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ORIGINAL ARTICLE

IL-17A induction of ADAMTS-5 in differentiated THP-1 cells is modulated by the ERK signaling pathway

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ABSTRACT. Atherosclerosis is initiated when lipoproteins are trapped by proteoglycans in the arterial intima. Macrophages play a vital role in this disease, especially in the formation of foam cells and the regulation of pro-inflammatory responses. They also participate in plaque stabilization through the secretion of matrix metalloproteinases. Studies have reported the role of ADAMTS proteases in osteoarthritis and atherosclerotic lesions.In the present study, we have studied the effect of interleukin-17A (IL-17A) on the expression of ADAMTS-5 in the macrophage cell line THP-1. The results show that the mRNA and protein expression levels of ADAMTS-5 were significantly upregulated when differentiated THP-1 cells were treated with 100 ng/mL of IL-17A for 24 h with maximum ADAMTS-5 mRNA expression levels obtained at 8 h of stimulation. Subsequent inhibition studies showed that IL-17A upregulation of ADAMTS-5 was mediated through ERK and JNK pathways in THP-1 cells. Phosphorylation studies revealed that the expression of ADAMTS-5 transcripts was upregulated by IL-17A through the activation of p-c-Raf (S338), p-MEK1/2 (Ser217/221), p-p44/42 MAPK (Thr202/Tyr204), and p-Elk1 (Ser383). ERK1/2 siRNA transfection further confirmed that the ERK pathway is involved in the expression of ADAMTS-5 in IL-17A-stimulated THP-1 cells.

Key words: ADAMTS-5, interleukin-17A, atherosclerosis, signaling

INTRODUCTION

Atherosclerosis is a chronic inflammatory disorder triggered by endothelial injury and the retention of modified lipids in the arteries. The disturbance of endothelial function, usually at the branching point and bends of arteries, are more likely to cause the development of atherosclerotic lesions. At the injured site, penetration and accumulation of lipoprotein particles will take place in the subendothelial proteoglycan-rich layer of the arterial wall [1]. Monocytes will then be attracted to the injured area, pass through the endothelial wall of arteries, and differentiate into macrophages for the clearance of lipoproteins. Cholesterol-laden foam cells will eventually form when macrophages are engorged with lipids, disrupting the metabolism of lipids. Atherosclerotic plaques usually consist of infiltration of immune cells, foam cells, necrotic cores, and modified low-density lipoprotein (LDL) [2]. In advanced atherosclerosis, plaques are more prone to rupture due to the secretion of matrix metalloproteinases (MMP) by macrophages which can disrupt the extracellular matrix (ECM) of fibrous cap [3]. A prothrombotic necrotic core will be formed when lipid contents are released from dying macrophages. Rupture of atherosclerotic plaques will cause the development of intravascular blood clots and this will eventually lead to myocardial infarction and stroke [4]. A disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) proteases are a family of proteins that consists of 19 secreted metalloproteinases. The 19 members are divided into subgroups based on their known substrate and ADAMTS-1, -4, -5, -8, -9, -15 and -20 are grouped under aggrecanases or proteoglycanases [5]. The mammalian ADAMTS family is structurally related to the MMP family and some were shown to be responsible for aggrecan degradation in osteoarthritis [6]. ADAMTS-1, -4, and -5 were found to be expressed within atherosclerotic plaques and macrophages, and played a prominent role in atherosclerosis [7]. A study has reported that ADAMTS-5 proteolytic activity on versican and biglycan (major LDL-binding proteoglycans) caused the release of LDL from human aortic lesion [8].

The expressions of ADAMTS proteases in macrophages are usually regulated by cytokines. Cytokines are wide range of low-molecular-weight proteins involved in the cell signaling pathways [9, 10]. They are involved either in promoting the development of atherosclerotic plaques or in attenuating plaque formation [11]. Interleukin 17 (IL-17), a proinflammatory cytokine, consists of six isoforms (IL-17A through

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IL-17F). The signaling pathways activated by IL-17A are most fully characterized as compared with other members in the IL-17 family. IL-17A activates nuclear factor-κB (NF-κB) and components of mitogen-activated protein kinase (MAPK) pathways, including c-Jun N-terminal kinase (JNK), p38 MAPK, and extracellular signal-regulated kinase (ERK) [12].

Several studies have shown the presence of IL-17A and its role in atherosclerosis. IL-17A was increased in the plasma and tissues of apolipoprotein E–deficient ($Apoe^{-/-}$) mice. When IL-17A in $Apoe^{-/-}$ mice was blocked using adenovirus-produced IL-17 receptor A, a decrease in plaque burden could be seen in $Apoe^{-/-}$ mice fed with Western diet for 15 weeks [13]. Besides, a study also showed the reduction of atherosclerotic lesion development and the decrease in plaque vulnerability, cellular infiltration, and tissue activation in apolipoprotein E-deficient mice when IL-17A was blocked [14].

In another study, a statistically significant increase was observed in ADAMTS-5 expression when differentiated THP-1 cells were treated with 200 ng/ml of IL-17A [15]. However, the underlying signal transduction pathways of IL-17A remain largely unknown. This study successfully elucidated the detailed ERK signaling pathway undertaken by IL-17A on mediating the expression of ADAMTS-5 in differentiated THP-1 cells.

MATERIALS AND METHODS

Cell culture

Experiments were carried out using human leukaemia monocytic cell line (THP-1) (AddexBio, USA). The cells were grown and maintained in complete RPMI-1640 medium (Mediatech Inc., USA) supplemented with 10% (v/v) fetal bovine serum (FBS) (Biowest, USA), penicillin (10,000 unit/mL) and streptomycin (10,000 µg/mL) (Millipore, USA) at 37 °C in a 5% (v/v) CO_2 humidified incubator. THP-1 monocytes were differentiated into macrophages using 1 µM of phorbol myristate acetate (PMA) (Nacalai Tesque, Japan). Culture medium in the flasks was replaced after 3 days of incubation and the cells were allowed to rest for another 5 days [16, 17].

Dose and time response

Differentiated macrophages were serum starved with 0.5 % (v/v) FBS for 2 hours before treatment. For dose response studies, THP-1-differentiated macrophages were treated with various concentrations of IL-17A (Abm, Canada) (0, 50, 100, 150, and 200 ng/mL) for 24 hours. For time response studies, a single dose of IL-17A (100 ng/mL) was used to treat differentiated macrophages for several time intervals (2, 4, 8, 16, and 24 hours). Total RNA and total protein were then extracted and quantified.

Inhibition study

To investigate the signaling pathways involved, differentiated THP-1 cells were pretreated with a panel of inhibitors (25 µM of PD98059, 5 µM of ERK inhibitor [3-(2-Aminoethyl)-5-((4-ethoxyphenyl)methylene)-2,4-thiazolidinedione], 150 nM of NF-κB activation inhibitor IV, 10 µM of IKK-2 inhibitor [SC-514], 35 nM of p38 MAP Kinase inhibitor [2-(4-Chlorophenyl)-4-(4-fluorophenyl)-5-pyridin-4-yl-1,2-dihydropyrazol-3-one] [Calbiochem, Germany], 20 nM of CEP 1347 [TOCRIS Bioscience, UK], 40 nM of SP600125 [Santa Cruz Biotechnology, USA] for 2 hours before treatment with 100 ng/ml of IL-17A for 8 hours. Concentrations of each inhibitor were determined by referring to their respective IC_{50}. The vehicle control used in this experiment was 0.1% (v/v) of DMSO (Thermo Fisher Scientific, USA). Total RNA was extracted and quantified.

Phosphorylation and protein study

For the mapping of the ERK signaling pathway undertaken by IL-17A, a phosphorylation study was carried out. Differentiated macrophages were treated with four different treatments (vehicle control, 100 ng/ml of IL-17A, 25 µM of PD98059, and 100 ng/ml of IL-17A with 25 µM of PD98059 simultaneously). The cells were then harvested for protein extraction. Extracted proteins were electrophoresed using SDS-PAGE, transferred onto Immobilon-P polyvinylidene fluoride (PVDF) membrane (Merck, Germany), and immunoblotted for the detection of phosphorylation activities. Protein concentration was measured using the Bio-Rad D_c Protein Assay reagent kit (Bio-Rad Laboratories, USA). Protein samples were electrophoresed at 100 V for 60 minutes. After SDS-PAGE, protein samples from polyacrylamide gel were transferred to the PVDF membrane through wet transfer. Membranes were then blocked in 1% BSA (Biowest, USA). The antibodies used are c-Raf, phospho-c-Raf (Ser259), phospho-c-Raf (Ser338) (56A6), MEK1, phospho-MEK1 (Thr286), phospho-MEK1 (Ser298), MEK1/2, phospho-MEK1/2 (Ser217/221), p44/42 MAPK (Erk1/2), phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204), phospho-Elk-1 (Ser383), c-Jun (60A8), phospho-c-Jun (Ser73), phospho-c-Jun (S63) II, anti-rabbit IgG, HRP-linked (Cell Signaling Technology, USA), ADAMTS-5 and β-actin (Invitrogen, USA). The quantification of immunodetected protein bands was performed using the Image Lab™ version 5.2.1 software (Bio-Rad, USA).

siRNA transfection

In this study, 4×10^5 cells/mL were seeded into 24-well plate and differentiated using 1 µM of PMA. After 3 days of incubation, the culture medium in each well was replaced and the cells were allowed to rest for another 3 days. On the last day of differentiation, the culture medium was removed and replaced with 500 µL of complete medium prior to transfection. The cells were incubated under normal growth condition (37 °C and 5% CO_2) until transfection. Five microliters of p44/42 MAPK (Erk1/2) siRNA (Cell Signaling Technology, USA) was diluted to a final concentration of 100 nM. A total of 6 µL of HiPerFect

Transfection Reagent (Qiagen, Germany) was added into the diluted siRNA and mixed well. The samples were incubated at room temperature for approximately 10 minutes for the formation of transfection complexes. The complexes were added drop-wise onto the cells and the plates were gently swirled to ensure uniform distribution of transfection complexes. Cells with transfection complexes were incubated under normal growth conditions for 48 hours prior to cell lysis. Eight hours before cell lysis, 100 ng/mL of IL-17A was added. The cells were then harvested for total protein extraction.

Total RNA extraction and quantification

The extraction of total RNA from differentiated macrophages was carried out according to the manufacturer's instruction (TRI-Reagent® LS). Bleach agarose gel electrophoresis was then performed to assess the integrity of extracted RNA before qRT-PCR is carried out [18]. The purity and concentration of the extracted RNA samples were measured using Nanodrop™ 1000 Spectrophotometer (Thermo Scientific, USA).

QUANTITATIVE REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (QRT-PCR)

The one-step QuantiTect SYBR Green RT-PCR kit (Qiagen, Germany) was used for this experiment, with β-actin as a housekeeping gene. The nucleotide sequences of forward and reverse primers are as follows: ADAMTS-5 (5′-CAC TGT GGC TCA CGA AAT CG-3′; 5′-CGC TTA TCT TCT GTG GAA CCA AA-3′) [15] and β-actin (5′-CGT ACC GGC ATC GTG AT-3′; 5′-CCA TCT CTT GCT CGA AGT CC-3′). The cycling conditions for the one-step RT-qPCR are as follows: Reverse transcription at 55 °C for 30 minutes, followed by 95 °C for 10 minutes and 35 cycles of 94 °C for 15 seconds, 60 °C for 30 seconds, and 72 °C (fluorescence) for 30 seconds. A melt curve analysis was also performed at 95 °C for 1 minute, 55 °C for 1 minute, followed by 30 seconds of melt curve fluorescence read with each 5 °C increment spanning from 65 °C to 95 °C.

STATISTICAL ANALYSIS

Numerical data obtained from the experiments were displayed as mean ± standard error mean (SEM). Analysis of variance (ANOVA) and t-test program in GraphPad Prism statistics software version 7.00 were used for statistical analysis of data obtained from qRT-PCR and western blot.

RESULTS

IL-17A induced the expression of ADAMTS-5 in differentiated macrophages

ADAMTS-5 mRNA expression exhibited no significant change at 50 ng/mL of IL-17A stimulation. However, the expression level peaked at 100 ng/mL and remained stagnant as the concentration increased [*figure 1A*]. Densitometric analysis indicated that the trend of ADAMTS-5 protein expression corresponded with its mRNA expression levels. Treatment of cells with 100 ng/mL of IL-17A demonstrated a significant increase in ADAMTS-5 protein expression as compared to the untreated sample. As the concentration of IL-17A was increased to 150 and 200 ng/mL, the protein expression reduced slightly. However, protein levels of β-actin were unaffected by the treatments [*figure 1B*].

In time course experiments, the cells were treated with a single dose of IL-17A (100 ng/mL) for a few different time frames (2, 4, 8, 16, and 24 hours). A slight increase in ADAMTS-5 expression was observed at 2 and 4 hours of incubation with IL-17A. At 8 hours of incubation, a significant upregulation of ADAMTS-5 was observed. However, the expression level of ADAMTS-5 decreased at 16 and 24 hours [*figure 1C*]. Hence, the incubation period of 8 hours with 100 ng/mL of IL-17A was chosen for subsequent experiments.

ERK and JNK pathway inhibitors attenuated the expression of ADAMTS-5

CEP 1347 and SP600125 are inhibitors of the JNK pathway, whereby ERK inhibitor and PD98059 are inhibitors of the ERK pathway. NF-κB activation inhibitor IV and IKK-2 inhibitor are inhibitors targeting the NF-κB pathway and p38 MAP kinase inhibitor targets the p38 MAPK pathway. These inhibitors were selected as they are involved in the pathways potentially undertaken by IL-17A to regulate ADAMTS-5 expression.

Cells treated with IL-17A only (control) showed an increase in ADAMTS-5 expression, relative to vehicle control (VC). Treatment with CEP 1347 and SP600125 led to a significant reduction in ADAMTS-5 expression, relative to control (*figure 2*). However, the most significant drop in ADAMTS-5 expression level was observed when the cells were treated with PD98059 in which the expression was reduced to the basal level. Treatment with ERK inhibitor also caused a significant decrease in ADAMTS-5 expression. This indicated that ERK and JNK could be the possible pathways undertaken by IL-17A to modulate ADAMTS-5 expression in THP-1 cells (*figure 2*). Cells treated with NF-κB activation inhibitor IV, IKK-2 inhibitor, p38 MAP kinase inhibitor displayed a nonsignificant reduction in ADAMTS-5 expression.

IL-17A-induced ADAMTS-5 expression was through the activation of a series of kinases of the ERK signaling pathway

To elucidate the possible signaling mechanism that led to a rise in ADAMTS-5 expression in IL-17A-stimulated cells, the changes in the level of protein kinases were investigated through western blot analysis. Since PD98059 (MEK inhibitor) exhibited the most significant reduction in the ADAMTS-5 mRNA expression level, this study was focused mainly on this pathway.

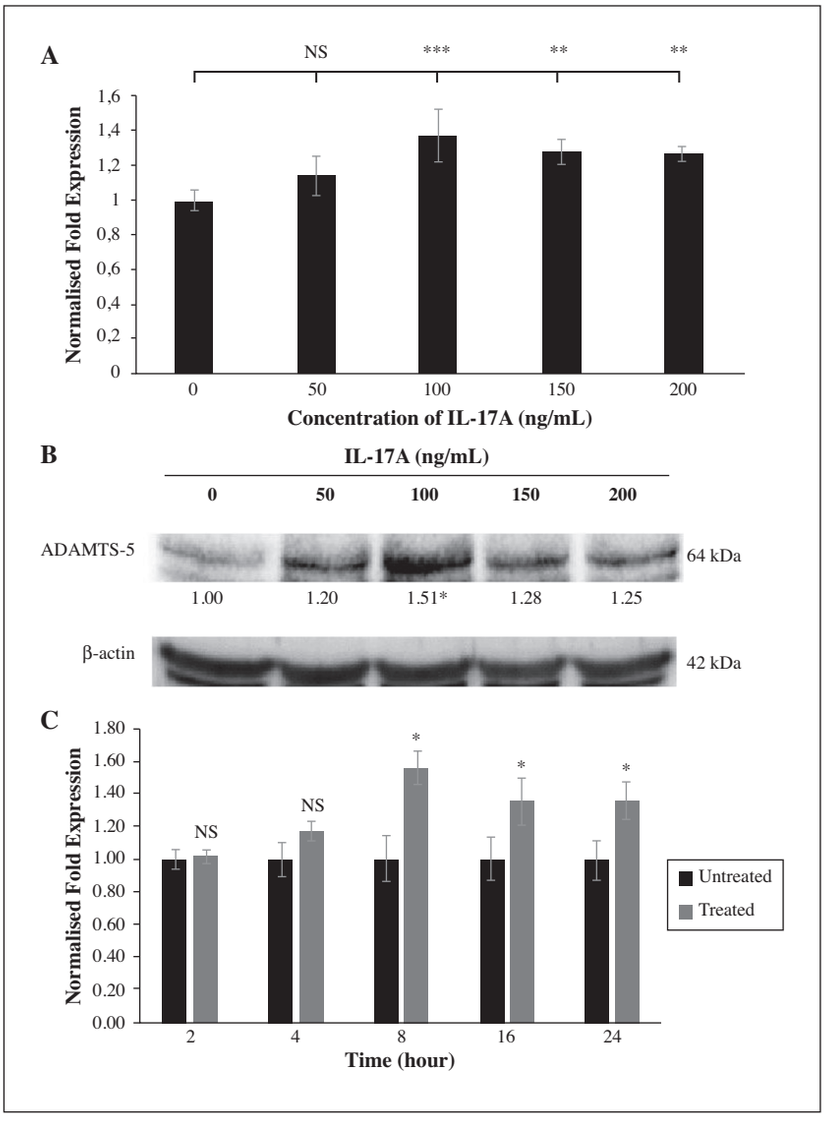

Figure 1

Dose and time response effect of IL-17A on ADAMTS-5 expression in THP-1-differentiated macrophages. For dose response study, THP-1-differentiated macrophages were treated with different concentrations of IL-17A for 24 hours before they were harvested for mRNA and protein analyses. **A)** ADAMTS-5 mRNA expression level was measured by qRT-PCR. Values represent means ± SEM of ADAMTS-5 mRNA from triplicate of three independent experiments (n = 9) normalized to β-actin mRNA and relative to untreated cells values (control). **B)** Western blot analyses of ADAMTS-5 and β-actin. All the lanes were loaded with 25 µg of proteins and probed with the respective antibodies. Secondary HRP-conjugated anti-rabbit IgG antibody was then used for detection. Values shown are the fold changes of ADAMTS-5 protein expression after normalization with β-actin and relative to untreated sample (1.00). **C)** For time response study, differentiated THP-1 cells were treated with a single dose of IL-17A (100 ng/mL) for several time frames. ADAMTS-5 mRNA expression level was measured by qRT-PCR. The mRNA expression levels were normalized to β-actin mRNA and then compared with their untreated sample at the respective time point. Values represent means ± SEM of ADAMTS-5 mRNA from triplicate of three independent experiments (n = 9). Statistical significance is indicated by $*P < 0.05$; $**P < 0.01$; $***P < 0.001$; NS, not significant.

Treatment of cells with IL-17A increased the expression level of ADAMTS-5 protein. However, the expression was downregulated when PD98059 was added before the treatment with IL-17A. The expression level of phospho-c-Raf (Ser338) protein was shown to be upregulated when IL-17A was added, but not phospho-c-Raf (Ser259). The addition of inhibitor significantly reduced the expression of phospho-c-Raf (Ser338) protein, whereas the latter did not show any significant changes (*figure 3b, c*).

Phospho-MEK1/2 (Ser217/221) protein was significantly upregulated when THP-1-differentiated macrophages were treated solely with IL-17A (*figure 3e*). Pretreatment with inhibitor caused a significant decrease in its expression. However, no significant changes were observed for both phospho-MEK1 (Thr 286) and phospho-MEK1 (Ser298) proteins.

As for phospho-p44/42 MAPK (Thr202/Tyr204) protein, a notable increase in the expression level for both phospho-p44 and -p42 MAPK was observed with the presence of IL-17A (*figure 3j*) Interestingly, pretreatment with PD98059 led to a dramatic decrease in band intensity as well as the expression level for both phospho-p44 and -p42 MAPK.

No significant changes were observed in the expression levels of phospho-c-Jun (Ser63) II and phospho-c-Jun (Ser73) protein. However, a significant rise in phospho-Elk1 (Ser383) protein level was seen in the presence of

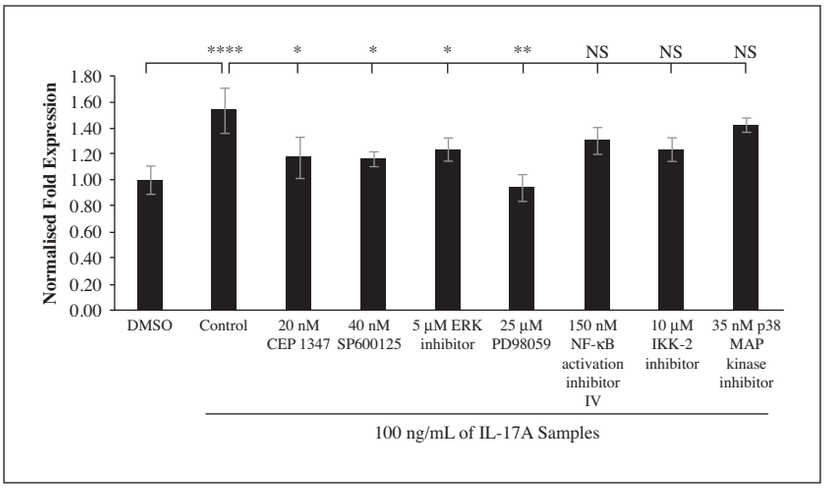

Figure 2
Inhibitory effect of different inhibitors toward ADAMTS-5 mRNA expression. THP-1-differentiated macrophages were serum-starved and then pretreated with inhibitors for 2 hours before 100 ng/mL of IL-17A was added for another 8 hours. ADAMTS-5 mRNA expression level was analyzed using qRT-PCR. Values represent means ± SEM of ADAMTS-5 mRNA from triplicate of three independent experiments (n = 3) normalized to β-actin mRNA, relative to the values of control (cells treated with IL-17A only). Statistical significance is indicated by $*P < 0.05$; $**P < 0.01$; $****P < 0.0001$; NS, not significant.

IL-17A and the expression level diminished when the cells were prestimulated with inhibitor.

Silencing of IL-17A-activated ERK1/2 pathway

To further confirm the results obtained from the phosphorylation study, p44/42 MAPK (ERK1/2) siRNA was employed to induce silencing of ERK pathway in THP-1-differentiated macrophages. Before the main experiment was conducted, time response was performed to determine the optimum time required for siRNA to induce its silencing effect. A statistically significant downregulation was observed in both p44 and p42 protein expression at 48 hours of treatment [*figure 4A*]. However, no significant changes were observed at other time points. Thus, the incubation period of 48 hours with 100 nM of ERK1/2 siRNA was chosen for the subsequent experiment. As shown in *figure 4B*, densitometry analysis showed the protein expression levels for both ADAMTS-5 and phospho-p44/42 (Thr202/Tyr204) were highly elevated with the presence of IL-17A. However, a sharp reduction in band intensity for both ADAMTS-5 and phospho-p44/42 (Thr202/Tyr204) was observed when the cells were pretreated with ERK1/2 siRNA. The ADAMTS-5 protein level was almost reduced to the basal level. Phospho-p44 and 42 MAPK expression also declined significantly.

DISCUSSION

IL-17A is the most well-studied subset in the IL-17 family. It is an inflammatory cytokine that plays a crucial host-defensive role in numerous infectious diseases [19]. IL-17 receptor A (IL-17RA) recruits adaptor molecules to activate and mediate downstream signaling [20]. Numerous studies have shown that cytokines such as IL-1β, TNF-α, and TGF-β directly enhanced the expression of ADAMTS-5 [15, 21]. Although there are reports of indirect effect on IL-17A production by TNF-α inhibition in psoriatic skin lesions or the indirect induction of other pro-inflammatory cytokines by IL-17A stimulation [22, 23], any information on the indirect effect of IL-17A on ADAMTS-5 expression is still lacking. This current study investigated the direct effects of IL-17A on the mRNA and protein expressions of ADAMTS-5 and the signaling pathway undertaken. A study published in 2013 reported a significant increase in ADAMTS-5 mRNA expression when THP-1 macrophages were treated with 200 ng/mL of IL-17A [15]. However, in this study, the ADAMTS-5 mRNA expression level was the highest when the cells were stimulated with 100 ng/mL of IL-17A. The possible explanation for this inconsistency is that different protocols were used in the differentiation of THP-1 cells. A previous study differentiated the cells with a much lower concentration of PMA for a shorter period. In addition, the same author also reported that IL-17A alone had a minimal effect on the expression of ADAMTS-5 while displaying a synergistic effect with TL-1A [15]. In this current study, ADAMTS-5 expression was also analyzed at its protein level. The trend of expression corresponded with its mRNA expression, where the highest ADAMTS-5 expression was observed at 100 ng/mL [*figure 1B*]. For a time response study, the most significant expression was observed at 8 hours of incubation with 100 ng/mL of IL-17A [*figure 1C*]. A recent study reported that ADAMTS-5 gene expression in condyle and fossa cells was enhanced at 6 and 24 hours, respectively, after treatment with 10 ng/mL of IL-1β [24]. Thus, it can be concluded that the type of cell lines and treatments are the important factors in determining the time required for gene expression.

IL-17A is said to activate several signaling pathways such as NF-κB and MAPKs to activate the expression of downstream target genes [25]. The MAPKs are grouped into three families, mainly ERKs, JNKs, and p38 MAPK [26]. In this study, the NF-κB and p38 MAPK pathways were ruled out because inhibitors targeting both pathways exhibited a nonsignificant

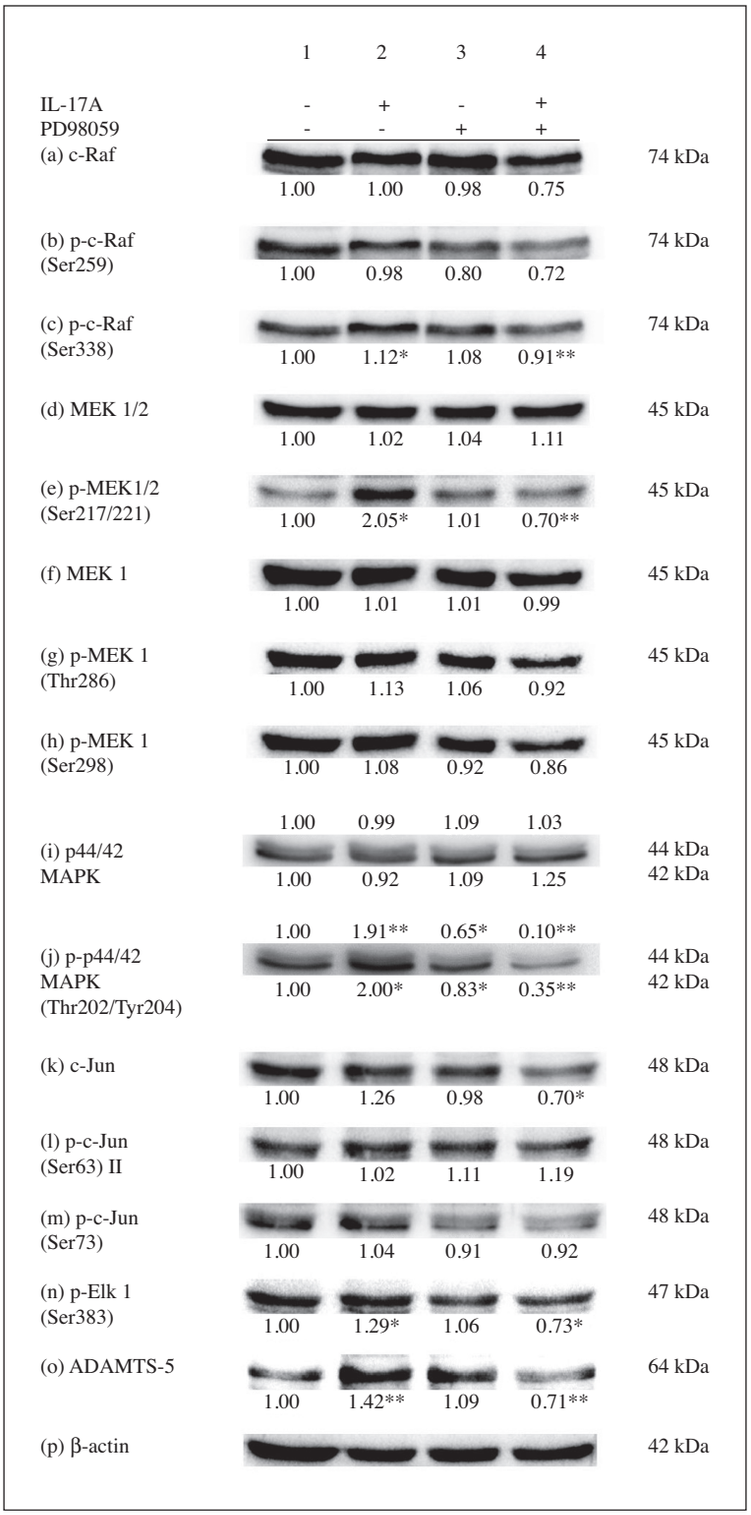

Figure 3

Representative western blots for all the protein kinases tested. THP-1-differentiated macrophages were pretreated with or without 25 μM of PD98059 for 2 hours before the addition of IL-17A (100 ng/mL) for 8 hours. Lanes 1 to 4 were loaded with 25 μg of proteins extracted from THP-1-differentiated macrophages under different treatment conditions. Lane 1 is the vehicle control (DMSO). Quantification for total protein was done by normalizing the density of each band against β-actin expression while phosphorylated proteins were first normalized against total proteins and then against β-actin. Normalized band densities are recorded beneath each blot. Values shown were the representation of three independent experiments (n = 3). $*P < 0.05$ and $**P < 0.01$ indicate significant difference from respective group. Lanes 2 and 3 were compared to Lane 1, whereas Lane 4 was compared to Lane 2 for statistical significance.

suppression of ADAMTS-5 mRNA expression in IL-17A-treated cells (*figure 2*). However, some studies have revealed that ADAMTS-5 expression was mediated through the NF-κB pathway in both human and mouse chondrocytes when stimulated with leptin and RelA/p65, respectively [27, 28]. This discrepancy may be due to treatment and cell line disparity. Following treatment with JNK (CEP 1347 and SP600125) and MEK (PD98059) inhibitors, ADAMTS-5 expression exhibited significant attenua-

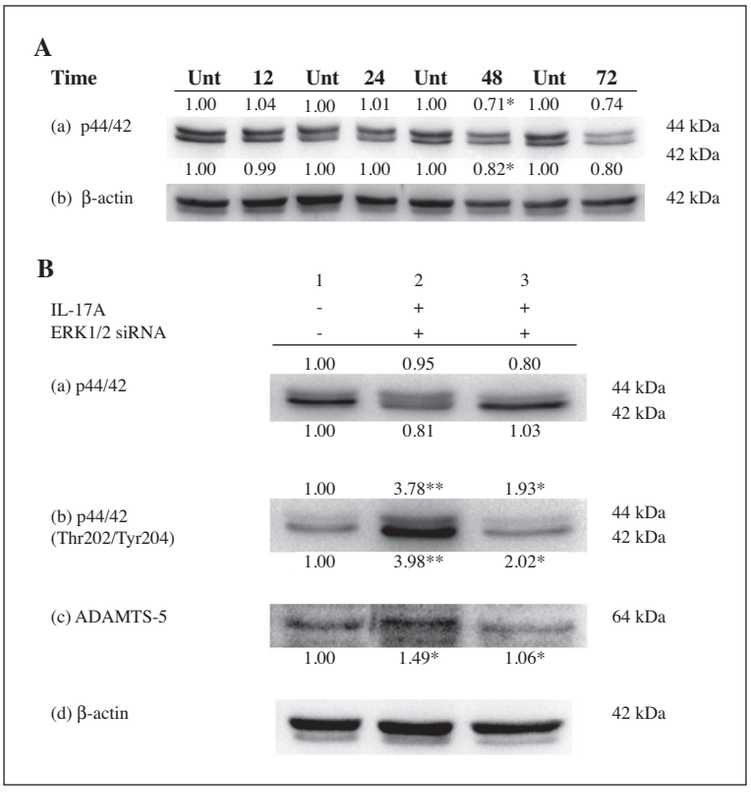

Figure 4

A) Representative western blots for (a) p44/42 and (b) β-actin expression in THP-1-differentiated macrophages in siRNA time course study. THP-1-differentiated macrophages treated with or without 100 nM of ERK1/2 siRNA for several time points (12, 24, 48, and 72 hours). Quantification was done by normalizing the density of each band against β-actin expression and then compared with their untreated sample at respective time points. Values shown were the representation of three independent experiments (n = 3). **B)** Representative western blots for (a) p44/42, (b) p-p44/42 (Thr202/Tyr204), (c) ADAMTS-5, (d) β-actin expression in THP-1-differentiated macrophages. THP-1-differentiated macrophages were pretreated with or without 100 nM of ERK1/2 siRNA for 48 hours prior to cell lysis. The cells were then treated with or without 100 ng/mL of IL-17A for 8 hours before they were lysed. Lanes 1 to 3 were loaded with 25 μg of proteins extracted from THP-1-differentiated macrophages under different treatment conditions. Quantification for total protein was done by normalizing the density of each band against β-actin expression while phosphorylated proteins were first normalized against total proteins and then against β-actin. Band densities are recorded beneath each blot. Values shown were the representation of four independent experiments (n = 4). $*P < 0.05$ and $**P < 0.01$ indicate significant difference from respective group. For statistical significance, Lane 2 was compared to Lane 1, whereas Lane 3 was compared to Lane 2.

tion in IL-17A-stimulated cells. A previous study showed similar findings whereby IL-1β-induced expression of ADAMTS-5 in rat articular chondrocytes was suppressed by the inhibitors of JNK (10 μM of SP600125) and ERK (50 μM of FR180204) pathway [29]. Osteoprotegerin-induced ADAMTS-5 expression was also shown to be altered when the ERK activity was suppressed by 10 μM of PD98059 [30]. Another study in 2004 also demonstrated that the IL-17 upregulations of MMP-3, MMP-13, and ADAMTS-4 in bovine chondrocytes were mediated through the ERK, JNK, and p38 MAPK pathways [31].

This research focused on the mapping of the ERK pathway because pretreatment with PD98059 (MEK inhibitor) exhibited the most significant inhibition in ADAMTS-5 expression. MEK is the upstream kinase of the ERK pathway. PD98059 acts by hindering the activation of MEK1 and MEK2 by upstream kinase such as c-Raf. The inhibition effect will eventually block the subsequent phosphorylation of kinases in the ERK pathway [32].

Our findings demonstrated that c-Raf was phosphorylated at Ser338 with the presence of IL-17A but not at Ser259. Phosphorylation at Ser338 also diminished significantly with PD98059 treatment (*figure 3c*). The result obtained here is supported by various findings highlighting the importance of c-Raf phosphorylation at Ser338 for c-Raf activation, whereas the phosphorylation at Ser259 interfered with c-Raf activation [33-35]. Besides, prominent upregulation in phosphorylation of MEK1/2 at Ser217 and Ser221 was observed (*figure 3e*). This indicated that the activation of c-Raf by IL-17A subsequently led to the activation of MEK1/2 in THP-1 macrophages. The result obtained here is supported by two recent findings that postulated the activation of MEK1/2 by c-Raf was through phosphorylation of two serine residues, Ser217 and Ser221 [36, 37]. ERK1 and ERK2 are members of the MAPK family. This pathway can be activated by extracellular stimuli such as cytokines and growth factors [38]. Data presented in this study showed that p44/42 MAPK (ERK1/2) was involved in the upregulation of ADAMTS-5 expression (*figure 3j*). Upstream kinase MEK1/2 activates ERK1 and ERK2 through dual phosphorylation of Thr202/Tyr204 and Thr185/Tyr187, respectively [39, 40].

ERK1/2 activates several downstream targets such as p90RSK and transcription factor Elk-1 [41]. Transcription factors play a crucial role in modulating the expression of a network of genes through binding to

their promoter region [42]. Elk-1 is a member of the E twenty-six (Ets) family of transcription factor. It is involved in numerous biological processes such as cellular growth, differentiation, hematopoiesis, inflammation, cancer, and angiogenesis [43]. In this study, Elk-1 was shown to be activated by IL-17A through phosphorylation at Ser383 (*figure 3n*). The Ets sequence GGA(A/T) can be found in the ADAMTS-5 promoter [44]. The presence of putative binding sites for the Ets family of transcription factors in the ADAMTS-5 promoter suggests that Elk-1 could be the primary regulatory factor in the regulation, but no decisive evidence is available in this present study. However, there is another study that reported that ERK1/2 upregulated ADAMTS-5 through RUNX2 transcription factor in human articular cartilage and intervertebral disc [45]. This shows that ERK1/2 may also govern the activation of multiple transcription factors. However, c-Jun transcription factor is not involved in the upregulation of ADAMTS-5 in THP-1 macrophages as it was not phosphorylated at both Ser63 and Ser73 with the presence of IL-17A (*figure 3l, m*).

ERK1/2 siRNA was employed to verify the results obtained from the phosphorylation study. The results from the optimization of the transfection period showed that ERK1/2 siRNA exhibited the most prominent ERK1/2 silencing at 48 hours [*figure 4 (i)*]. This result corroborated with Si and colleagues who used the same period of incubation time and concentration of ERK1/2 siRNA in osteosarcoma cells to suppress ERK1/2 expression [46]. A previous study showed efficient knockdown of ERK1/2 at the protein level when treated with ERK1/2 siRNA, but the ADAMTS-5 expression was not examined [47]. The data obtained from the silencing experiment clearly revealed that the ERK signaling cascade was undertaken by IL-17A in the induction of ADAMTS-5 expression [*figure 4(ii)*].

In conclusion, we have demonstrated that IL-17A significantly induced the mRNA and protein expression of ADAMTS-5 in differentiated THP-1 cells. Besides, we also showed that IL-17A-induced ADAMTS-5 expression was through the activation of a series of kinases of the ERK cascade. The study provides novel insights on the regulation of ADAMTS-5 protease by the key cytokine implicated in atherosclerosis. For future study, an overexpression study can be performed to further verify the involvement of the pathway. Other than that, ADAMTS-5 promoter-driven luciferase reporter assay, ChIP, or EMSA analysis could be carried out to show the effect of ERK pathway inhibitor (PD98059) on the IL-17A-induced ADAMTS-5 promoter activity. As this study was carried out *in vitro* using cell line, it is unclear whether the effects of IL-17A on ADAMTS-5 gene expression and signaling pathways involved are reproducible in *in vivo* conditions. Hence, this study could be further conducted in *in vivo* conditions by producing an ERK knockout mice model. These experiments should further improve our understanding and provide more insights on the complexities of signaling mechanisms of IL-17A on ADAMTS-5 gene expression.

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