Constitutive Activation of Phosphatidylinositol 3-Kinase Signaling Pathway Down-regulates TLR4-mediated Tumor Necrosis Factor-α Release in Alveolar Macrophages from Asymptomatic HIV-positive Persons in Vitro*

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Alveolar macrophages represent critical effector cells of innate immunity to infectious challenge in the lungs and recognize bacterial pathogens through pattern recognition receptors such as Toll-like receptors (TLRs). Phosphatidylinositol 3-kinase (PI3K) regulates TLR-mediated cytokine release, but whether HIV infection influences PI3K signaling pathway and alters TLR4-mediated macrophage response has not been investigated. In the current study, surface TLR4 pathway and activates TLR4-mediated macrophage response has not been investigated. In the current study, surface TLR4 expression were similar but TLR4 activation (lipid A, 10 μg/ml) resulted in lower TNF-α release by HIV+ human macrophages compared with healthy cells. Pharmacological inhibition of PI3K (LY294002) normalized TNF-α release in HIV+ macrophages and augments ERK1/2 mitogen-activated protein kinase phosphorylation in response to lipid A. Importantly, HIV+ macrophages demonstrated increased constitutive phosphatidylinositol 3,4,5-trisphosphate formation, increased phosphorylation of downstream signaling molecules Akt and glycogen synthase kinase-3β (GSK-3β) at Ser9, and reduced PTEN protein expression. As a functional assessment of GSK-3β phosphorylation, TLR4-mediated interleukin-10 release was significantly higher in HIV+ human macrophages compared with healthy cells. Incubation of human macrophages with exogenous HIV Nef protein induced phosphorylation of Akt and GSK-3β (whereas phosphorylation was reduced by PI3K inhibition) and promoted interleukin-10 release. Taken together, these data demonstrate increased constitutive activation of the PI3K signaling pathway in HIV+ macrophages and support the concept that PI3K activation (by HIV proteins such as Nef) may contribute to reduced TLR4-mediated TNF-α release in HIV+ human macrophages and impair host cell response to infectious challenge.

Respiratory tract infections remain frequent and serious complications in persons with chronic human immunodeficiency virus (HIV)² infection. HIV+ persons have up to a 25-fold greater risk of bacterial pneumonia compared with the general population (1), but the underlying mechanisms contributing to this exceptional high rate are not well understood. Importantly, HIV-infected individuals remain at an elevated clinical risk for bacterial pneumonia despite relatively preserved peripheral blood CD4+ T-lymphocyte counts (2), which suggests the possibility that dysfunction of other components of immunity (3) may contribute to the pathogenesis of pneumonia. Although the recovery of CD4+ T-lymphocytes associated with highly active antiretroviral therapy (HAART) may reduce the risk of opportunistic infections (4), the use of HAART may not be associated with a significant reduction in the risk for common bacterial pneumonia (5). These observations strengthen the notion that other factors may contribute to pneumonia pathogenesis in HIV+ persons.

AM are the most abundant immune cells in the alveolar air space and represent critical effector cells in the innate immune response to infectious challenge in the lungs, including bacterial pathogens (6). AM constitutively express a number of surface receptors involved in pathogen recognition, including Toll-like receptors (TLRs) for Gram-positive bacteria (example, TLR2) and Gram-negative bacteria (example, TLR4) (7). TLR activation by bacterial products triggers intracellular signaling cascades that activate antimicrobial pathways (such as reactive oxygen species) and host defense genes (including pro-inflammatory cytokines such as TNF-α) that promote pathogen control and elimination of invading bacteria (8). HIV-1 can infect AM (9) and may result in specific defects in macrophage innate immune function such as mannose receptor-mediated phagocytosis (10) and NF-κB activation in response to Pneumocystis (11) and impairs TNF-α mediated macrophage apoptotic response to Mycobacterium tuberculosis (12). Recent investigation of AM from HIV+ persons demonstrated significantly reduced TNF-α release in response to TLR4 stimula-

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² The abbreviations used are: HIV, human immunodeficiency virus; AM, alveolar macrophages; PI3K, phosphoinositide 3-kinase; PTEN, phosphatase and tensin homologue deleted on chromosome 10; GSK3-β, glycogen synthase kinase 3-beta; TLR4, toll-like receptor 4; ERK, extracellular signal-regulated kinase; TNF, tumor necrosis factor; MAP, mitogen-activated protein; UL-10, interleukin-10; ELISA, enzyme-linked immunosorbent assay; PIP₃, phosphatidylinositol 3,4,5-trisphosphate; siRNA, small interfering RNA.
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PI3K is a member of the lipid kinase family and elicits TLR-mediated signaling. PI3K activation links to downstream intracellular signaling events that influence innate immune responses including cytoskeletal reorganization and inflammatory gene expression (14). Recognition of pathogen-associated molecular patterns by TLRs can activate PI3K, which in turn activates Akt, a key effector of the PI3K pathway (15). In contrast to the activating role that PI3K/Akt play in immune receptor signaling, these enzymes have been shown to inhibit lipopolysaccharide-induced activation of MAP kinases and NF-κB driven gene transcription (16). In addition, activated Akt phosphorylates glycogen synthase kinase-3β (GSK-3β), resulting in GSK-3β inactivation and a dampened pro-inflammatory immune response mediated by enhanced expression of anti-inflammatory cytokines IL-10 and transforming growth factor-β (17, 18), thus suppressing the TLR-mediated inflammatory response (19). However, whether the PI3K signaling pathway is intact in HIV+ AM and the determination of its role in regulating TLR4-mediated signaling events have not been previously investigated. Using human macrophage cell lines and clinically relevant human AM, we show that reduced TLR4-mediated TNF-α release in AM from HIV+ persons at risk for bacterial pneumonia is attributable to increased constitutive activation of PI3K signaling pathway. Importantly, reduced TLR4-mediated TNF-α release in HIV+ macrophages can be partially restored by inhibiting PI3K, identifying PI3K as a potential pharmacological target in restoring macrophage innate immune response in HIV+ persons.

EXPERIMENTAL PROCEDURES

Study Subjects—Healthy and asymptomatic HIV+ individuals were recruited without evidence of active pulmonary disease and with normal spirometry. Healthy individuals were without known risk factors for HIV infection and were confirmed to be HIV-negative by ELISA, performed according to the manufacturer’s instructions (Abbot Diagnostics, North Chicago, IL). Demographic characteristics for all participants were recorded on standardized forms and included age, gender, smoking status, HIV risk factor, medical history, and prescribed antiretroviral medications.

Reagents—Lipid A (the biologically active component of lipopolysaccharide, and specific TLR4 ligand) from Escherichia coli F583 Rd mutant, protease inhibitor mixture, phorbol myristic acid, and SB16763 were purchased from Sigma, and wortmannin and LY294002 was purchased from Calbiochem. Recombinant Nef was from Trinity Biotech (Dublin, Ireland) and from the National Institutes of Health AIDS Research and Reference Reagent Program (Bethesda, MD).

Antibodies—Anti-TLR4, anti-phospho-GSK-3β, anti-phospho-ERK1/2, and anti-ERK1/2 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), anti-phospho-Akt, anti-Akt, and anti-PTEN antibodies were purchased from Cell Signaling (Beverley, MA), cytokine ELISA kits were from R & D (McKinley Place, Minneapolis, MN), and anti-β-actin antibody was from Sigma.

Macrophage Cell Lines—Macrophages were differentiated from human promonocytic U937 (American Type Culture Collection) and HIV-infected U1 cell lines (AIDS Research and Reference Reagent Program; Bethesda, MD). U1 cells (HIV-infected subclone of U937 cells) contain two integrated copies of HIV-1 proviral DNA and are characterized by low levels of constitutive virus expression that can be modulated by cytokines and pharmacological agents (20). For experiments, U937 and U1 cells were harvested during exponential growth phase, washed, and then incubated in complete medium (RPMI 1640 containing 10% heat-inactivated fetal calf serum (fetal calf serum), 2 mM glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. To allow differentiation to macrophages, U937 and U1 cells were incubated with 100 nM phorbol myristic acid at 37 °C in a humidified atmosphere containing 5% CO₂ for 24 h. Adherent cells were then washed 3× with phosphate-buffered saline (to remove phorbol myristic acid) and incubated in complete media (without phorbol myristic acid) for an additional 24 h before use in experiments.

Human Alveolar Macrophages—To determine clinical relevance of the study, select experiments were performed using human AM. Recruited healthy and asymptomatic HIV+ individuals were without evidence for active pulmonary disease and had normal spirometry. Healthy individuals were confirmed to be HIV-seronegative by ELISA and had no known risk factors for HIV infection. For the HIV+ subjects, peripheral blood CD4 lymphocyte counts were >200 cells/mm³, and HIV risk factors included intravenous drug use and homosexual exposures. All were prescribed highly active antiretroviral therapy, all had undetectable serum viral load (<50 HIV-1 RNA copies/ml), and none experienced a prior opportunistic pneumonia. Using standard techniques, bronchoalveolar lavage was performed to obtain lung immune cells (21). All procedures were performed on consenting adults following protocols approved by Beth Israel Deaconess Medical Center institutional review board and the Committee for Clinical Investigations. Cells were cultured for 24–48 h, and non-adherent cells were washed away followed by the addition of fresh media before experimentation (13).

Western Blot Analyses—Adherent human macrophages were washed 2× with ice-cold phosphate-buffered saline (pH 7.4). Cells were lysed in lysis buffer containing 25 mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% Nonidet P-40, 1% sodium deoxycholate, 0.1% SDS, and protease inhibitor mixture (Sigma) and placed on ice for 20 min (22). Cells were harvested by scraping followed by centrifugation at 4 °C for 15 min at 14,000 rpm. Equal amounts of cell lysates were subjected to SDS/PAGE and Western blot analysis with designated antibodies and detected by enhanced chemiluminescence (ECL) detection system (Amersham Biosciences). Resolved bands were quantified by densitometry (Amersham Biosciences).
PI3K Assay and Quantitation of Phosphatidylinositol 3,4,5-Trisphosphate (PIP₃) Formation—PI3K enzymatic assay and PIP₃ formation were measured using a PI3K ELISA kit following the manufacturer’s instructions (Echelon Biosciences, Salt Lake City, UT). In brief, cell lysates (500 µg) were immunoprecipitated with anti-p85 antibody and incubated with phosphatidylinositol 4,5-bisphosphate substrate and incubated for 1 h at room temperature. Enzymatic reactions were stopped by centrifugation and were incubated with PIP₃ detector and incubated at room temperature for 1 h. After incubation, reacted mixtures were transferred to the detection plate and incubated for 1 h at room temperature. The plate was washed 3 times with Tris-buffered saline-Tween 20, immediately secondary detection was added, and the color was allowed to develop for 1–15 min in the dark. Color reaction was stopped with the addition of 1 N H₂SO₄ stop solution. Absorbance was recorded at 450 nm, and the enzyme activity was interpolated from the standard curve generated following the manufacturer’s instructions.

Small Interfering RNA (siRNA)-mediated Knockdown in AM—To determine the functional relevance of PI3K pathway in TLR4 signaling, RNA-mediated interference-mediated knockdown of PTEN was performed using synthetic duplex RNA oligonucleotides. We used On-Target plus SMART pool siRNA PTEN (catalog #L-003023-00, Dharmacon, Boulder, CO). Target sequences were GACCUAGACUUGACCUAUA, GAUCGCAUCACAAAUUA, GAUCUGACCGAUGCUGUA, and CGAUAAGCUUUGCAGUAU. On-target plus non-targeting siRNA were used as controls (catalog #D-001810-01-05, Dharmacon). The target sequence was UGGUUUAACUUGCAGUAA. Macrophages were electroporated with 100 nM siRNA using Amazxa system following the manufacturer’s protocol (Amazxa GmbH, Cologne, Germany). PTEN siRNA-mediated knockdown was determined by Western blot probed with anti-PTEN antibody 24–48 h after transfection.

Flow Cytometry Analysis—Cell surface expression of TLR4 was determined by Epics XL flow cytometer (Beckman Coulter, Miami, FL) with a laser power of 5.76 milliwatts. The instrument was calibrated before each measurement with standardized fluorescent particles (Immunocheck; AMAC, Inc. Westbrook, ME). Fluorescent signals of the cells were measured simultaneously by three photomultiplier tubes and optical filters and shown as the mean of the log fluorescence intensity of simultaneously by three photomultiplier tubes and optical filters. Cells were then fixed in Optlyse (Beckman/Coulter, Miami, FL) at room temperature for 5–10 min, and analyzed by flow cytometry. Human macrophages were first identified by the characteristic forward and side scatter parameters on unstained cells and confirmed by staining with phosphatidylethanolamine-conjugated primary anti-human HLA-DR (Beckman Coulter). Data were expressed as a mean relative fluorescence units and the percentage of cells staining positive. Isotype primary conjugated antibodies served as a negative control. Samples were prepared and analyzed in duplicate, and a minimum of 5000 cells was counted for each sample.

ELISA—Cultured supernatants were collected, centrifuged to remove cellular debris, and assayed immediately or stored at −80 °C until assayed. Cytokine measurements were performed using commercially available ELISA (R&D Systems, Minneapolis, MN) following the manufacturer’s instructions, and absorbance was measured at 450 nm on a Bio kinetic Elisa reader (Bio-Tek Instruments; Winooski, VT). The detection limit for TNF-α was 4.4 pg/ml. All measurements were performed in duplicate, and the mean values of the two measurements were used for statistical analysis.

Statistical Analysis—Group comparisons were performed using Student’s t test (two sample tests) or one-way analysis of variance. Calculations were performed with StatView (SAS Institute, Inc; Cary, NC) and INSTAT2 (GraphPad Software, San Diego, CA) software package. Results are given as the mean ± S.E. Statistical significance was accepted for p < 0.05.

RESULTS

Inhibition of PI3K Is Associated with Enhanced TLR4-mediated TNF-α Release in HIV+ Macrophages—TLR4-mediated TNF-α release in HIV+ AM is reduced, and the mechanism was in part due to constitutive activation of MAP kinase phosphatase-1 (13). To determine the influence of other signal transduction pathways, we examined TLR4-mediated PI3K activation in these cells. PI3K activation is known to limit the release of pro-inflammatory cytokines from monocytes and macrophages (19, 23), but whether PI3K signaling influences TLR-mediated TNF-α release in HIV+ AM has not been established. Human macrophage U937 cells exhibited robust TNF-α release in response to lipid A, whereas this response in HIV+ U1 macrophages is severely reduced (13) (Fig. 1A, left panel). This reduction was not attributable to lower surface TLR4 expression comparing these two cell lines (Fig. 1A, right panel). Such invariance also exists between clinically relevant human alveolar macrophages obtained from healthy persons and asymptomatic HIV+ individuals (Fig. 1B, left and right panels). In separate experiments preincubation of human macrophage U937 cell lines with a PI3K inhibitor (50 µM LY294002) did not influence TLR4-mediated TNF-α release, whereas preincubation of HIV+ U1 macrophages with the PI3K inhibitor increased TLR4-mediated TNF-α release (Fig. 1C). A similar pattern was also observed in AM obtained from healthy and asymptomatic HIV+ persons (Fig. 1D). These results suggest that constitutive PI3K activation may in part account for the reduced TLR4-mediated TNF-α release in HIV+ macrophages as TNF-α release is restored in the presence of PI3K inhibitor.

PI3K Signaling Pathway Is Constitutively Active in HIV+ Macrophages—PI3K is a lipid kinase that induces the transfer of phosphate group to the D-3 position of myoinositol ring of phosphoinositides that leads to the formation of PIP₃. PIP₃ is a critical second messenger that targets the downstream molecule Akt and influences innate immune responses such as inflammatory gene expression (14). Using PIP₃ formation as a measure of PI3K activity, levels of PIP₃ were detected in unstimulated human U937 macrophages and significantly increased in response to TLR4 agonist, lipid A (Fig. 2A). In
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Contrast, constitutive release of PIP₃ was higher in unstimulated HIV+ U1 macrophages compared with unstimulated U937 cells, increased significantly in response to lipid A, and returned to unstimulated conditions in the presence of LY294002 (Fig. 2A). As another measure of PI3K activity, Akt phosphorylation was elevated in unstimulated HIV+ human AM and further increased in response to TLR4 agonist and returned to a basal state in the presence of LY294002 (Fig. 2B). PTEN is a major lipid phosphate antagonist of PI3K signaling pathway. PTEN dephosphorylates PIP₃, converting to phosphatidylinositol 4,5-bisphosphate. Thus, PTEN antagonizes the effect of PI3K, favoring the accumulation of Akt over phospho-Akt and suppressing phosphorylated Akt formation leading to increased activation of PI3K (24). Because overexpression of HIV tat protein into human fetal microglial cell line decreases expression of PTEN (25), we hypothesized that PTEN levels would be reduced in HIV+ macrophages. This is in agreement with our finding that constitutive PTEN protein expression is lower in AM from asymptomatic HIV persons compared than those obtained from healthy persons (Fig. 2C). Taken together these findings support the concept of constitutive PI3K activation (as measured by elevated levels of PIP₃, increased constitutive phosphorylation of Akt, and lower levels of PTEN) in HIV+ macrophages) and suggest that elevated PI3K activity may contribute to reduced TLR4-mediated TNF-α release in HIV+ macrophages.

Enhanced Constitutive GSK-3β Phosphorylation in HIV+ Macrophages—GSK-3β is an important downstream signaling molecule of the PI3K signaling pathway. Akt-mediated phosphorylation of GSK-3β on serine 9 (26) results in GSK-3β inactivation and TLR4-mediated suppression of pro-inflammatory cytokine release while promoting anti-inflammatory cytokine release such as IL-10 (18). In the current study, constitutive phosphorylation of GSK-3β in HIV+ AM was increased compared with healthy AM and was reduced in the presence of GSK-3β inhibitor (Fig. 3A). Phosphorylation of constitutively active GSK-3β at position Ser9 results in inhibition of GSK-3β and, consequently, an increase in TLR4-mediated IL-10 release (18). Consistent with enhanced GSK-3β phosphorylation in HIV+ alveolar macrophages, TLR4-mediated IL-10 release was higher in alveolar macrophages from asymptomatic HIV+ persons compared with alveolar macrophages from healthy individuals (Fig. 3B). These data demonstrate enhanced GSK-3β phosphorylation and enhanced TLR4-mediated IL-10 release (a functional consequence of enhanced GSK-3β phosphorylation) and are consistent with increased constitutive PI3K activation in AM from HIV+ persons.

PI3K Attenuates TLR4-induced TNF-α Release by Limiting ERK1/2 MAP Kinase Activation in HIV+ Macrophages—Ligation of TLR4 by lipopolysaccharide induces an immune response through activation of MAP kinases (27), with subsequent release of critical host defense molecules such as TNF-α in vitro (28). We have shown that ERK1/2 MAP kinase activity is down-regulated in HIV+ macrophages with concomitant down-regulation of TNF-α in response to TLR4 activation (13). To investigate the role of PI3K activity on TLR4-mediated TNF-α release in HIV+ macrophages, we examined the effects
of PI3K inhibitor LY294002 on ERK1/2 MAP kinase phosphorylation in U1 cells in response to TLR4 activation. We observed that pretreatment of U1 cells with LY294002 resulted in markedly enhanced and prolonged phosphorylation ERK1/2 MAP kinase in response to lipid A (Fig. 3C). Such enhanced ERK1/2 activity was noted at 15 min, appeared maximal at 30 min, and gradually declined by 60 min after lipid A treatment, consistent with enhancement of lipid A–induced TNF-α release in HIV+ macrophages in the presence of PI3K inhibitor (Figs. 1, C and D). In contrast to its effect on ERK1/2 augmentation, LY294002 treatment did not affect phosphorylation of p38 MAP kinase, indicating that PI3K blockade did not affect p38 MAP kinase within this time frame (data not shown). These results suggest that blockade of PI3K results in augmented and prolonged ERK1/2 MAP kinase phosphorylation in HIV+ macrophages.

**Gene Silencing of PTEN Results in Diminished TNF-α Release in Response to Lipid A in HIV+ Macrophages—**Consumption of the 3-phosphorylated product of PI3K resulted from an increase in inositol phosphatase activity of PTEN (3). To determine whether PTEN regulates TLR4-mediated TNF-α release, functional assays were performed using targeted functional gene silencing of PTEN. A reduced PTEN level was demonstrated in human AM after PTEN siRNA gene silencing by ∼50% (Fig. 4A). TLR4-mediated TNF-α release was robust in healthy AM with non-silencing siRNA, whereas in PTEN-silenced cells, the lipid A–mediated TNF-α release was significantly diminished (Fig. 4B). Consistent with these data in clinically relevant human AM, similar results were observed in our model U937 human macrophages (Fig. 4, C and D). After electroporation and siRNA transfection, replated macrophage cell viability was 76%, as determined by trypan blue dye exclusion (n = 2).

**HIV rNef Is Sufficient to Induce Akt Phosphorylation and GSK-3β Phosphorylation and Induce IL-10 Release in Human Macrophages—**HIV Nef protein was detected in the serum of HIV-infected subjects in the range of 5–10 ng/ml (29), and antibodies directed against Nef have been found in a large proportion of infected subjects (30). Exogenous Nef has been found to enter cells by adsorptive endocytosis after nonspecific binding to the surface of CD4 T-cells, primary macrophages, and U937 cells and to activate the signaling pathway such as STAT-1 in human monocyte macrophages (31). In vitro HIV infection promotes Akt recruitment to the plasma membrane leading to Akt phosphorylation in primary human macrophages (32). Furthermore, Nef has been shown to bind to the C terminus of p85 component of PI3K (33), and exogenous addition of Nef cul-

**Constitutive PI3K Activation in HIV+ Macrophages**

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Constitutive PI3K Activation in HIV+ Macrophages

To determine whether a viral-secreted protein is sufficient to induce the activation of PI3K signaling pathway, U937 cells were incubated with recombinant HIV Nef (rNef) protein and analyzed for PI3K induction. Exogenous HIV rNef promoted Akt phosphorylation in a dose-dependent manner, and Akt phosphorylation was inhibited in the presence of either PI3K inhibitors, LY294002, or wortmannin (Fig. 5A). Similarly, HIV rNef enhanced GSK-3β phosphorylation at Ser^9^ in a dose-dependent manner, and phosphorylation was inhibited by PI3K inhibitors (Fig. 5B), suggesting that the response is specific to PI3K activity. Furthermore, exogenous HIV rNef induced IL-10 release in human U937 macrophages, and HIV rNef-mediated IL-10 release was reduced in the presence of PI3K inhibitor (Fig. 5C). Importantly, rNef failed to induce the release of TNF-α when added alone, whereas when added in the presence of lipid A, rNef inhibits lipid A-induced TNF-α in a dose-dependent manner (Fig. 5D). Furthermore, we observed that Nef protein is expressed in HIV+ U1 cells but not in U937 cells (Fig. 5E). Taken together, these data show that HIV rNef is sufficient to induce phosphorylation of Akt and GSK-3β and promote IL-10 release but failed to induce the release of TNF-α. Furthermore, Nef is expressed in HIV+ U1 cells. These data support the concept that rNef may in part contribute to the reduced TNF-α release and enhanced IL-10 release observed in HIV+ macrophages following TLR4 stimulation.

**DISCUSSION**

In this study we demonstrated that the PI3K signaling pathway is constitutively active in HIV+ macrophages, and enhanced constitutive PI3K activation may in part contribute to reduced TLR4-mediated TNF-α release in alveolar macrophages from HIV+ persons. Up-regulated PI3K signaling pathway in HIV+ macrophages is supported by our finding that after pharmacological inhibition of PI3K in HIV+ macrophages, the effects of TLR4 activation on TNF-α release were normalized. Importantly, measurement of the PI3K signaling pathway showed that HIV+ macrophages demonstrated increased constitutive PI3K activation, increased constitutive phosphorylation of downstream signaling molecules Akt and GSK-3β, and reduced PTEN protein expression. As a functional assessment of GSK-3β phosphorylation, TLR4-mediated IL-10 release was significantly higher in HIV+ human macrophages compared with healthy cells. In addition, targeted gene silencing of U937 cells and AM PTEN reduced TLR4-mediated TNF-α release, suggesting that PTEN plays a role in modulating innate immune responses mediated by TLR4. Taken together, these data demonstrate increased constitutive activation of the PI3K signaling pathway in HIV+ macrophages and support the concept that PI3K activation may contribute to reduced TLR4-mediated TNF-α release in HIV+ human macrophages and impair host cell innate immune response to bacterial cell wall products.

The mechanism by which constitutively enhanced PI3K activity reduces TLR4-mediated TNF-α release in HIV+ macrophages appears in part due to regulation of GSK-3β activity. PI3K can be activated by TLRs, which in turn activates (phosphorylates) Akt, a key effector of the PI3K in alveolar macrophages (15). Activated Akt in turn phosphorylates GSK-3β, resulting in GSK-3β inactivation and a consequent dampened pro-inflammatory response (23). Our data show that HIV infection is associated with constitutive Akt phosphorylation and GSK-3β phosphorylation, which is associated with reduced TLR4-mediated release of TNF-α and enhanced IL-10 release in alveolar macrophages from asymptomatic HIV+ persons. Furthermore, inhibition of the PI3K pathway rescues TLR4-mediated ERK1/2 MAP kinase activity, which could lead in part to restoration of TLR4-mediated TNF-α release in HIV+ human macrophages.

Enhanced constitutive PI3K activity may in part be related to specific HIV proteins such as HIV Nef. Nef-mediated effects on cell survival (35) and blocked platelet-derived growth factor receptor-mediated signaling by interfering with PI3K (36). Direct association between Nef and the regulatory p85 subunit of PI3K induced the activation of p21-activated kinase (PAK) leading to increased production of HIV-1 (33). In the current study incubation of human macrophages with exoge-
nous HIV Nef protein induced phosphorylation of Akt and GSK-3β (whereas phosphorylation was reduced by PI3K inhibition), and promoted IL-10 release in response to TLR4 stimulation. In addition, the exogenous addition of rNef alone did not induce the release of TNF-α but impaired TNF-α release in response to lipid A. These data support the mechanism that HIV Nef may be sufficient to induce Akt phosphorylation and phosphorylation of GSK-3β at Ser8 and promote IL-10 release.

This is the first study to report that HIV Nef induces the phosphorylation of GSK-3β. As a consequence of phosphorylation, GSK-3β activity is inhibited, leading to diminished TLR4-mediated TNF-α release and enhanced IL-10 production. This finding is consistent with the report that exogenous Nef induces the production of IL-10 in U937 cells and peripheral blood mononuclear cells (34). In addition, this finding is consistent with the report that inhibition of GSK-3β stimulates IL-10 production (18). IL-10 has been shown to exert anti-inflammatory properties (37), which through a negative feedback mechanism deactivates macrophages (38, 39). IL-10 also reduces the formation of TNF-α in vivo (40) and the intensity of cellular recruitment at the lung level upon lipopolysaccharide challenge (41). The mechanism by which Nef induces phosphorylation and inactivation of GSK-3β is currently under investigation.

In the current study the results of in vitro experiments may not accurately reflect signaling pathways in vivo. However, the use of clinically relevant primary human AM may allow more direct translation to human disease. Although HIV Nef was sufficient to promote Akt and GSK-3β phosphorylation in human U937 macrophages, the role of other HIV proteins was not specifically investigated. Other limitations of the study include that other pathways and effector molecules downstream of PI3K activation were not specifically investigated, and whether the PI3K pathway dysregulation is limited to TLR4 or may be applicable to other TLRs was not determined.

In conclusion, the current study shows that AM from asymptomatic HIV+ persons exhibit constitutively activated PI3K signal transduction pathway, as evidenced by elevated levels of Akt phosphorylation and GSK3-β phosphorylation, reduced levels of PTEN, and enhanced IL-10 release in response to TLR4 stimulation. Enhanced PI3K activity may in part contribute to impaired TLR4-mediated TNF-α release, as PI3K inhibition restores TLR4-mediated TNF-α release in HIV+ macrophages. Taken together with recent studies, HIV infection is associated with impaired TLR4-mediated host cell response and may be the consequence of targeted activation of inhibitory intracellular pathways such as MAP kinase phosphatase-1 (13) and PI3K (this study). Furthermore, PI3K inhibition may represent a potential therapeutic target to restore the macrophage host cell response to bacterial product challenge in HIV+ persons.

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REFERENCES

1. Feikin, D. R., Feldman, C., Schuchat, A., and Janoff, E. N. (2004) *Lancet Infect. Dis.* 4, 445–455
2. Wallace, J. M., Hansen, N. I., Lavange, L., Glassroth, J., Brody, B. L., Rosen, M. J., Kvale, P. A., Mangura, B. T., Reichman, L. B., and Hopewell, P. C. (1997) *Am. J. Respir. Crit. Care Med.* 155, 72–80
3. Cao, X., Wei, G., Fang, H., Guo, J., Weinstein, M., Marsh, C. B., Ostrowski, M. C., and Trandadapini, S. (2004) *J. Immunol.* 172, 4851–4857
4. Kolber, M. A., Saenz, M. O., Tanner, T. J., Arheart, K. L., Pahwa, S., and Liu, H. (2008) *Clin. Immunol.* 126, 315–321
5. Benito, N., Rano, A., Moreno, A., Gonzalez, J., Luna, M., Agusti, C., Danes, C., Pumarola, T., Miro, J. M., Torres, A., and Gatell, J. M. (2001) *J. Acquired Immune Defic. Syndr.* 27, 35–43
6. Sibille, Y., and Reynolds, H. Y. (1990) *Am. Rev. Respir. Dis.* 141, 471–501
7. Maris, N. A., Dressing, M. C., de Vos, A. F., Bresser, P., van der Zei, J. S., Jansen, H. M., Spek, C. A., and van der Poll, T. (2006) *Eur. Respir. J.* 28, 622–626
8. Brightbill, H. D., and Modlin, R. L. (2000) *Immunology* 101, 1–10
9. Park, I. W., Koziel, H., Hatch, W., Li, X., Du, B., and Groupman, I. E. (1999) *Am. J. Respir. Cell Mol. Biol.* 20, 864–871
10. Koziel, H., Eichbaum, Q., Kruskal, B. A., Pinkston, P., Rogers, R. A., Armstrong, M. Y., Richards, F. F., Rose, R. M., and Ezekowitz, R. A. (1998) *J. Clin. Invest.* 102, 1332–1344
11. Zhang, J., Zhu, J., Imrich, A., Cushion, M., Kinane, T. B., and Koziel, H. (2004) *Infect. Immun.* 72, 3147–3160
12. Patel, N. R., Zhu, J., Tachado, S. D., Zhang, J., Wan, Z., Saukkonen, J., and Koziel, H. (2007) *J. Immunol.* 179, 6973–6980
13. Tachado, S. D., Zhang, J., Zhu, J., Patel, N., and Koziel, H. (2005) *Am. J. Respir. Cell Mol. Biol.* 33, 610–621.
14. Canteley, L. C. (2002) *Science* 296, 1655–1657
15. Monick, M. M., Carter, A. B., Robeck, P. K., Flaherty, D. M., Peterson, M. W., and Hunningleh, G. W. (2001) *J. Immunol.* 166, 4713–4720
16. Guha, M., and Mackman, N. (2002) *J. Biol. Chem.* 277, 32124–32132
17. Fukao, T., Tanabe, M., Terauchi, Y., Ota, T., Matsuda, S., Asano, T., Kadawaki, T., Takeuchi, T., and Koyasu, S. (2002) *Nat. Immunol.* 3, 875–881
18. Martin, M., Rehani, K., Jope, R. S., and Michalek, S. M. (2005) *Nat. Immunol.* 6, 777–784
19. Fukao, T., and Koyasu, S. (2003) *Trends Immunol.* 24, 358–363
20. Folks, T. M., Justement, J., Kinter, A., Schnittman, S., Orenstein, J., Poli, G., and Fauci, A. S. (1988) *J. Immunol.* 140, 1117–1122
21. Fraser, I. P., Koziel, H., and Ezekowitz, R. A. (1998) *Semin. Immunol.* 10, 363–372
22. Tachado, S. D., Zhang, J., Zhu, J., Patel, N., Cushion, M., and Koziel, H. (2007) *J. Leukocyt. Biol.* 81, 205–211
23. Martin, M., Schifferle, R. E., Cuesta, N., Vogel, S. N., Katz, J., and Michalek, S. M. (2003) *J. Immunol.* 171, 717–725
24. Osaki, M., Oshima, M., and Ito, H. (2004) *Apoptosis* 9, 667–676
25. Chugh, P., Fan, S., Planelles, V., Maggirwar, S. B., Dwhurst, S., and Kim, B. (2007) *J. Mol. Biol.* 366, 67–81
26. Cross, D. A., Alessi, D. R., Cohen, P., Andjelkovich, M., and Hemmings, B. A. (1995) *Nature* 378, 785–789
27. Beutler, B. (2000) *Curr. Opin. Immunol.* 12, 20–26
28. Aggarwal, B. B. (2003) *Nat. Rev. Immunol.* 3, 745–756
29. Fujii, Y., Otake, K., Tashiro, M., and Adachi, A. (1996) *FEBS Lett.* 393, 105–108
30. Ameisen, J. C., Guy, B., Chamaret, S., Loche, M., Mouton, Y., Neyrinck, J. L., Khalife, J., Leprevost, C., Beauchet, G., and Boutillon, C. (1989) *AIDS Res. Hum. Retroviruses* 5, 279–291
31. Federico, M., Percario, Z., Olivetta, E., Fiorucci, G., Muratori, C., Micheli, A., Romeo, G., and Affabris, E. (2001) *Blood* 98, 2752–2761
32. Chugh, P., Bradel-Trethewy, B., Monteiro-Filho, C. M., Planelles, V., Maggirwar, S. B., Dwhurst, S., and Kim, B. (2008) *Apoptosis* 11, 246–255
33. Brigno, E., Haraguchi, S., Koutsonikolis, A., Cianciollo, G. J., Owens, U., Good, R. A., and Day, N. K. (1997) *Proc. Natl. Acad. Sci. U. S. A.* 94, 3178–3182
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35. Wolf, D., Witte, V., Laffert, B., Blume, K., Stromer, E., Trapp, S., d’Aloja, P., Schurmann, A., and Baur, A. S. (2001) Nat. Med. 7, 1217–1224
36. Graziani, A., Galimi, F., Medico, E., Cottone, E., Gramaglia, D., Bocaccio, C., and Comoglio, P. M. (1996) J. Biol. Chem. 271, 6590–6593
37. Lalani, I., Bhol, K., and Ahmed, A. R. (1997) Ann. Allergy Asthma Immunol. 79, 469–483
38. Fiorentino, D. F., Zlotnik, A., Mosmann, T. R., Howard, M., and O’Garra, A. (1991) J. Immunol. 147, 3815–3822
39. Bogdan, C., Vodovotz, Y., and Nathan, C. (1991) J. Exp. Med. 174, 1549–1555
40. Gerard, C., Bruyns, C., Marchant, A., Abramowicz, D., Vandenabeele, P., Delvaux, A., Fiers, W., Goldman, M., and Velu, T. (1993) J. Exp. Med. 177, 547–550
41. Rogy, M. A., Auffenberg, T., Espat, N. J., Philip, R., Remick, D., Wollenberg, G. K., Copeland, E. M., III, and Moldawer, L. L. (1995) J. Exp. Med. 181, 2289–2293