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Mammalian Target of Rapamycin Inhibition in Macrophages of Asymptomatic HIV$^+$ Persons Reverses the Decrease in TLR-4–Mediated TNF-α Release through Prolongation of MAPK Pathway Activation

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TLR-4–mediated signaling is significantly impaired in macrophages from HIV$^+$ persons, predominantly owing to altered MyD88-dependent pathway signaling caused in part by constitutive activation of PI3K. In this study we assessed in these macrophages if the blunted increase in TLR-4–mediated TNF-α release induced by lipid A (LA) is associated with PI3K-induced upregulation of mammalian target of rapamycin (mTOR) activity. mTOR inhibition with rapamycin enhanced TLR-4–mediated TNF-α release, but suppressed anti-inflammatory IL-10 release. Targeted gene silencing of mTOR in macrophages resulted in LA-induced TNF-α and IL-10 release patterns similar to those induced by rapamycin. Rapamycin restored MyD88/IL-1R–associated kinase interaction in a dose-dependent manner. Targeted gene silencing of MyD88 (short hairpin RNA) and mTOR (RNA interference) inhibition resulted in TLR-4–mediated 70-kDa ribosomal protein S6 kinase activation and enhanced TNF-α release, whereas IL-10 release was inhibited in both silenced and nonsilenced HIV$^+$ macrophages. Furthermore, mTOR inhibition augmented LA-induced TNF-α release through enhanced and prolonged phosphorylation of ERK1/2 and JNK1/2 MAPK, which was associated with time-dependent MKP-1 destabilization. Taken together, impaired TLR-4–mediated TNF-α release in HIV$^+$ macrophages is attributable in part to mTOR activation by constitutive PI3K expression in a MyD88-dependent signaling pathway. These changes result in MAPK phosphatase 1 stabilization, which shortens and blunts MAPK activation. mTOR inhibition may serve as a potential therapeutic target to upregulate macrophage innate immune host defense responsiveness in HIV$^+$ persons. ***The Journal of Immunology*****, 2011, 187: 6052–6058.**

Increased risk of opportunistic lung infections remains a serious problem in patients infected with HIV (1). Despite reconstitution of adaptive immunity, asymptomatic HIV-infected persons have up to 25-fold higher incidence of bacterial pneumonia, compared with a healthy population (2). Innate immunity is impaired in asymptomatic HIV$^+$ persons (3), as TLR-4–mediated TNF-α release is reduced in alveolar macrophages (AMs), suggesting a suboptimal host defense response (4, 5). We previously demonstrated that the PI3K signaling pathway is constitutively active in HIV$^+$ macrophages and negatively regulates TNF-α release in HIV$^+$ AM exposed to a specific TLR-4 ligand, lipid A (LA)(6). Furthermore, we recently demonstrated that MyD88-dependent TLR-4 signaling is selectively impaired in AM from asymptomatic HIV$^+$ persons (7).

Mammalian target of rapamycin (mTOR) is a Ser/Thr protein kinase in a signaling pathway downstream from PI3K (8). mTOR regulates the phosphorylation of proteins required for translation, such as 70-kDa ribosomal protein S6 kinase (p70s6K) and eIF4E-binding protein 1 (9). The mTOR signaling pathway regulates proinflammatory cytokine production mediated by different bacterial stimuli in innate immune cells, such as monocytes, macrophages, dendritic cells, and other immune cells (10–13). However, in HIV$^+$ macrophages, it is unknown whether mTOR signaling is altered and selectively blunts LA-induced TLR-4 activation and increases in TNF-α release through disruption of MyD88-dependent signaling pathway.

Using healthy human U937 and HIV$^+$ U1 macrophage cell lines, and clinically relevant human AMs, we show in this article that reduced TLR-4–mediated TNF-α release in AMs from asymptomatic HIV$^+$ persons at risk for bacterial pneumonia is attributable in part to increased mTOR activation. Elevated mTOR signaling impairs TLR-4–induced MAPK control of TNF-α release by disrupting MyD88-dependent signaling. Inhibited MAPK signaling is associated with increased levels of the phosphorylated form of MAPK phosphatase 1 (p-MKP-1) expression, which suppresses TNF-α release. As rapamycin reverses such suppression, mTOR could be a potential therapeutic target to overcome suppression of TLR-4–induced increase in TNF-α release in HIV$^+$ macrophages.

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Abbreviations used in this article: AM, alveolar macrophage; HAART, highly active antiretroviral therapy; IRAK, IL-1R–associated kinase; LA, lipid A; MKP-1, MAPK phosphatase 1; mTOR, mammalian target of rapamycin; p70s6K, 70-kDa ribosomal protein S6 kinase; p-MKP-1, phosphorylated form of MKP-1; siRNA, small interfering RNA.

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Materials and Methods

Reagents

LA (the biologically active component of LPS, as well as specific TLR-4 ligand) from *Escherichia coli* F583 Rd mutant, protease inhibitor mixture, and PMA were purchased from Sigma Chemical (St Louis, MO). Rapamycin was purchased from Calbiochem (San Diego, CA).

Abs

Anti-pERK1/2, -p38, -JNK1/2, and -GAPDH Abs used for multiplex assay were purchased from Millipore (Billerica, MA). Anti–p-MKP-1, anti–MKP-1, and anti-p70s6K Abs were purchased from Cell Signaling (Beverley, MA). Cytokine ELISA kits were from R&D (Minneapolis, MN).

Study subjects

Recruited healthy individuals were confirmed to be HIV negative by ELISA and have no known risk factors for HIV infection (Abbot Diagnostics, Chicago, IL). Healthy and HIV$^+$ subjects presented no evidence of active pulmonary disease and had normal findings on spirometry. 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.

Human AMs

To determine the clinical relevance of the study, selected experiments were carried out using human AMs. Recruited healthy and asymptomatic HIV$^+$ individuals had no active pulmonary disease and had normal findings on 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 $>$200 cells/mm^3, HIV risk factors included intravenous drug use and homosexual exposures, and all were prescribed highly active antiretroviral therapy (HAART). They also had undetectable serum viral load ($<$50 HIV-1 RNA copies/ml), and none experienced a prior opportunistic pneumonia. With standard techniques, bronchoalveolar lavage was performed to obtain lung immune cells (14). All procedures were performed on consenting adults following protocols approved by Beth Israel Deaconess Medical Center institutional review board and Committee for Clinical Investigations. Cells were separated from the pooled bronchoalveolar lavage fluid, and AMs were isolated as described (15). AMs were isolated by adherence to culture plates and yielded cells that were $>$98% viable, as determined by trypan blue dye exclusion, and demonstrated $>$95% positive nonspecific esterase staining.

Macrophage cell lines

Macrophages were differentiated from human promonocytic U937 (American Tissue and Cell Company, Manassas, VA) 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 (16). U937 and U1 cells were harvested during exponential growth phase, washed, and then incubated in complete medium (RPMI 1640 containing 10% heat-inactivated FCS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin). To induce differentiation to macrophages, U937 and U1 cells were incubated with 100 nM PMA at 37°C in a humidified atmosphere containing 5% CO_2 for 24 h. Adherent cells were then washed 3 times with PBS (to remove PMA) and incubated in complete medium (without PMA) for an additional 24 h prior to use.

Western blot analyses

Western blotting was performed as described (17). Briefly, adherent human macrophages were treated with the indicated dose of LA, washed two times with ice-cold PBS (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 Chemicals), and then placed on ice for 20 min. 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 Abs and detected by the ECL detection system (Amersham Biosciences, Piscataway, NJ). Resolved bands were quantified by densitometry (Amersham Biosciences).

Immunoprecipitation

Immunoprecipitation studies were performed as described (4). In brief, equivalent amounts of proteins were precleared by incubation with protein A/G Sepharose (Amersham Biosciences) for 1 h at 4°C. Samples were spun briefly, and supernatant was collected. Ab was then added to the samples and incubated overnight at 4°C. The immune complexes were precipitated with 50 μl protein A/G Sepharose (50% suspension) and incubated for 1 h at 4°C. Nonspecific bound proteins were removed by washing the Sepharose beads three times with modified radioimmunoprecipitation buffer and once with PBS. The immune complexes bound to the beads were solubilized in 40 μl 2× Laemmli buffer and further analyzed by Western blotting, as described above.

Small interfering RNA-mediated knockdown in macrophages

To determine the functional relevance of the PI3K/mTOR pathway in regulating TLR-4–mediated induction of innate immune responses in the context of HIV, knockdown of mTOR was performed using synthetic duplex RNA oligonucleotides. We used On-Target plus smart pool small interfering RNA (siRNA) mTOR (Dharmacon, Boulder, CO). Target sequences were 5′-GGCCAUAGCUAGCCUCAUA-3′, 5′-CAAAGGACUUCGCCCAUAA-3′, 5′-GCAGAAUUGUCAAGGGAUA-3′, and 5′-CCAAAGCACUACACUACAA-3′. On-target plus nontargeting siRNAs were used as controls (Dharmacon). The target sequence was 5′-UGGUUUACAUGUCGACUAA-3′. Macrophages were electroporated with 100 nM siRNA using the Amaxa system following the manufacturer's protocol (Amaxa, Cologne, Germany). mTOR-mediated knockdown was determined by Western blot with anti-mTOR Ab 24–48 h after transfection.

ELISA

Cell-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), following the manufacturer's instructions, and absorbance was measured at 450 nm on a Biokinetic ELISA plate reader (Bio-Tek Instruments, Winooski, VT). The detection limit for TNF-α was 4.4 pg/ml. All measurements were performed in duplicate, and mean values of the three independent measurements were used for statistical analysis.

Multiplex MAPK cell signaling phosphoassay

Macrophages were plated and pretreated with rapamycin for 1 h, followed by LA incubation for different times. Cells were then lysed, and samples collected according to instructions provided in the Multiplex MAP Cell Signaling Buffer and Detection Kit (Millipore). Briefly, the multiplex assay was performed in a 96-well filter-bottom plate. Assay buffer (100 μl) was added onto the plate, followed by the addition of 25 μl standard/samples and 25 μl beads. The plate was incubated at 4°C overnight, and the beads were washed two times. Biotinylated detection Ab was added and incubated at room temperature for 1 h. After washing, streptavidin-PE was added and incubated for 15 min at room temperature, followed by the addition of amplification buffer, and then the plate was read on Luminex instrumentation.

Statistical analysis

Group comparisons were performed using the Student *t* test (two-sample test) or one-way ANOVA. Calculations were performed with StatView (SAS Institute, Cary, NC) and INSTAT2 (GraphPad Software, San Diego, CA) software packages. Results are given as mean ± SEM. Statistical significance was accepted for $p < 0.05$.

Results

mTOR inhibition restores TLR-4–mediated MyD88-dependent TNF-α release in HIV$^+$ AM

We recently reported that MyD88-dependent TLR-4 signaling in AMs from asymptomatic HIV$^+$ persons is selectively impaired (7). Furthermore, we previously demonstrated constitutive activation of PI3K signaling pathway in HIV$^+$ macrophages. Such perturbations may in part account for the downregulation of TLR-4–mediated TNF-α release (6). To determine whether constitutive PI3K activation leads to reduced TLR-4–mediated TNF-α release through altered mTOR activation, we compared the effects of rapamycin on LA-induced increases in TNF-α in HIV$^+$ AMs and in healthy macrophages.

Rapamycin, a prototypic mTOR inhibitor, is a bacterial macrolide that exhibits immunosuppressive and antitumor effects by

inhibiting the mTOR protein activity (12). AMs from healthy individuals exhibited robust TNF-α release, whereas TNF-α release by AMs from asymptomatic HIV$^+$ persons was markedly reduced in response to LA (Fig. 1), consistent with our previous reports and others (4–7). Pretreatment of AMs with rapamycin increased LA-mediated TNF-α release in AMs from asymptomatic HIV$^+$ persons, but had no significant effect on TNF-α release from healthy persons (Fig. 1*A*). In addition, treatment of AMs with rapamycin by itself did not induce the release of TNF-α (Fig. 1*B*). In contrast, in asymptomatic HIV$^+$ and noninfected (i.e., healthy) AM, IL-10 release was similar in unstimulated conditions, but in the presence of LA, IL-10 release is significantly higher in HIV$^+$ AMs (Fig. 1*C*), consistent with our previous report (6). Furthermore, mTOR inhibition with rapamycin reduced IL-10 release in AMs from both groups (Fig. 1*C*). These data demonstrate that whereas mTOR similarly influences IL-10 release in AMs isolated from healthy and HIV$^+$ persons, mTOR inhibition instead selectively restores in AMs from HIV$^+$ persons LA-mediated TNF-α release toward levels in AMs from healthy persons.

Targeted gene silencing of mTOR stimulates LA-mediated TNF-α release in HIV$^+$ macrophages

As p70s6K is downstream from mTOR, changes in its phosphorylation status provide a readout of mTOR activation (18).

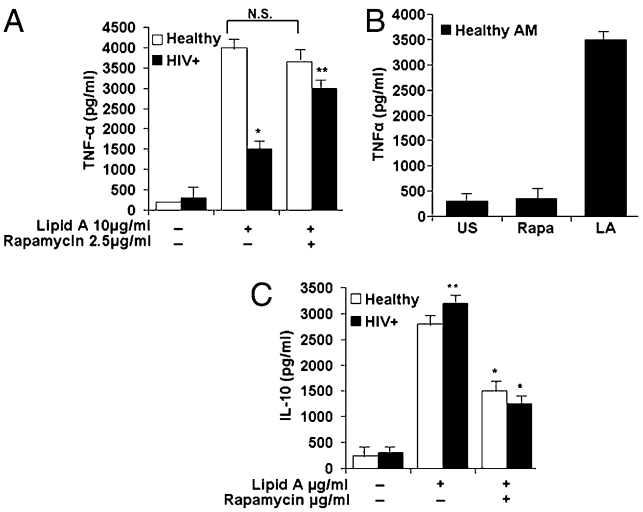

FIGURE 1. Restoration of TLR-4–mediated increases in MyD88-dependent TNF-α release in HIV$^+$ AM by rapamycin. *A*, mTOR inhibition by rapamycin reversed blunted increases in TLR-4–mediated MyD88-dependent TNF-α release in HIV$^+$ AMs. Healthy and HIV$^+$ AMs were pretreated with 2.5 μg/ml rapamycin for 1 h, followed by incubation with 10 μg/ml LA for 24 h, and cell-free supernatant was analyzed for TNF-α by ELISA. Data shown are mean ± SEM of three independent experiments done in triplicate. *$p < 0.01$ compared with healthy with LA, **$p < 0.05$ compared with HIV$^+$ in the absence of rapamycin. N.S., not significant compared with healthy AMs with LA alone. *B*, Rapamycin alone did not stimulate the release of TNF-α from healthy human AMs. Healthy AMs were treated with and without rapamycin for 24 h, and cell-free supernatant was analyzed for TNF-α by ELISA. Data shown are mean ± SEM of three independent experiments done in triplicate. *C*, TLR-4–mediated MyD88-independent IL-10 release was preserved in HIV$^+$ AMs and inhibited by rapamycin. Healthy and HIV$^+$ AMs were treated with 10 μg/ml LA in the presence or absence of 2.5 μg/ml rapamycin for 24 h, and cell-free supernatant was analyzed for IL-10 by ELISA. $n = 3$ subjects for each group. Data shown are mean ± SEM of three independent experiments done in triplicate. *$p < 0.01$ compared with both HIV$^+$ and healthy AMs in the absence of rapamycin, **$p < 0.05$ compared with healthy AMs with LA alone.

Accordingly, in human AMs, we measured changes in p70s6K phosphorylation status to monitor mTOR activation. In AMs from healthy persons, LA increased baseline p70s6K phosphorylation by ~20%, and it remained unchanged with rapamycin (Fig. 2*A*). In contrast, in AMs from HIV$^+$ persons constitutive p70s6K phosphorylation was ~2-fold higher than that in AMs from healthy persons. Phosphorylation increased approximately another 30% in response to LA. This LA-mediated increase was reduced to unstimulated levels in the presence of rapamycin. This finding is in agreement with the effect of rapamycin in dendritic cells exposed to HIV-1 (19). Furthermore, phosphorylation of mTOR was determined as a measure of mTOR activity, and levels of mTOR phosphorylation were detected in unstimulated human U937 cells and markedly increased in the presence of LA (Fig. 2*B*). In contrast, constitutive activation of mTOR (phosphorylation) was significantly higher in HIV$^+$ U1 macrophages compared with U937 macrophages and increased levels in the presence of LA (Fig. 2*B*). These data demonstrate that the mTOR signaling pathway is more active in AMs from HIV$^+$ persons and HIV$^+$ U1 macrophages under constitutively PI3K-activated conditions.

siRNA gene silencing was performed to validate by an independent method the role of mTOR in LA-mediated signaling in healthy U937 and HIV$^+$ U1 human macrophage cell lines. Targeted gene silencing of mTOR in human U937 macrophages resulted in ~60% reduction of mTOR protein expression (Fig. 2*C*). Compared to nonsilencing conditions, mTOR gene silencing did not influence constitutive TNF-α release, but augmented LA-induced increase in TNF-α release in U937 cells (Fig. 2*D*) and U1 cells (Fig. 2*E*). Constitutive IL-10 release was not affected by mTOR gene silencing, but, instead, LA-induced increase in IL-10 release was suppressed (Fig. 2*F*). Similarly, targeted mTOR gene silencing in U1 macrophages reduced LA-induced increase in IL-10 release (Fig. 2*G*). These response patterns to LA were similar to those observed following exposure to rapamycin, and validate the reciprocal influence of mTOR inhibition on LA-mediated TNF-α and IL-10 release in human HIV$^+$ AMs.

mTOR influences MyD88 signaling in human macrophages

Intracellular TLR-4 signaling can be triggered through an interaction between MyD88 and IL-1R–associated kinase (IRAK) after receptor engagement (20). Our recent results showed that such interaction is impaired in AMs from HIV$^+$ persons (7). Such impairment could, in part, play a role in downregulating TLR-4–mediated TNF-α release (4). We now tested whether mTOR inhibition could restore MyD88/IRAK interaction. Pretreatment of HIV$^+$ U1 human macrophages with rapamycin promoted LA-mediated MyD88/IRAK interaction in a dose-dependent manner (Fig. 3*A*). In contrast, rapamycin pretreatment had no effect on LA-induced activation of IFN regulatory factor 3, a component of the MyD88-independent pathway (data not shown). These results suggest that inhibiting mTOR in HIV$^+$ macrophages restores LA-induced TNF-α release in a MyD88-dependent manner.

Next, to validate that mTOR inhibition reverses HIV-induced blunting of LA-mediated TNF-α release through enhanced MyD88/IRAK interaction, MyD88 shRNA targeted gene silencing was performed in human U937 macrophages. Targeted gene silencing of MyD88 in human U937 macrophages led to marked reduction of MyD88 protein expression (7). Monitoring changes in the phosphorylation status of p70s6K provides a readout of mTOR activation (18). Constitutive p70s6K phosphorylation was low, and its levels were similar in both MyD88-silenced and nonsilenced U937 macrophages (Fig. 3*B*). In nonsilenced macrophages, p70s6K phosphorylation status remained unchanged in response to TLR-4 stimulation or mTOR inhibition. In contrast, in

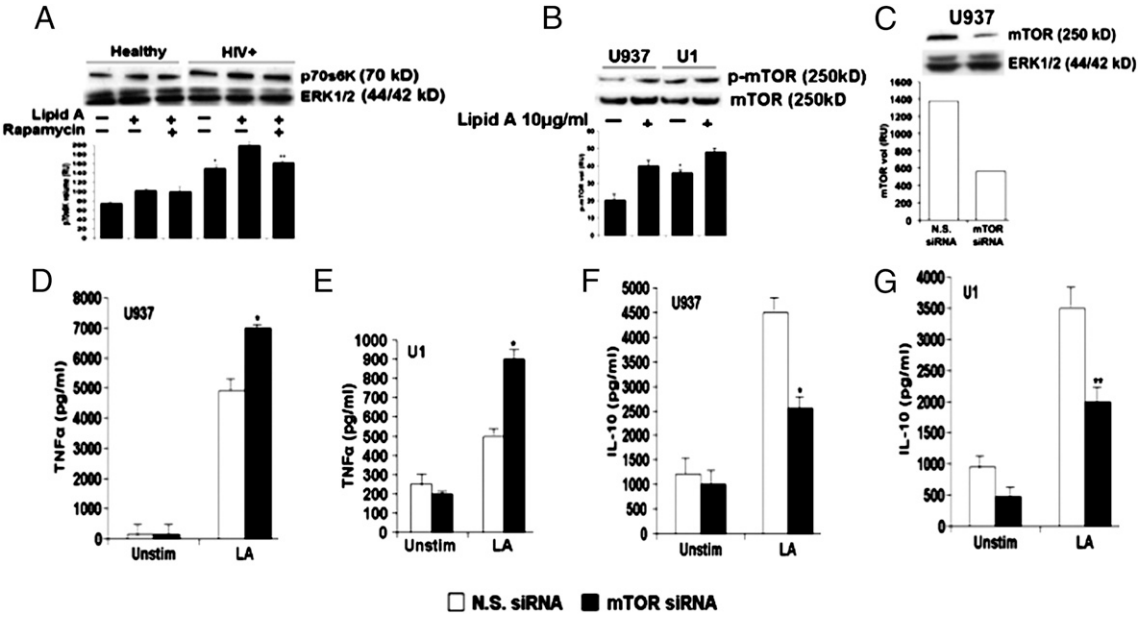

FIGURE 2. Dependence of LA-mediated TNF-α release in HIV$^+$ macrophages on changes in mTOR activity. *A*, Western blot analysis of healthy and HIV$^+$ AMs following inhibition of activated mTOR with rapamycin for 1 h and incubated with LA for 15 min. Membranes were probed with anti-p70s6K Ab, and immunoreactive bands were detected with HRP-conjugated secondary Ab. Total ERK1/2 was used to validate protein-loading equivalence after membrane stripping. Densitometric analysis is displayed beneath Western blots. The Western blot is a representative experiment of three independent experiments from three different individuals for each group with similar results ($n = 3$). *$p = 0.01$ compared with unstimulated healthy AMs, **$p = 0.05$ compared with HIV$^+$ AM in the presence of LA alone. *B*, mTOR is constitutively active in HIV$^+$ macrophages. Western blot analysis of U937 cells and U1 cells in the presence or absence of LA for 15 min. Membranes were probed with anti–phospho-mTOR Ab, and immunoreactive bands were detected with HRP-conjugated secondary Ab. Total mTOR was used to validate protein-loading equivalence after membrane stripping. Densitometric analysis is displayed beneath the Western blots. Western blot is a representative experiment of one of four independent experiments with similar results. *$p = 0.01$ compared with unstimulated U937 cells. *C*, Western blot analysis of human mTOR after gene silencing using siRNA. Total ERK1/2 was used to validate protein-loading equivalence after membrane stripping. A representative blot shows results from one of four independent experiments with similar results. Densitometric analysis is displayed under the blot. *D–G*, Human U937 and U1 macrophages were pretreated with either mTOR siRNA or scrambled (N.S.) siRNA and were then incubated with or without 10 μg/ml LA for 24 h; and the cell-free supernatant was analyzed for TNF-α release (*D* and *E*), *$p = 0.05$ compared with N.S siRNA with LA, or IL-10 (*F* and *G*), *$p = 0.03$ compared with N.S. siRNA with LA, **$p = 0.05$ compared with N.S. siRNA with LA by ELISA. Data shown are mean ± SEM of three independent experiments done in triplicate with similar results.

MyD88 gene-silenced macrophages, p70s6K phosphorylation increased 3-fold in response to TLR-4 stimulation, and mTOR inhibition reduced p70s6K phosphorylation to baseline levels (Fig. 3*B*).

LA-mediated TNF-α release was robust in MyD88 nonsilenced U937 macrophages, and not altered by mTOR inhibition (Fig. 3*C*). In silenced cells, LA-induced TNF-α release was significantly reduced compared with nonsilenced cells and enhanced significantly in the presence of rapamycin (Fig. 3*C*). In comparison, LA-mediated IL-10 release was similar in both MyD88-silenced and nonsilenced U937 cells, and mTOR inhibition reduced IL-10 release equally in both cell groups (Fig. 3*D*). These data demonstrate that mTOR influences both MyD88-dependent and -independent TLR-4–mediated signaling, but mTOR inhibition selectively restores TLR-4–induced MyD88-dependent TNF-α release in HIV$^+$ human macrophages.

mTOR inhibition promotes MAPK phosphorylation and reduces MKP-1 phosphorylation in HIV$^+$ macrophages

LA promoted a rapid but transient ERK1/2 activation (phosphorylation) in human U937 macrophages, whereas LA-mediated ERK1/2 phosphorylation was markedly reduced in HIV$^+$ U1 macrophages (4). We next examined whether mTOR inhibition modulates LA-mediated TNF-α release in U1 macrophages through mimicking the pattern of ERK1/2 and JNK MAPK activation in U937 cells. In HIV$^+$U1 macrophages, rapamycin pretreatment delayed the onset of LA-induced ERK1/2 phosphorylation at 15 min, but later it was prolonged compared with HIV$^+$ U1 macrophages without rapamycin (Fig. 4*A*). It reached, instead, a maximal level at 30 min, with a gradual decline toward baseline levels occurring between 45 and 60 min. A similar trend was observed for JNK1/2 MAPK phosphorylation, except that the onset for phosphorylation did not occur until 60 min (Fig. 4*B*). Furthermore, rapamycin had no significant effect on either the kinetics or the magnitude of p38 MAPK phosphorylation (data not shown). Taken together, these data suggest that in HIV$^+$ macrophages mTOR activation is enhanced and blunts MAPK signaling, whereas mTOR inhibition partially reverses the altered ERK1/2 and JNK1/2 signaling pattern induced by HIV.

The protein phosphatase MKP-1 has been shown to play a role in regulating innate immune responses by mediating a negative feedback effect on MAPK activation (21, 22). We previously demonstrated that reduced ERK1/2 MAPK activity in AM from asymptomatic HIV$^+$ persons was associated with reciprocal sustained elevated levels of MKP-1 protein expression, and that targeted gene silencing of MKP-1 rescued TLR-4–mediated TNF-α release (4). Next, to determine whether mTOR inhibition can modulate MKP-1 stability (i.e., phosphorylation status), HIV$^+$ U1 macrophages were incubated with LA in the presence and absence of rapamycin. mTOR inhibition was associated with biphasic changes in LA-mediated MKP-1 phosphorylation (Fig. 4*C*). Furthermore, p-MKP-1 expression was much higher and invariant in U1 macrophages in response to LA in the absence of rapamycin (Fig 4*D*).

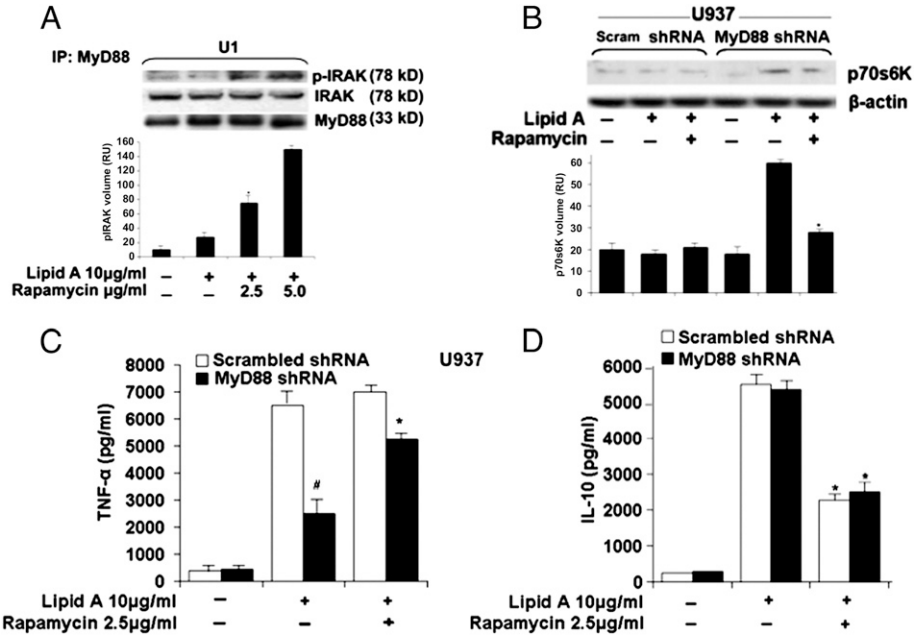

FIGURE 3. Restoration by mTOR inhibition of MyD88-dependent signaling in human macrophages. *A*, MyD88/IRAK interaction restoration as a function of dose-dependent increases in rapamycin accounted for reversal of blunting effect of activated mTOR on LA-induced increases in TNF-α. U1 cells were preincubated in the presence of 2.5 μg/ml rapamycin for 1 h followed by LA for 15 min. Detergent soluble extracts were immunoprecipitated with anti-MyD88 Ab and immunoblotted with anti–p-IRAK Ab. Densitometric analysis for p-IRAK bands is displayed beneath the blot. Blot is representative of three independent experiments with similar results. $*p = 0.05$ compared with LA without rapamycin. *B*, Functional silencing of MyD88 led to increased phosphorylation of p70s6K in macrophages in response to LA. U937 cells were pretreated with shRNA MyD88 and N.S. shRNA. Cells were differentiated with phorbol ester, challenged with LA in either the presence or the absence of rapamycin. Total cell lysates were isolated and probed with anti–phospho-p70s6K Ab, and the immunoreactive band was detected with HRP-conjugated secondary Ab. β-actin expression was used to validate protein-loading equivalence after stripping membrane. Densitometric analysis is displayed beneath the Western blots. The Western blot is a representative experiment of four independent experiments with similar results. $*p = 0.01$ compared with MyD88$^{-/-}$ cells in the presence of LA. *C* and *D*, Inhibition of mTOR augmented LA-mediated TNF-α release in MyD88$^{-/-}$ macrophages, but IL-10 release was inhibited. U937 cells were pretreated with either shRNA MyD88 or N.S. shRNA, and differentiated with phorbol ester, challenged with LA in the presence or absence of rapamycin, and incubated for 24 h. Cell-free supernatant was assayed for TNF-α release (*C*) and IL-10 release (*D*) by ELISA. Results are mean ± SEM of three independent experiments performed in triplicate with similar results. $\#p < 0.01$ compared with N.S. shRNA with LA, $*p < 0.05$ compared with shRNA MyD88 with LA (*C*). $*p < 0.01$ compared with both shRNAs with LA (*D*).

Taken together, these results show that in LA-stimulated HIV$^+$ U1 macrophages, mTOR inhibition caused p-MKP-1 levels to decrease because the transient increase in ERK1/2 phosphorylation was delayed. Such an effect was not seen in untreated HIV$^+$ U1 macrophages. The delay in ERK1/2 activation may account for lack of MKP-1 stabilization because ERK1/2 activation is requisite for stabilizing MKP-1 by inducing MKP-1 phosphorylation (23). Less MKP-1 expression in rapamycin-treated HIV$^+$ U1 macrophages may ultimately account for enhanced ERK1/2 phosphorylation, which is sufficient to trigger adequate increases in transcription factor activity needed to elicit increase in TNF-α gene and protein expression levels.

In summary, Fig. 5 shows a working model describing how, in HIV$^+$ macrophages, constitutively activated PI3K elicits blunting of LA-induced increases in TNF-α release through sustained mTOR activation.

Discussion

In this study, we demonstrated that activation of mTOR, a signaling mediator downstream of PI3K, contributes to impaired LA-mediated TNF-α release in HIV$^+$ macrophages. Critical involvement of mTOR is supported by the findings that mTOR is constitutively upregulated in HIV$^+$ macrophages (as measured by p70S6k phosphorylation and mTOR phosphorylation). mTOR inhibition by rapamycin restored MyD88/IRAK interaction and rescued TLR-4–mediated TNF-α release toward levels in healthy macrophages. Similarly, targeted gene silencing of mTOR resulted in enhanced TNF-α release. mTOR inhibition in macrophages following targeted gene silencing of MyD88 (as a model of impaired MyD88-mediated signaling in HIV$^+$ macrophages) resulted in reduction in LA-mediated p70s6K activation and enhanced TNF-α release, similar to the response observed in HIV$^+$ macrophages. Furthermore, mTOR inhibitor-mediated rescue of TNF-α release was specific to HIV$^+$ macrophages, as mTOR inhibition did not influence TNF-α release in healthy macrophages. These effects suggest that constitutively enhanced mTOR activity could be in part responsible for suboptimal LA-mediated TNF-α release in AMs from asymptomatic HIV$^+$ persons.

The current study shows in macrophages that mTOR activation suppresses a TLR-4–mediated net immune response. Supportive data include the observations that constitutive mTOR activation in HIV$^+$ macrophages was associated with reduced LA-mediated TNF-α release, whereas rapamycin significantly increased LA-mediated TNF-α release, which was validated by showing that targeted gene silencing of mTOR significantly augmented LA-mediated TNF-α release in healthy macrophages. Furthermore, the increase in LA-mediated TNF-α release with mTOR inhibition did not represent a general augmentation of macrophage cytokine release, as the patterns of IL-10 release were distinct and often in the opposite direction to TNF-α release. Furthermore, the rescue of LA-mediated TNF-α release was specific to HIV$^+$ macrophages. mTOR inhibition was associated with increases in TNF-α release to levels approaching those secreted by healthy macrophages. Taken together with our prior findings (4, 6, 7, 15), these

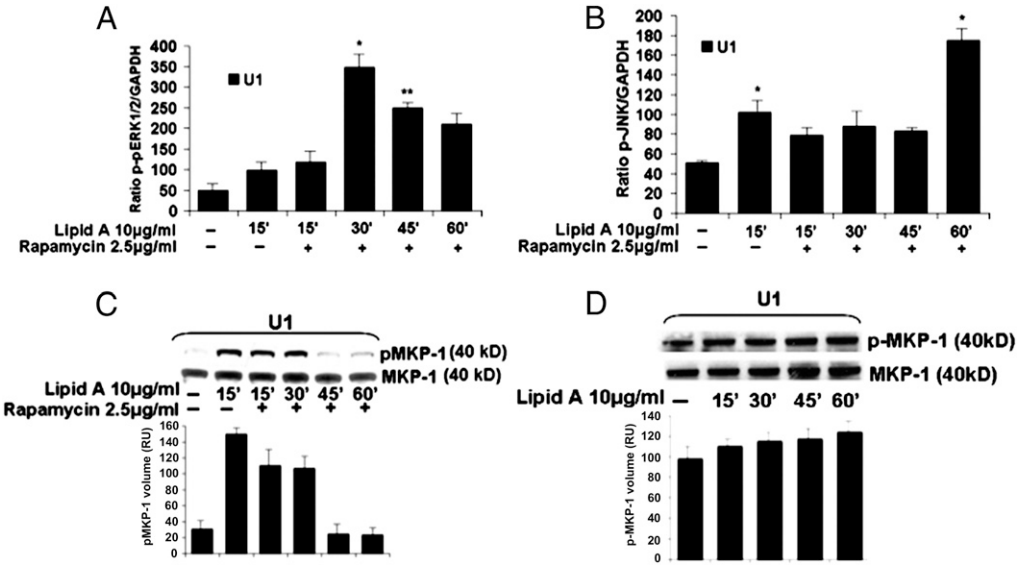

FIGURE 4. ERK1/2 and JNK1/2 phosphorylation pattern altered by mTOR inhibition through declines in MKP-1 phosphorylation in HIV$^+$ macrophages. HIV$^+$ macrophages (U1 cells) were differentiated with phorbol ester and pretreated with 2.5 μg/ml rapamycin for 1 h, followed by LA incubation for different times. Cell lysates were analyzed by Milliplex Human MAP Kit panel. Results are representative of three independent experiments performed in duplicate. *A*, Phospho-ERK1/2; $*p < 0.01$ compared with LA alone for 15 min, $**p < 0.005$ compared with LA for 15 min. *B*, Phospho-JNK1/2; $*p < 0.05$ compared with unstimulated cells and compared with LA alone for 15 min. *C*, U1 cells were differentiated with phorbol ester and pretreated with 2.5 μg/ml rapamycin for 1 h followed by LA incubation for different times. Membranes were probed with anti–phospho-MKP-1 Ab, and immunoreactive bands were detected with HRP-conjugated secondary Ab. Total MKP-1 expression was used to validate protein-loading equivalence after membrane stripping. Densitometric analysis is displayed beneath the Western blots. The Western blot is a representative experiment of four independent experiments with similar results. *D*, U1 cells were differentiated with phorbol ester and challenged with LA (10 μg/ml), incubated for different times. Membranes were probed with anti–phospho-MKP-1 Ab, and immunoreactive bands were detected with HRP-conjugated secondary Ab. Total MKP-1 expression was used to monitor protein-loading equivalence after membrane stripping. The Western blot is a representative experiment of four independent experiments with similar results.

data demonstrate that active use of HAART (associated with immune reconstitution of CD4$^+$ T-cells) is not sufficient to rescue or restore macrophage innate immune function, as macrophages used in this study were from asymptomatic HIV$^+$ volunteers prescribed HAART and with preserved CD4 T cell counts. These in vitro data suggest that the defect in macrophage host defense function associated with HIV infection is reversible, and potentially amenable to other pharmacological agents to rescue or restore macrophage innate immune function.

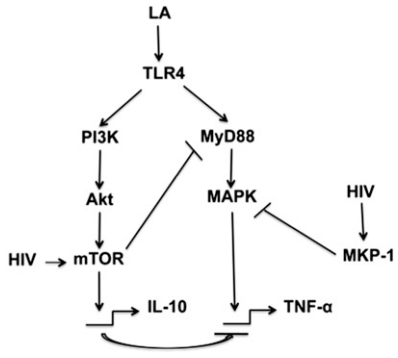

FIGURE 5. Working model describing how in HIV$^+$ macrophages constitutively activated PI3K elicits blunting of LA-induced increases in TNF-α release through sustained mTOR activation. The PI3K/Akt/mTOR signaling pathway is constitutively active in HIV$^+$ macrophages and inhibits the expression of TNF-α by (1) direct mTOR-dependent inhibition of MyD88-dependent signaling pathway, (2) IL-10 production through the activation of mTOR, and (3) destabilization of a pan-specific MKP-1 expression through declines in its phosphorylation status. Inhibition of MKP-1 elicited negative feedback on ERK1/2, and JNK1/2 phosphorylation prolonged ERK1/2 and JNK1/2 activation by declines in its dephosphorylation.

Our finding in HIV$^+$ macrophages that mTOR, along with its downstream signaling mediator p70s6K, is constitutively activated is consistent with our report describing constitutive PI3K activation in these cells (6). These changes agree with the notion that mTOR is downstream from PI3K and could account for reduced LA-mediated TNF-α release in HIV$^+$ AMs and U1 macrophages. Another indication that HIV infection suppresses LA-induced increases in TNF-α release through enhanced mTOR activation is that in U937 macrophages, compared with nonsilenced macrophages, gene silencing of mTOR enhanced LA-mediated TNF-α release. In contrast, silencing of both U937 and U1 cells led to inhibition of IL-10 release in response to TLR-4 activation. These data suggest that mTOR plays a dominant role in regulating innate immune responses because mTOR can influence both TLR-4–mediated MyD88-dependent and MyD88-independent signaling pathways.

The mechanism(s) by which enhanced mTOR activity reduces TLR-4–mediated TNF-α release in HIV$^+$ macrophages was not definitively established in the current study. However, data suggest the influence of mTOR on TLR-4–mediated TNF-α release is, in part, MyD88 dependent, a finding consistent with our prior publication (7), and mTOR inhibition may promote MyD88/IRAK interaction. Furthermore, mTOR inhibition in HIV$^+$ U1 macrophages was associated with delayed, but enhanced, phosphorylation of ERK1/2 and JNK1/2 MAPKs. These changes were associated with time-dependent variation of MKP-1 phosphorylation status that was reciprocal with ERK1/2 activation, whereas in untreated macrophages, p-MKP-1 expression was invariant and higher than in the presence of rapamycin. Acetylation may be a better indicator of cellular phosphatase activity (24), although this was not specifically investigated. Finally, it is still uncertain whether our in vitro findings accurately reflect macrophage function in vivo. Nevertheless, the similarity between results ob-

tained with primary human AMs and those obtained with relevant cell lines may allow for direct translation of findings to treatment of human disease.

In conclusion, collectively these data suggest that elevated mTOR activation in HIV$^+$ macrophages may represent a critical signaling pathway that impairs TLR-4–mediated TNF-α release. Importantly, mTOR inhibition restores TLR-4–mediated TNF-α release by HIV$^+$ macrophages, through a mechanism that is in part MyD88 dependent, and may involve enhanced ERK1/2 MAPK phosphorylation occurring because of a decline in negative feedback induced by MKP-1 as a result of delayed pERK1/2-induced MKP-1 phosphorylation (Figs. 4*A*, 4*C*). These data identify mTOR as a potential therapeutic target in HIV$^+$ macrophages to restore critical host defense cytokine responses to bacterial challenge.

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Disclosures

The authors have no financial conflicts of interest.

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