM LY294002 treatment. These results demonstrated that the PI3K pathway is necessary for FOXO3a phosphorylation in human macrophages.

To further confirm FOXO3a regulation by the PI3K pathway, we delivered constitutively active Akt-1 with an adenoviral vector (Ad-myr-Akt) to control and HIV-1-infected MDM. First, we measured Akt-1 expression through Western blotting after 72 h of infection with Ad-myr-Akt and confirmed Akt-1 was overexpressed (Fig. 7B). Then, we tested the phosphorylation of FOXO3a with or without Ad-myr-Akt infection. Densitometrical quantification of FOXO3a phosphorylation were shown in Fig. 7C. Compared with Ad-GFP vehicle control, HIV-1 infection reduced the phosphorylation of FOXO3a by 30%. Ad-myr-Akt infection increases the phosphorylation of FOXO3a in both the control and HIV-1-infected MDM (p < 0.05) (Fig. 7C). More importantly, there was no significant decrease of phosphorylated FOXO3a in HIV-1-infected MDM after Ad-myr-Akt infection. These results further demonstrated that HIV-1 reduced phosphorylation of FOXO3a via down-regulating the PI3K/Akt-1 pathway and FOXO3a is a downstream factor of Akt-1 in human MDM.

**Discussion**

Eliminating HIV reservoirs and combating long-term viral persistence is critical to the treatment of AIDS (30–32). In the present study, the regulation and function of FOXO3a in HIV-induced macrophage cell death was investigated. First, we demonstrated that phosphorylation of FOXO3a was decreased in HIV-1-infected macrophages facilitating FOXO3a translocation to the nucleus (Figs. 1–4). Second, we applied a gene delivery system implementing adenoviral delivery of constitutively active FOXO3a. This rFOXO3a contains three mutated phosphorylation sites maintaining a transcriptionally active FOXO3 and was found to induce apoptosis in macrophages (Fig. 5). Third, both DN FOXO3a and FOXO3a knockdown with siRNA prevented macrophages from HIV-induced cell death (Fig. 6). Fourth, the PI3K inhibitor LY294002 inhibited FOXO3a phosphorylation, whereas constitutively active Akt-1 increased FOXO3a phosphorylation (Fig. 7). Together, these results suggest HIV infection increases the activity of transcription factor FOXO3a, and elevated FOXO3a activity promotes HIV-1-infected macrophage cell death. Although FOXO3a has been shown to influence cell death signaling, to our knowledge, we are the first to report that FOXO3a is a pivotal transcription factor involved in HIV-1-induced human macrophage death.

The physiological relevance of FOXO3a-mediated macrophage death is significant because a large number of macrophages in the brain and lung are infected with HIV-1 during late stage disease.
Clearance of activated or HIV-1-infected macrophages from inflamed tissue is important to the host-mediated immune response. Understanding the signaling pathways regulating these processes, such as Akt-1 inhibition and FOXO3a activation, provides important information about macrophage survival and is relevant to HIV-1 pathogenesis, and could potentially identify therapeutic avenues for the treatment of HIV-1 infection.

Forkhead transcription factors play key roles in immunoregulation. Members of the FOXO subfamily have been implicated in the regulation of the cell cycle and/or apoptosis. However, the function of FOXO3a in peripheral immune cells, particularly in macrophages, is not clear. Gene knockout of FOXO3a led to spontaneous lymphoproliferation associated with inflammation of several organs in mice, suggesting FOXO3a regulates homeostasis of immune cells (19, 20). Indeed, FOXO3a appears to be a critical factor regulating apoptosis in lymphocytes (33–35). Our results support a similar role for FOXO3a in human macrophages. Exogenously expressed FOXO3a induced apoptosis in human macrophages and was found to significantly reduce viability of macrophages in vitro (Fig. 5).

In mammals, FOXO3a contains a highly conserved motif that is posttranslationally modified by phosphorylation. PI3K/Akt activation is the major kinase for phosphorylation of FOXO3a on T32, S253, and S315 or on homologous sites in other family members (29, 36, 37). Akt-1-mediated phosphorylation of FOXO proteins leads to cytoplasmic retention and impairment of nuclear transcription (38–40). Our data demonstrated an increase of nuclear translocation of FOXO3a by HIV-1 infection (Figs. 3 and 4). We demonstrated that inhibition of the PI3K/Akt pathway caused a decrease of FOXO3a phosphorylation in human macrophages and overexpression of constitutive Akt-1 blocked the HIV-1-mediated decrease of FOXO3a phosphorylation (Fig. 7). Notably, phosphorylated Akt-1 is also critical for macrophage survival (41). We recently demonstrated that Akt-1 is inhibited by HIV-1 infection and this inhibition contributes significantly to TRAIL-induced apoptosis (17). Together, these observations suggest the decrease of FOXO3a phosphorylation is due to inhibition of Akt-1 after HIV-1 infection. Although downstream factors of Akt-1 inhibition in our model are not clear, our study suggests the Akt-1-related signaling pathway plays a critical role in macrophage survival and is relevant to HIV-1 pathogenesis.

Akt-1-associated pathways and signaling cross-talk between Akt-1 and different apoptotic molecules remain a complex story. Recent studies have identified interactions between molecules downstream of Akt-1 and apoptosis pathways (see review in Ref. 42). For example, phosphorylation of BCL2-antagonist of cell death at Ser136 by phospho-Akt-1 prevents mitochondrial translocation of BCL2-antagonist of cell death (43, 44); phospho-Akt-1 phosphorylates procaspase-9 at Ser196 (45); phosphorylation of the Forkhead family of transcription factors by phospho-Akt-1 leads to decreased transcriptional activity for proapoptotic genes including Fas, TRAIL, Bim, or Puma (46–48). Furthermore, Akt-1 phosphorylation activates NF-κB, a transcription factor promoting the transcription of many essential survival genes (49). In our study, we demonstrated the inhibition of FOXO3a phosphorylation during HIV-1 infection of human macrophage is at least in part responsible for HIV-1-mediated cell death. Thus, FOXO3a appears to be one downstream factor of Akt-1 mediating apoptosis in HIV-1-infected macrophages.

HIV-gp120 has been reported to up-regulate the PI3K pathway and, thus, enhanced viral replication (50). We observed phosphorylation of Akt-1 decreased following 3 days of HIV-1 infection in human macrophages (17). Activation of FOXO3a by productive HIV-1 infection further supports this earlier observation. Variation in Akt-1 phosphorylation status is likely due to the state of HIV-1 infection. In addition, although we found HIV-1-infection decreased Akt-1 phosphorylation, and concurrently inhibited FOXO3a phosphorylation, there are other kinases that regulate the phosphorylation and translocation of FOXO3a, such as serum/glucocorticoid regulated kinase and dual-specificity regulated kinase 1A. Among those kinases, only phosphorylation of the Akt site in the forkhead domain on FOXO3a protein is indispensable for FOXO3a translocation (38). The exact roles of other kinases involved in the FOXO3a regulation require further investigation. How downstream factors of FOXO3a are involved in HIV-1-mediated macrophage death is also an interesting and important topic. FOXO3a has been reported to induce proapoptotic signaling by regulating the expression of TRAIL, FasL, Bim, and Puma (23, 48, 51), and regulates cell cycle arrest by activation of p27, p130, and p21, repression of cyclin D expression (G1/S arrest) (29, 52), or activation of cyclin G2 (G2/M arrest) (53). Intriguingly, recent reports suggest FOXO3a also promotes cell type-specific antioxidant stress of manganese superoxide dismutase, catalase, and growth arrest and DNA damage inducible gene 45 in addition to promoting apoptosis (29, 54). Our study supports the role of FOXO3a in the induction of Puma in human MDM (Fig. 5B), and this would likely shift the cellular equilibrium of survival-apoptosis, and promote an apoptotic phenotype in macrophage. The specific mechanism by which Puma participates in FOXO3a- or HIV-1-mediated macrophage apoptosis is certainly interesting and remains further investigation.

In summary, the evidence presented in this study demonstrates that HIV-1 infection inhibits the phosphorylation and increases the activity of transcription factor FOXO3a activation, resulting in cell death of human macrophages. Our study may help unveil the mechanism of macrophage cell death in HIV-1 pathology, which facilitates the elimination of HIV-1 reservoirs during disease, making this pathway a potential therapeutic target during HIV-1 infection.

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