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# HIV protease inhibitor lopinavir-induced TNF-α and IL-6 expression is coupled to the unfolded protein response and ERK signaling pathways in macrophages

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ABSTRACT

HIV protease inhibitor (PI)-associated cardiovascular risk, especially atherosclerosis, has become a major concern in the clinic. Macrophages are key players in the inflammatory response and atherosclerosis formation. We have previously shown that HIV PIs induce endoplasmic reticulum (ER) stress, activate the unfolded protein response (UPR), and increase the synthesis of the inflammatory cytokines, TNF-α and IL-6, by regulating the intracellular translocation of RNA binding protein HuR in macrophages. However, the underlying signaling mechanisms remain unclear. We show here that the HIV PI lopinavir significantly activated the extracellular-signal regulated protein kinase (ERK), but not c-Jun *N*-terminal kinase (JNK) and p38 MAPK. Lopinavir-induced cytosolic translocation of HuR and TNF-α and IL-6 synthesis was attenuated by specific chemical inhibitor of MEK (PD98058) or over-expression of dominant negative mutant of MEK1. In addition, we demonstrated that lopinavir-induced ERK activation and TNF-α and IL-6 expression were completely inhibited in macrophages from CHOP null mice. Taken together, these results indicate activation of the UPR plays an essential role in HIV PI-induced inflammatory cytokine synthesis and release by activating ERK, which increases the cytosolic translocation of HuR and subsequent binding to the 3′UTR of TNF-α and IL-6 mRNAs in macrophages.
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## 1. Introduction

HIV protease inhibitors (PIs) are the most effective agents in current HIV therapy. Since the introduction of the first HIV PI in the early 1990s, HIV-induced morbidity and mortality have been significantly reduced and the lifespan of HIV patients is prolonged [1]. However, the benefits of HIV PIs are compromised by serious side effects, such as dyslipidemia, insulin resistance, lipodystrophy and hepatotoxicity [2–5]. HIV PI-associated cardiovascular complications have become a matter of particular concern in the clinic. Although the underlying cellular/molecular mechanisms of HIV PI-induced cardiovascular complications remain to be fully identified, numerous studies have suggested that multiple mechanisms may be involved and individual HIV PIs may have different effects. Our previous studies have shown that activation of the endoplasmic reticulum (ER) stress response and the unfolded protein response (UPR) play an important role in HIV PI-induced disruption of lipid metabolism in hepatocytes and foam cell formation in macrophages [6,7]. The activation of the UPR may represent a key molecular mechanism of HIV PI-associated side effects.

Atherosclerosis is a complex and chronic inflammatory disease and macrophages are the key players in the initiation and progression of atherosclerotic lesions [8,9]. In addition to the accumulation of oxidized lipid and free cholesterol in macrophages, another key characteristic of atherosclerotic lesions is the presence of abundant inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin (IL)-6. Posttranscriptional regulation of mRNA stability is emerging as an important control mechanism regulating inflammatory cytokine synthesis. Most short-lived inflammatory cytokines have adenine and uridine (AU) rich stability elements (ARE) within the 3′-untranslated regions (3′UTR) [10]. Modulation of mRNA stability has a significant impact on inflammatory cytokine synthesis. Our recent studies also have shown that HIV PIs increase TNF-α and IL-6 expression by increasing mRNA stabilities through regulating the

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intracellular translocation of RNA binding protein HuR and enhancing the binding of HuR to 3′UTR of TNF-α and IL-6 in macrophages [11]. However, the cellular signaling mechanisms by which HIV PIs regulate TNF-α and IL-6 mRNA stability and synthesis remain to be identified.

The mitogen-activated protein kinase (MAPK) signaling pathways are highly conserved in mammalian cells and can be activated by various extracellular stimuli. MAPKs are involved in many facets of cellular regulation including regulating the expression of many inflammatory genes [12]. Three major MAPK pathways, extracellular-signal regulated protein kinase (ERK), p38 MAPK and c-Jun *N*-terminal kinase (JNK)/stress activated protein kinase (SAPK), have been found to be involved in posttranscriptional regulation of mRNAs encoding TNF-α, IL-6 and cyclooxygenase (COX)-2 [13–15]. It has been reported that p38 MAPK plays a critical role in lipopolysaccharide (LPS)-induced expression of TNF-α and IL-6 by increasing mRNA stability in macrophages [12,15,16]. Recent studies reported by Feng et al. and Li et al. indicate that accumulation of free cholesterol in macrophages induces ER stress and increases TNF-α and IL-6 expression by activating JNK, p38 and ERK signaling pathways [17,18]. However, the possible links and hierarchical relationship among HIV PI-induced UPR activation, MAPK signaling pathways, and expression of TNF-α and IL-6 in macrophages remain to be identified.

In the present study, we investigated the implications of MAPK signaling pathways for HIV PI-induced increase of TNF-α and IL-6 expression in macrophages. Herein we present evidence that ERK signaling pathway is involved in lopinavir-induced TNF-α and IL-6 expression and the HIV PI-induced ER stress response is responsible for ERK activation and subsequent increase of TNF-α and IL-6 mRNA stability and protein expression in macrophages.

## 2. Materials and methods

### 2.1. Materials

Mouse J774A.1 macrophage cells and human monocytic leukemia THP-1 cells were purchased from American Type Culture Collection (ATCC) (Manassas, VA). Cell culture reagents, Dulbecco's modified Eagle's medium (DMEM) and penicillin–streptomycin–glutamine (100×), were obtained from Invitrogen (Carlsbad, CA). Fetal bovine serum was from Atlanta Biologicals (Norcross, GA) and was heat-inactivated at 56 °C for 30 min. Criterion XT Bis–Tris Gels, XT MOPS running buffer, 4× XT sample buffer, Precision Plus Protein Kaleidoscope Standards and Bio-Rad protein assay reagent were obtained from Bio-Rad (Hercules, CA). Antibodies against phospho-ERK1/2, ERK1/2, phospho-JNK, JNK, phospho-p38, p38, HuR, β-actin, lamin B, horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, HRP-conjugated donkey anti-goat IgG and HRP-conjugated goat anti-mouse IgG were from Santa Cruz Biotechnology (Santa Cruz, CA). Western Lightning Chemiluminescence Plus was purchased from PerkinElmer (Boston, MA). High capacity cDNA Reverse Transcription Kit was obtained from Applied Biosystem (Foster City, CA). Amprenavir was generous gift from GlaxoSmithKline (Barnard Castle, Co. Durham, UK). Lopinavir was obtained from NIH AIDS Research & Reference Reagent Program. PD98059 was purchased from Calbiochem (San Diego, CA). Blue Lite Autorad Films were purchased from ISC Bioexpress (Kaysville, UT). The SV Total RNA Isolation System came from Promega (Madison, WI). Recombinant mouse TNF-α and IL-6 were obtained from BioLegend (San Diego, CA). Anti-mouse TNF-α and IL-6 antibodies, biotinylated anti-mouse TNF-α and IL-6, and avidin–HRP were from eBioscience (San Diego, CA).

*Mice*: Male mice, 8–10 weeks of age were used in this study. C57BL/6 mice and CHOP knock out mice ($CHOP^{-/-}$) on C57BL/6 background were from Jackson Laboratories.

### 2.2. Cell culture and HIV PIs treatment

Mouse J774A.1 cells were cultured in DMEM medium containing 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C with 5% $CO_2$. Cells were split using cell scrapers when they reached 100% confluence. Human THP-1 monocytes were cultured in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C with 5% $CO_2$. THP-1 monocytes were treated with PMA (100 ng/ml) for 4 days to facilitate differentiation into macrophages. HIV PIs, amprenavir and lopinavir, were dissolved in dimethyl sulfoxide (DMSO). HIV PIs or DMSO as a vehicle control were directly added into the culture medium with final concentration of 15 or 25 μM and incubated for different time periods (0–24 h). ERK inhibitor (PD98059) was added into the medium 30 min prior to HIV PIs treatment. For over-expression of MEK1 dominant mutant (DN-MEK1), cells were incubated with recombinant DN-MEK1 adenovirus or control CMV adenovirus (MOI = 50) for 24 h, then treated with HIV PIs [19].

### 2.3. Isolation of mouse peritoneal macrophages

C57BL/6 wild type or $CHOP^{-/-}$ (male, 8-week old) was injected intraperitoneally with 0.5 ml of phosphate-buffered saline (PBS) containing 40 μg concanavalin A. The macrophages were harvested 72 h after injection by peritoneal lavage. The harvested cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 20% L-cell-conditioned medium [20]. The medium was replaced every 24 h until the macrophages were confluent.

### 2.4. Western blot analysis

After HIV PIs treatment, culture medium was collected and cells were washed with ice-cold PBS. Whole cell lysate was prepared using lysis buffer containing 150 mM NaCl, 1.0% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, pH 8.0, and protease and phophatase inhibitors. Nuclear and cytosolic extracts were isolated as previously described [7]. Protein concentration was measured using Bio-Rad protein assay reagent. The total cell lysate (30 μg of proteins), cytoplasmic proteins (30 μg of proteins) or nuclear proteins (20 μg of proteins) were resolved on 10% Bis–Tris Criterion XT gels and transferred onto nitrocellulose membranes. Immunoblots were blocked with 5% non-fat milk in TBS buffer for 1 h at room temperature then incubated with primary antibodies against p-ERK1/2, ERK1/2, p-JNK, JNK, p-p38, p-38, HuR, lamin B or β-actin at 4 °C overnight. Immunoreactive bands were detected using HRP-conjugated secondary antibodies with the Western Lightning Chemiluminescence Plus reagent. The density of the immunoreactive bands was analyzed using Image J computer software (NIH) [7].

### 2.5. Enzyme-linked immunosorbent assay (ELISA) of TNF-α and IL-6

Mouse J774A.1 macrophages were pre-treated with individual MAPK inhibitor for 30 min, then treated with HIV PIs or vehicle control for 24 h. At the end of the treatment, the culture media were collected and centrifuged at 14,000 rpm for 1 min. The supernatants were stored in aliquots at −70 °C. TNF-α and IL-6 levels in the media were determined by ELISA using anti-mouse TNF-α and anti-mouse IL-6 as capture antibodies and biotinylated anti-mouse TNF-α and biotinylated anti-mouse IL-6 as detection antibodies (eBioscience). Recombinant mouse TNF-α and IL-6 were used as standards (BioLegend). The total protein concentrations of the viable cell pellets were determined using Bio-Rad Protein Assay reagent. Total amounts of TNF-α and IL-6 in media

were normalized to the total protein amounts of the viable cell pellets.

### 2.6. RNA isolation and real-time quantitative RT-PCR

Total cellular RNA was isolated from mouse J774A.1 macrophages after treatment with MAPKs inhibitors and HIV PIs, or vehicle control (DMSO) for 24 h, using the Promega SV Total RNA Isolation System. Total RNA (5 μg) was used for first-strand cDNA synthesis using the High-Capacity cDNA Archive Kit. The mRNA levels of TNF-α and IL-6 were quantified using the following primers: TNF-α forward primer 5′ TGGAACTGGCAGAAGAGG 3′; TNF-α reverse primer 5′ AGACAGAA GAGCGTGGTG 3′; IL-6 forward primer 5′ GAGGATACCA CTCCCAACAGACC 3′; IL-6 reverse primer 5′ AAGTGCATCATCGTTGT TCATACA 3′; β-actin forward primer 5′ ACCACACCTTCTACAATGAG 3′; β-actin reverse primer 5′ ACGAC CA GAGGCATACAG 3′. iQ™ SYBR Green Supermix (Bio-Rad Laboratories) was used as a fluorescent dye to detect the presence of double-stranded DNA. The mRNA values for each gene were normalized to internal control β-actin mRNA. The ratio of normalized mean value for each treatment group to vehicle control group was calculated.

### 2.7. Assessment of TNF-α and IL-6 mRNA stability

Mouse J774A.1 macrophages were pretreated with ERK inhibitor, PD98059 (30 μM) for 30 min, then treated with HIV PI (lopinavir, 15 μM) or vehicle control for 16 h before addition of actinomycin D (5.0 μg/ml) (time 0). Total cellular RNA was extracted 0.5, 1, 2, and 4 h after actinomycin D addition. TNF-α and IL-6 mRNA levels were determined by qRT-PCR as described in the previous section and results are expressed as the percentage of the mRNA amount at the time of actinomycin D addition.

### 2.8. Immunoprecipitation of endogenous HuR–mRNA complexes

To assess the association of endogenous HuR with endogenous TNF-α and IL-6 mRNAs, immunoprecipitation (IP) of endogenous HuR–mRNA complexes was performed as described previously [11]. Briefly, after treatment with HIV PIs or vehicle control (DMSO), cells were harvested, counted, and the same number of cells per condition ($3 \times 10^7$) were pelleted and suspended in approximately two cell pellet volumes of polysome lysis buffer containing 100 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES (pH 7.0), 0.5% Nonidet P-40 with 1 mM DTT, 100 U/ml RNaseOUT, 0.2% vanadyl-ribonucleoside complex, 0.2 μM PMSF, 1 μg/ml pepstatin A, 5 μg/ml bestatin, and 20 μg/ml leupeptin. Cell lysates were centrifuged at 16,000 × *g* for 10 min at 4 °C. For IP, protein A-Sepharose beads were swollen 1:1 (v/v) in NT2 buffer [50 mM Tris (pH 7.4), 150 mM NaCl, and 1 mM $MgCl_2$/0.05% Nonidet P-40] supplemented with 5% BSA. A 100-μl aliquot of the preswollen protein A bead slurry was used for each IP reaction and incubated for 4 h at room temperature with excess immunoprecipitating Ab (30 μg), using either a mouse mAb specific for HuR, or an $IgG_1$ isotype control Ab. RNA in IP materials was reverse transcribed and used to detect the presence of TNF-α and IL-6 mRNAs by real-time PCR.

### 2.9. Statistical methods

Student's *t*-test was employed to analyze the differences between sets of data. Statistics were performed using GraphPad Pro (GraphPad, San Diego, CA).

## 3. Results

### 3.1. Effect of HIV PIs on TNF-α and IL-6 expression and ERK activation in macrophages

Our previous studies showed that individual HIV PIs activate the UPR and induce TNF-α and IL-6 expression to varying degrees in macrophages. Lopinavir showed the most potent effects on UPR activation and induction of TNF-α and IL-6 expression and thus was the focus of current study; while amprenavir has the least potential to activate the UPR and induce TNF-α and IL-6 expression and was used as negative control [7,11]. As shown in Fig. 1A, lopinavir time-dependently increased both TNF-α and IL-6

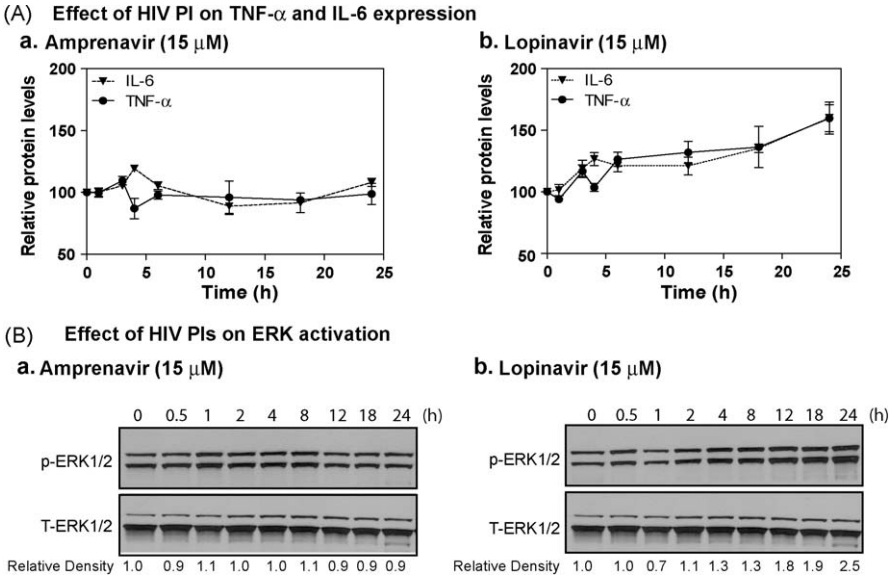


**Fig. 1.** Time course of HIV PI-induced TNF-α and IL-6 expression and ERK activation in macrophages. (A) Time course of HIV PI-induced TNF-α and IL-6 expression in mouse J774A.1 cells. Cells were treated with 15 μM of amprenavir (a) or lopinavir (b) for 0, 0.5, 1, 2, 4, 6, 12, 18 or 24 h. At the end of treatment, the culture media and cells were collected separately. The amounts of TNF-α and IL-6 released to the media were analyzed by ELISA and normalized to the total protein amounts of the viable cell pellets as described in Section 2 , and expressed as a percent of the 0 time point control. Values are mean ± SE of three independent experiments. (B) Time course of HIV PI-induced ERK activation in mouse J774A.1 cells. Representative immunoblots from three independent experiments for phospho (p)-ERK and total (T)-ERK from the total cell lysates of mouse J774A.1 cells treated with individual HIV PIs (15 μM) for 0, 0.5, 1, 2, 4, 8, 12, 18, and 24 h. (a) Amprenavir and (b) lopinavir. The density of the immunoreactive bands was analyzed using Image J software. Relative density was shown.

expression, but amprenavir had no significant effect. To determine whether individual HIV PIs also have different effects on MAPKs' activation in macrophages, mouse J774A.1 cells were treated with vehicle control or individual HIV PIs (amprenavir or lopinavir) at a clinically relevant concentration of 15 μM for different time periods (0–24 h). The activation of ERK, JNK or p38 MAPK was detected by Western blot analysis using phospho-specific antibodies against p-ERK1/2, p-p38 and p-JNK1/2 and normalized to the total protein levels of ERK1/2, JNK1/2 and p38. As shown in Fig. 1B, lopinavir significantly induced ERK activation in a time-dependent manner. The earliest response was observed at the 2 h time point and the highest response was observed at 24 h. However, amprenavir has no significant effect on ERK activation. Interestingly, although numerous studies have shown that JNK and p38 MAPK are involved in stress responses, none of the tested HIV PIs significantly induced JNK or p38 MAPK activation (online supplement data, Fig. S1). Similar results were obtained in human THP-1-derived macrophages (data not shown).

### 3.2. Effect of MAPKs activation on HIV PI-induced expression of TNF-α and IL-6 in macrophages

Our previous studies showed that lopinavir and atazanavir markedly induced TNF-α and IL-6 expression in macrophages, but amprenavir had no effect [11]. To determine if HIV PI-induced ERK activation is correlated to the corresponding increase of TNF-α and IL-6 expression in macrophages, J774A.1 cells were pre-treated with specific chemical inhibitor of MEK (PD98059) for 30 min, then treated with amprenavir and lopinavir for 24 h. TNF-α and IL-6 levels in the culture media were measured by ELISA as described in Section 2. Total amounts of TNF-α and IL-6 in media were normalized to the total protein amounts of the viable cell pellets. The results indicated that inhibition of ERK activation by specific chemical inhibitor of MEK significantly reduced lopinavir-induced increase of TNF-α and IL-6 expression in macrophages (online supplement data, Fig. S2). To further confirm the effect of activation of ERK on lopinavir-induced TNF-α and IL-6 expression in macrophages, cells were infected with the recombinant adenovirus expressing the dominant negative mutant DN-MEK1 [19]. After 24 h, the cells were treated with HIV PIs for 24 h. As shown in Fig. 2, lopinavir-induced TNF-α and IL-6 expression was inhibited by over-expression of DN-MEK1. The inhibition of ERK activation by PD98059 and over-expression of DN-MEK was confirmed by Western blot analysis (online supplement data, Fig. S3).

### 3.3. Effect of ERK activation on lopinavir-induced increase of TNF-α and IL-6 mRNA expression and stability in macrophages

In order to further examine whether ERK signaling pathways are involved in the regulation of TNF-α and IL-6 at the mRNA level, mouse J774A.1 macrophages were treated with PD98059 as described above followed by treatment with individual amprenavir or lopinavir for 24 h. Total cellular RNA was isolated. The mRNA levels of TNF-α and IL-6 were measured by real-time quantitative RT-PCR. The results indicate that inhibition of ERK activation showed similar effects on lopinavir-induced increase of TNF-α and IL-6 mRNA levels as seen with lopinavir-induced increase of TNF-α and IL-6 protein levels (data not shown).

Our previous studies have shown that HIV PIs induce TNF-α and IL-6 expression by increasing mRNA stabilities through regulating the translocation of RNA binding protein HuR from nucleus to cytosol and the binding of HuR to the 3′UTR of TNF α and IL-6 mRNAs [11]. We further examined whether ERK signaling pathways are involved in regulating TNF-α and IL-6 mRNA stabilities in macrophages. As shown in Fig. 3, lopinavir-induced increase of TNF-α and IL-6 mRNA stabilities was significantly inhibited by MEK inhibitor.

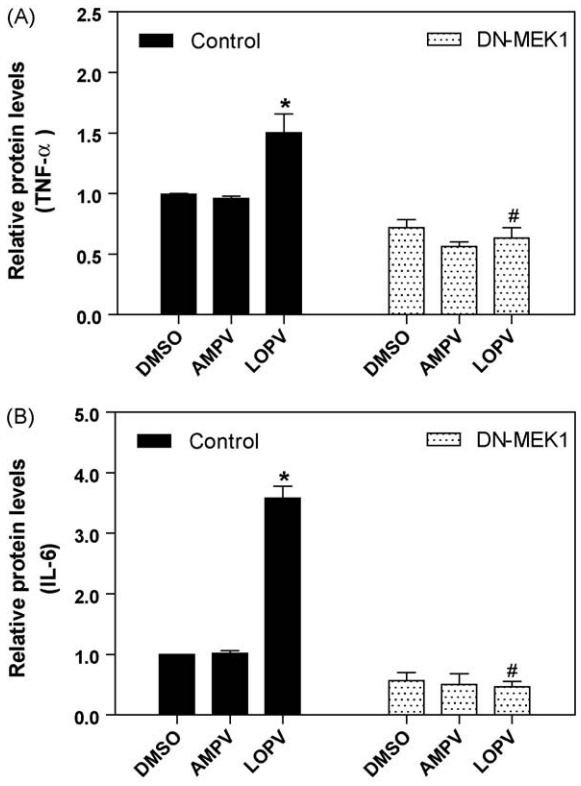


**Fig. 2.** Effect of over-expression of dominant negative mutants of MEK1 on HIV PI-induced TNF-α and IL-6 protein expression in macrophages. Mouse J774A.1 cells were infected with a recombinant adenovirus expressing a dominant negative mutant MEK1 or CMV control virus (MOI = 50). After 24 h, cells were treated with individual HIV PIs (15 μM), amprenavir (AMPV), lopinavir (LOPV) or vehicle control (DMSO) for 24 h. At the end of treatment, the culture media and cells were collected separately. The amounts of TNF-α and IL-6 released to the media were analyzed by ELISA and normalized to the total protein amounts of the viable cell pellets as described in Section 2, and expressed as a percent of the vehicle control of control virus group. Values are mean ± SE of three independent experiments. Statistical significance relative to DMSO control within each treatment group, $^{*}p < 0.05$. Statistical significance relative to control group, $^{\#}p < 0.05$.

### 3.4. Inhibition of ERK activation prevents HIV PI-induced increase of cytoplasmic levels of HuR and binding of HuR to 3′UTRs of TNF-α and IL-6

We have previously shown that RNA binding protein HuR is involved in HIV PI-induced TNF-α and IL-6 expression in macrophages [11]. HIV PIs not only induce HuR translocation from nucleus to cytosol, but also increase the binding of HuR to the 3′UTR of TNF-α and IL-6 expression in macrophages. We sought to further determine whether HIV PI-induced ERK activation plays a role in regulating HuR intracellular translocation and binding to 3′UTRs of TNF-α and IL-6. Results presented in Fig. 4 show that lopinavir-induced increase of cytosolic HuR levels and decrease of nuclear HuR levels were blocked by over-expression of DN-MEK1. But the total HuR protein levels remained unchanged. By using immunoprecipitation of HuR under conditions that preserved its association with target mRNAs in ribonucleoprotein (RNP) complexes, we examined the effect of ERK inhibition on lopinavir-induced increase of HuR binding to TNF-α and IL-6 mRNAs. As shown in Fig. 5, lopinavir-induced increase of HuR binding to TNF-α and IL-6 mRNAs was completely prevented by over-expression of DN-MEK1. These findings suggest that ERK

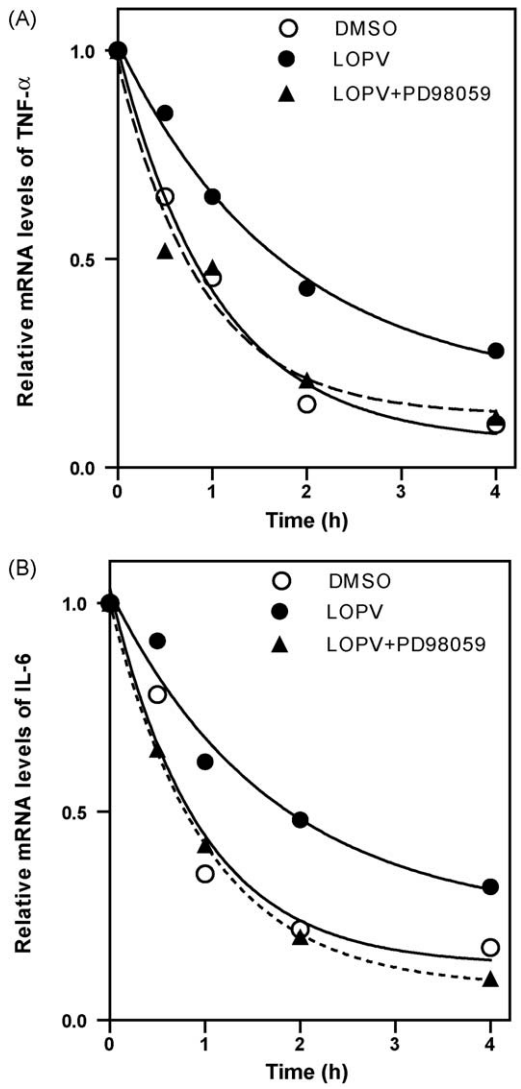


**Fig. 3.** Effect of ERK inhibitor on HIV PI-induced TNF-α and IL-6 mRNA stability in macrophages. Mouse J774A.1 cells were pretreated with individual PD98059 (30 μM) for 30 min, and then treated with lopinavir (LOPV, 15 μM) or vehicle control (DMSO) for 16 h before addition of actinomycin D (5.0 μg/ml) (time 0). Total cellular RNA was extracted at 0, 0.5, 1, 2, and 4 h after actinomycin D addition. TNF-α (A) and IL-6 (B) mRNA levels were determined by real-time RT-PCR as described in Section 2. Values are mean ± SE of three independent experiments.

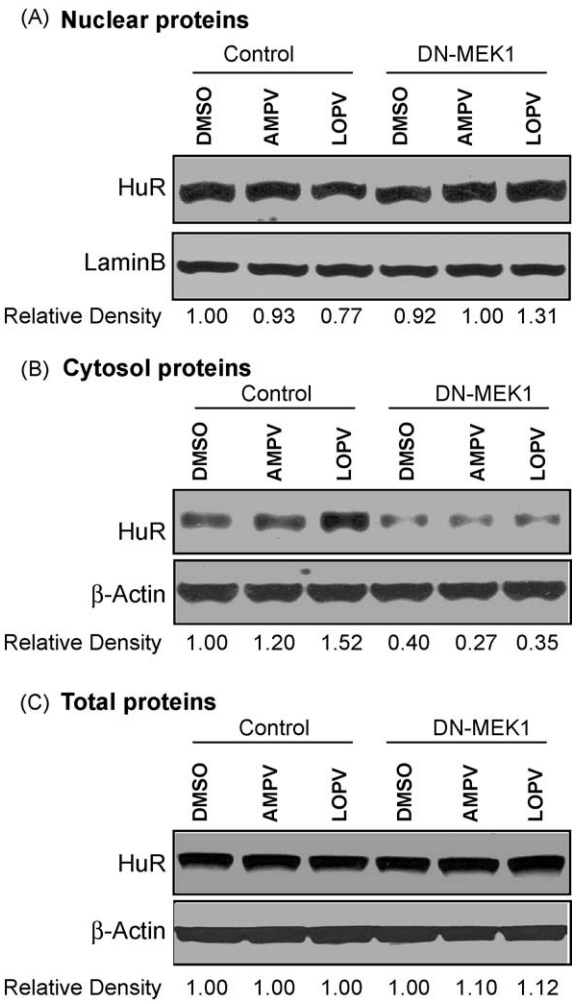


**Fig. 4.** Effect of over-expression of DN-MEK1 on HIV PI-induced cytoplasmic translocation of RNA binding protein HuR in mouse macrophages. Mouse J774A.1 cells were infected with CMV control adenovirus or a recombinant DN-MEK1 adenovirus for 24 h and then treated with DMSO, amprenavir (AMPV, 15 μM), or lopinavir (LOPV, 15 μM) for 24 h. The nuclear proteins and cytoplasmic proteins were isolated as described in Section 2. Three independent experiments were performed. (A) Representative immunoblot images against HuR and lamin B for nuclear proteins. (B) Representative immunoblot images against HuR and β-actin for cytoplasmic proteins. (C) Representative immunoblot images against HuR and β-actin for total proteins. The density of the immunoreactive bands was analyzed using Image J software and normalized to lamin B or β-actin control.

activation is required for HIV PI-induced HuR translocation and binding to the 3′UTR of TNF-α and IL-6 mRNAs.

### 3.5. Effect of ERK activation on HIV PI-induced UPR activation

Our previous studies have demonstrated that HIV PIs induce ER stress and activate the UPR in macrophages and primary hepatocytes [6,7]. Studies from our laboratories and others' suggest that HIV PI-induced ER stress represents an important cellular mechanism underlying HIV PI-associated various side effects [6,7,21]. To determine whether activation of ERK signaling pathways is required for HIV PI-induced UPR activation in macrophages, J774A.1 cells were over-expressed with DN-MEK1, then treated with amprenavir or lopinavir for 24 h. The expression of CHOP was determined by Western blot analysis. As shown in Fig. 6, over-expression of DN-MEK1 had no effect on lopinavir-induced CHOP expression, suggesting that HIV PI-induced ERK activation is not required for the UPR activation in macrophages.

### 3.6. CHOP is required for HIV PI-induced ERK activation and TNF-α and IL-6 expression

It has been reported that free cholesterol loading activates the UPR signaling pathway and all three MAPK pathways in macrophages. Free cholesterol-induced ERK activation requires the presence of CHOP, but free cholesterol-induced p38 and JNK activation is not CHOP-dependent [18]. In order to identify whether the UPR activation was implicated in HIV PI-induced ERK activation and expression of TNF-α and IL-6, primary macrophages isolated from wild type and CHOP$^{-/-}$ mice were used to address this question. We found that both lopinavir-induced ERK activation and TNF-α and IL-6 expression were diminished in CHOP$^{-/-}$ macrophages (Fig. 7). These results indicate that CHOP expression is responsible for HIV PI-induced ERK activation and subsequent increase of TNF-α and IL-6 expression in macrophages.

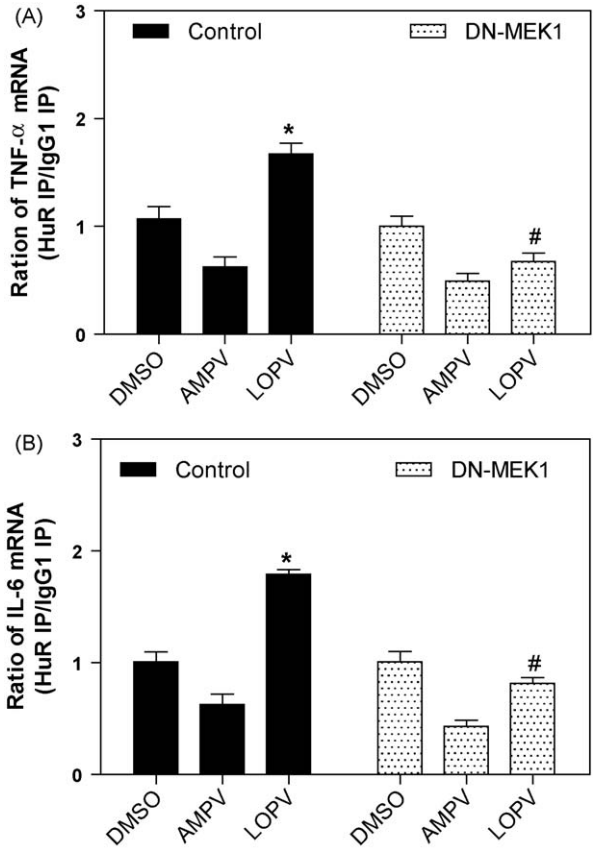


**Fig. 5.** Effects of over-expression of DN-MEK1 on HIV PI-induced increase of binding of HuR to TNF-α and IL-6 mRNAs in mouse macrophages. Mouse J774A.1 cells were infected with CMV control adenovirus or a recombinant DN-MEK1 adenovirus for 24 h and then treated with DMSO, amprenavir (AMPV, 15 μM), or lopinavir (LOPV, 15 μM) for 24 h. Total cell lysates are prepared and subjected to immunoprecipitation (IP) using either anti-HuR antibody or an isotype-matched control IgG1. mRNA levels of TNF-α and IL-6 in the IP material were detected by real-time RT-PCR using gene specific primers as described in Section 2 and expressed as relative ratio of mRNA in HuR IP over IgG1 IP. Values are mean ± SE of three independent experiments. $^{*}p < 0.05$, statistical significance relative to vehicle control, DMSO. $^{\#}p < 0.05$, statistical significance of the same HIV PI treatment between control group and DN-MEK1 group.

## 4. Discussion

HIV infection is associated with an increased risk of coronary artery disease [22]. Incorporation of HIV PIs in highly active antiretroviral therapy (HAART) has dramatically reduced mortality and morbidity of HIV-infected patients. However, increasing clinical evidence indicates that HIV PIs are the major contributors of HAART-associated atherosclerotic cardiovascular disease [23,24]. Although the exact mechanism of HIV PI-induced atherosclerosis remains largely unknown, both *in vitro* and *in vivo* studies have demonstrated that multiple mechanisms may be involved and individual HIV PIs may have different effects [1,23–25].

Atherosclerosis is associated with multiple risk factors including inflammation, endothelial dysfunction, dyslipidemia, and insulin resistance [2,26]. Inflammation plays critical roles in all the stages of atherosclerotic formation [8]. In addition to the accumulation of free cholesterol and lipids, the presence of abundant inflammatory cytokines, such as TNF-α and IL-6, in the atherosclerotic lesion is a major characteristic of atherosclerosis. It

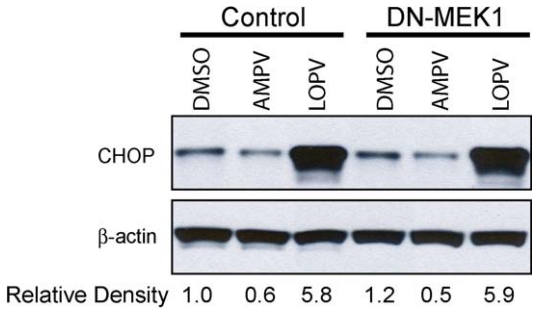


**Fig. 6.** Effect of over-expression of dominant negative mutant of MEK1 on HIV PI-induced UPR activation in macrophages. Mouse J774A.1 cells were infected with a recombinant adenovirus expressing a dominant negative mutant MEK1 (DN-MEK1) or CMV control virus. After 24 h, cells were treated with individual HIV PIs (15 μM), amprenavir (AMPV), lopinavir (LOPV) or vehicle control (DMSO) for 24 h. Total cell lysates were prepared as described in Section 2. Three independent experiments were performed. Representative immunoblots against CHOP and β-actin from each treatment group are shown.

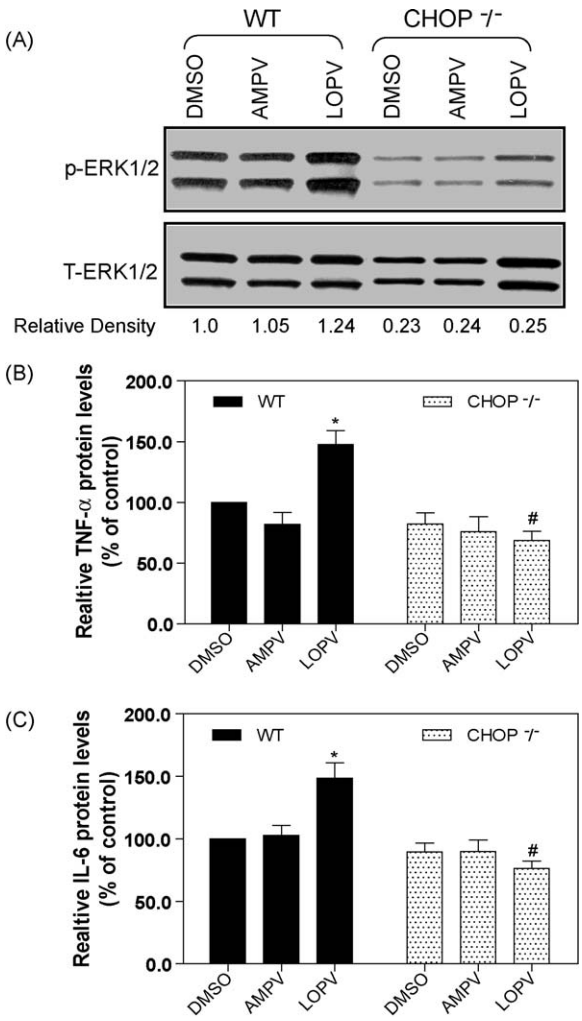


**Fig. 7.** Effect of CHOP knock out on HIV PI-induced ERK activation and expression of TNF-α and IL-6 in primary mouse macrophages. (A) Representative immunoblot images from three independent experiments against p-ERK and T-ERK from mouse primary peritoneal macrophages isolated from wild type (WT) and CHOP knock out ($CHOP^{-/-}$) and treated with vehicle control (DMSO), amprenavir (AMPV, 15 μM) or lopinavir (LOPV, 15 μM) for 24 h. The density of the immunoreactive bands was analyzed using Image J software. Relative density of p-ERK is shown. (B)–(C) Effect of $CHOP^{-/-}$ on HIV PI-induced TNF-α and IL-6 expression. Mouse primary peritoneal macrophages isolated from WT and $CHOP^{-/-}$ were treated with vehicle control (DMSO), amprenavir (AMPV, 15 μM) or lopinavir (LOPV, 15 μM) for 24 h. At the end of treatment, the culture media and cells were collected separately. The amounts of TNF-α and IL-6 released to the media were analyzed by ELISA and normalized to the total protein amounts of the viable cell pellets as described in Section 2. Values are mean ± SE of three independent experiments. $^{*}p < 0.05$, statistical significance relative to DMSO control within each group; $^{\#}p < 0.05$, statistical significance of the same treatment between WT and $CHOP^{-/-}$ groups.

is clear now that most of the inflammatory cytokines are released from macrophages in the advanced atherosclerotic lesions. Our previous studies have reported that HIV PIs activated the UPR and increased TNF-α and IL-6 expression by stabilizing mRNA levels through regulating the RNA binding protein HuR in macrophages [7,11]. However, the underlying cellular signaling mechanisms of the HIV PI-induced inflammatory response are still unknown.

In the present study, we have provided the first evidence showing that activation of ERK by HIV PIs represents a key signaling pathway involved in HIV PI-induced expression of inflammatory cytokines, TNF-α and IL-6, in macrophages. Our data also indicated that activation of ER stress response, the UPR, is required for HIV PI-induced ERK activation and subsequent increase of TNF-α and IL-6 expression in macrophages. Although it is unclear how HIV PIs activate the UPR, previous studies from our laboratories and others' suggest that depletion of ER calcium store and accumulation of intracellular free cholesterol contribute to HIV PI-induced ER stress [7]. Most recent studies reported that ER stress also can be induced by oxidative stress [27,28]. HIV PIs significantly increased generation of reactive oxygen species (ROS) in cerebral endothelial cells [29], macrophages and adipocytes [30]. Our preliminary studies also indicate that ritonavir and lopinavir markedly increase ROS production in mouse macrophages at 5–10 μM, while amprenavir had no effect at these concentrations (data not shown), suggesting that HIV PI-induced oxidative stress contributes to the activation of the UPR at least partially. However, the underlying mechanism remains to be identified and is the focus of our current ongoing research.

Regulation of the expression of inflammatory cytokines is mainly controlled at posttranscriptional levels. Modulation of mRNA stabilities is the key control point for TNF-α and IL-6 biosynthesis. Numerous studies have demonstrated that RNA binding protein HuR is a major player involved in regulating TNF-α and IL-6 mRNA stabilities during various inflammatory responses [31,32]. It also has been reported that MAPK signaling pathways are involved in regulating the expression of inflammatory cytokines [33]. Inhibition of p38 MAPK accelerated the degradation of LPS-induced increase of inflammatory cytokine mRNA levels, such as TNF-α, IL-6, and macrophage inflammatory protein-1 (MIP-1) [12,16]. MAPK p38 regulates LPS-induced COX-2 expression by modulating mRNA stability and transcription in human monocytes [34]. It also has been shown that prostaglandin A2 stabilizes p21 mRNA through an ERK-dependent pathway by increasing binding of HuR to p21 mRNA, but did not affect the cytoplasmic HuR protein levels in human non-small cell lung cancer cells [35]. Recent studies done by Skinner et al. demonstrated that LPS-induced ERK activation is responsible for regulation of TNF-α nuclear export through modulating exon junction complex proteins, TAP and NxT1, in macrophages [36]. Whether HIV PIs also regulate the nuclear export of TNF-α mRNA needs to be determined. In the current studies, we demonstrate that HIV PI-induced ER stress response and subsequent activation of ERK signaling pathway is the principal event involved in HIV PI-induced expression of TNF α and IL-6 in macrophages. Increasing evidence indicates that HuR-mediated mRNA stabilization is closely linked to its cytoplasmic presence [37]. Regulation of the subcellular localization of HuR and its binding to 3′UTR of target genes plays an important role in several types of cellular stress response including the inflammatory response [38]. The results reported here show that activation of ERK by HIV PIs not only increased the cytoplasmic HuR levels, but also increased the association of endogenous HuR with TNF-α and IL-6 mRNAs (Figs. 4 and 5). However, the underlying mechanism by which ERK regulates HuR export from the nucleus and its binding activity in response to HIV PI-induced ER stress response has not been determined. It has been reported that AMP-activated protein kinase (AMPK) is involved in regulation of HuR localization in response to metabolic stress [35]. Whether AMPK is also involved in HIV PI-induced HuR translocatioin in response to ER stress has not been examined and will be investigated in our future studies. These results also indicate that MAPKs regulate RNA binding proteins through different mechanisms in different cell types and in response to different stress signals. Although it has been previously demonstrated that MAPKs play important roles in the regulation of inflammatory cytokine expression in macrophages, the upstream mediators of MAPKs activation remain largely unknown. Recently, it has been shown that PKC-zeta is involved in mycobacteria-induced ERK activation in macrophages [39]. Whether PKC-zeta also plays a role in HIV PI-induced ERK activation will be further examined in future studies.

In summary, the findings reported in current studies suggest that ERK activation in response to ER stress signal is the key signaling pathway involved in HIV PI-induced increase of TNF-α and IL-6 expression. Based on our previous studies and current studies, we propose that HIV PI-induced oxidative stress and ER stress both contribute to HIV PI-induced ERK activation (online supplement data, Fig. S4). Activation of ERK not only affects the intracellular distribution of HuR, but also increases the RNA binding activity of HuR. Since HuR is involved in the stabilization of a number of short-lived mRNAs, HIV PI-induced changes in HuR intracellular distribution and RNA binding activity may also increase the production of other cytokines and inflammatory mediators which further increase the inflammation response. An increased awareness of the significance of cellular signaling mechanisms in HAART-associated various adverse side effects will lead to new strategies in anti-HIV therapies. Our results also suggest that inhibition of ERK may be used as a therapeutic option to prevent HIV PI-induced inflammation and atherosclerosis.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2009.03.022.

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