The International Journal of Biochemistry & Cell Biology 46 (2014) 113–123

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

The anti-atherogenic cytokine interleukin-33 inhibits the expression of a disintegrin and metalloproteinase with thrombospondin motifs-1, -4 and -5 in human macrophages: Requirement of extracellular signal-regulated kinase, c-Jun N-terminal kinase and phosphoinositide 3-kinase signaling pathways

Tim G. Ashlin[a], Melanie L. Buckley[a], Rebecca C. Salter[b], Jason L. Johnson[b], Alvin P.L. Kwan[a], Dipak P. Ramji[a,*]

[a] Cardiff School of Biosciences, Cardiff University, Sir Martin Evans Building, Museum Avenue, Cardiff CF10 3AX, United Kingdom
[b] Laboratory of Cardiovascular Pathology, School of Clinical Sciences, University of Bristol, Bristol Royal Infirmary, Bristol BS2 8HW, United Kingdom

ARTICLE INFO

Article history:
Received 25 June 2013
Received in revised form 18 October 2013
Accepted 5 November 2013
Available online 22 November 2013

Keywords:
ADAMTS
Cytokine action
Cardiovascular disease
Macrophages
Signal transduction

ABSTRACT

Atherosclerosis is an inflammatory disorder of the vasculature regulated by cytokines. Amongst the cytokines, IL-33 attenuates the development of atherosclerosis in mouse model systems *via* several mechanisms, including inhibition of macrophage foam cell formation and promotion of a Th1 to Th2 shift. Proteases produced by macrophages, such as matrix metalloproteinases and members of ADAMTS (a disintegrin and metalloproteinase with thrombospondin motifs) family, play potential roles in regulating atherosclerotic plaque stability. Despite such importance, the action of IL-33 on the expression of such proteases has not been analyzed. We have therefore investigated the effect of IL-33 on the expression of ADAMTS-1, -4 and -5 in human macrophages. Immunohistochemical analysis showed that these three proteases were expressed in human atherosclerotic lesions, particularly by macrophages and, to a lesser extent, by smooth muscle cells and endothelial cells. The expression of ADAMTS-1, -4 and -5 in human macrophages was specifically inhibited by IL-33. The action of IL-33 on the expression of these ADAMTS members was mediated through its receptor ST2. IL-33 activated ERK1/2, JNK1/2 and c-Jun, but not p38 MAPK or Akt, in human macrophages. RNA interference assays using a combination of adenoviral encoding small hairpin RNA and small interfering RNA showed a requirement of ERK1/2, JNK1/2, c-Jun, PI3Kγ and PI3Kδ, but not p38α, in the IL-33-inhibited expression of these ADAMTS isoforms. These studies provide novel insights into the expression of ADAMTS-1, -4 and -5 in human atherosclerotic lesions and the regulation of their expression in human macrophages by the key anti-atherogenic cytokine IL-33.

© 2013 The Authors. Published by Elsevier Ltd. Open access under CC BY-NC-ND license.

1. Introduction

Atherosclerosis, an inflammatory disease of the vasculature, is responsible for most deaths in the western world. Although the discovery of statins has had a major impact in reducing mortality from atherosclerosis and its complications, many clinical studies have highlighted the significance of residual cardiovascular risk in patients on statin therapy (McLaren et al., 2011; Michael et al., 2012a). It is therefore important to target other risk factors and to understand the molecular basis of the disease in detail. Macrophages play a pivotal role in all stages of atherosclerosis, including the production of proteases that control the remodeling of the extracellular matrix (ECM) and, thereby, plaque stability (McLaren et al., 2011; Michael et al., 2012a). From these proteases,

Abbreviations: ADAMTS, a disintegrin and metalloproteinase with thrombospondin motifs; apoE, apolipoprotein E; BMDM, bone marrow-derived macrophages; DAB, 3,3′-diaminobenzidine; ECM, extracellular matrix; HMDM, human monocyte-derived macrophages; IHC, immunohistochemistry; MMP, matrix metalloproteinase; RT-qPCR, real-time quantitative PCR; shRNA, small hairpin RNA; siRNA, small interfering RNA.

* Corresponding author. Tel.: +44 2920876753; fax: +44 2920874116.
E-mail address: Ramji@cardiff.ac.uk (D.P. Ramji).

1357-2725 © 2013 The Authors. Published by Elsevier Ltd. Open access under CC BY-NC-ND license.
http://dx.doi.org/10.1016/j.biocel.2013.11.008

the role of the matrix metalloproteinase (MMP) family in atherogenesis has been extensively investigated (Newby, 2012). However, recent studies are also indicating a potentially important role for a disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) family in atherosclerosis (Salter et al., 2010).

The ADAMTS proteases are secreted enzymes able to interact with, and degrade, components of the ECM, such as procollagen, aggrecan, versican, cartilage oligomeric matrix protein, biglycan, decorin and fibromodulin (Salter et al., 2010). Recent evidence suggests important roles for some members in atherosclerosis and cardiovascular disease (Salter et al., 2010). For example, several ADAMTS members are expressed in atherosclerotic plaques (Jonsson-Rylander et al., 2005; Wagsater et al., 2008; Salter et al., 2010; Lee et al., 2011, 2012; Didangelos et al., 2012). In addition, genome wide association studies have linked ADAMTS-7 to coronary atherosclerosis and pediatric stroke (Reilly et al., 2011; Arning et al., 2012). Furthermore, elevated levels of ADAMTS-4 have been found in the plasma and peripheral monocytes of patients with acute coronary syndrome (Zha et al., 2010). Moreover, an association between serum ADAMTS-4 levels and the severity of coronary artery disease has been observed (Chen et al., 2011).

Because atherosclerosis is an inflammatory disorder orchestrated by cytokines (McLaren et al., 2011; Michael et al., 2012a), it is important to fully understand their impact on the expression of the ADAMTS family members. The expression of ADAMTS-4, -7, -8 and -9 mRNA was first shown to be induced in macrophages by the cytokines IFN-γ or TNF-α (Wagsater et al., 2008). In contrast, the expression of ADAMTS-1 was inhibited by IFN-γ (Wagsater et al., 2008). The anti-atherogenic cytokine TGF-β inhibited the expression of ADAMTS-4 in human macrophages and small interfering RNA (siRNA)-mediated knockdown studies identified important roles for Smads, p38 MAPK and c-Jun in this response (Salter et al., 2011). However, the effect of other key anti-atherogenic cytokines on the expression of ADAMTS family in macrophages along with the underlying mechanisms has not yet been investigated.

IL-33 is a recently characterized member of the IL-1 family of cytokines and promotes T_h2 type immune responses by signaling through the ST2 and IL-1RAcP dimeric receptor complex (Kakkar and Lee, 2008). The activation of the ST2 receptor by IL-33 has been shown to stimulate multiple signaling pathways including NF-κB, MAPK and PI3K (Brint et al., 2002; Funakoshi-Tago et al., 2008, 2011; Choi et al., 2009; Tare et al., 2010; Yagami et al., 2010; Kamekura et al., 2012). Recent studies indicate a protective role for the IL-33-ST2 axis in atherosclerosis (Miller and Liew, 2011). Thus, exogenous administration of IL-33 induced a T_h1 to T_h2 shift within the plaques of an apolipoprotein E (apo$E^{-/-}$) mouse model of diet-induced atherosclerosis (Miller et al., 2008). In addition, injection of IL-33 resulted in significantly smaller atherosclerotic lesions compared to vehicle treated controls (Miller et al., 2008). Furthermore, IL-33 treatment significantly reduced the accumulation of macrophage-derived foam cells in atherosclerotic plaques (McLaren et al., 2010b). IL-33 also reduced macrophage foam cell formation *in vitro* by decreasing acetylated and oxidized LDL uptake, reducing intracellular total and esterified cholesterol content and enhancing cholesterol efflux (McLaren et al., 2010b). These changes were associated with IL-33-mediated reduction in the expression of key genes involved in modified LDL uptake, such as CD36, and simultaneous increase in the expression of genes involved in cholesterol efflux, including apoE (McLaren et al., 2010b). The use of bone marrow-derived macrophages (BMDM) from $ST2^{-/-}$ mice demonstrated that this receptor is integral to the action of IL-33 on macrophage foam cell formation (McLaren et al., 2010b).

We present here studies on the expression of ADAMTS-1, -4 and -5 within atherosclerotic lesions from human coronary arteries, and the effect of IL-33 on their levels in human macrophages. We demonstrate for the first time that IL-33 reduces the expression of ADAMTS-1, -4 and -5 and this requires the ST2 receptor. In addition, using a combination of biochemical analysis and RNA interference assays, we show a requirement for ERK, JNK and PI3K signaling in the response.

2. Methods

2.1. Reagents

All chemicals were purchased from Sigma–Aldrich (Poole, UK) unless otherwise stated. Recombinant human and mouse IL-33 were supplied by Peprotech (London, UK).

2.2. Cell culture

The human acute leukemia cell line THP-1 and human monocyte-derived macrophages (HMDM) were grown in complete RPMI-1640 supplemented with 10% (v/v) heat-inactivated FCS, penicillin (100 U/ml), streptomycin (100 μg/ml) and L-glutamine (2 mmol/L) at 37 °C in a humidified atmosphere containing 5% (v/v) CO_2. HMDM were differentiated from monocytes isolated from buffy coats supplied by the Welsh Blood service using Ficoll-Hypaque purification described elsewhere (McLaren et al., 2010a; Michael et al., 2012b). THP-1 monocytes were differentiated into macrophages using 0.16 μM PMA for 24 h. In all experiments, unless otherwise stated, macrophages were incubated with IL-33 (10 ng/ml) for 24 h. Recombinant IL-33 was reconstituted in PBS/0.1% (w/v) BSA that was subsequently used as a vehicle control.

2.3. Human coronary artery sections

Human coronary artery specimens were collected from cadaveric heart donors to the Bristol Coronary Artery Biobank under National Research Ethics Service approval from Frenchay Research Ethics Committee reference 08/H0107/48. The left and right coronary arteries were dissected within 48 h of death and pressure fixed at 100 mmHg with 4% (v/v) paraformaldehyde for 24 h at 4 °C. After paraffin embedding, serial 5 μm sections were used for immunohistochemistry (IHC) (Huang et al., 2012).

2.4. Real-time quantitative PCR (RT-qPCR)

RNA extraction, reverse transcription and qPCR analysis were performed as described elsewhere (McLaren et al., 2010a,b; Michael et al., 2012b). Oligonucleotide sequences are shown in Supplementary Table I and were purchased from Sigma–Aldrich. Fold changes in expression were calculated using $2^{-(\Delta Ct1-\Delta Ct2)}$, where ΔCt represents the difference between the threshold cycle (Ct) for each target gene and housekeeping mRNA transcript levels (McLaren et al., 2010a). Melting curve analysis was performed on each primer set to confirm amplification of a single product and all amplicons were sequenced to ensure reaction specificity (data not shown).

2.5. Western blotting

Total cell lysates were size-fractionated and analyzed by western blotting as previously described (McLaren et al., 2010a,b; Michael et al., 2012b). Samples were subjected to electrophoresis alongside comparative molecular weight markers (GE Healthcare, Wisconsin, USA) to determine the size of the protein product. Antibodies were from Cell Signaling Technologies (Danvers, MA, USA) [phospho p44/p42 Thr202/Tyr204 (9101), total p44/p42 (9102), phospho p38 Thr180/Tyr182 (9211), total p38 (9212), phospho Akt Ser473 (9271) and total Akt (9272)], Santa Cruz Biotechnology (Santa Cruz, CA, USA) [phospho-c-Jun Ser63 (sc-822) and total c-Jun (sc-1694)], and Sigma–Aldrich [β-actin (A2228)].

2.6. RNA interference

siRNA transfections were carried out using validated siRNAs against human c-Jun (SI00300580), JNK-1 (SI02758637), JNK-2 (SI02222920) and ERK-2 (SI00300755) from Qiagen (Manchester, UK). Negative control siRNA (AM4611) was from Invitrogen (Paisley, UK). Stock solutions were prepared from lyophilized siRNAs according to the manufacturer's instructions (Qiagen and Invitrogen) and used at a final concentration of 7.5 nM. siRNA transfection was carried out in THP-1 cells using INTERFERin^{TM} prior to differentiation with 0.16 μM PMA according to the manufacturer's instructions (Polyplus Transfection) (Source Biosciences LifeSciences, Nottingham, UK) as previously described (Salter et al., 2011; Michael et al., 2012b). Cells were then subjected to cytokine stimulation for the requisite time period before being harvested for RNA extraction.

The production of recombinant adenovirus encoding small hairpin RNA (shRNA) that target ERK-1 or -2 along with scrambled sequence has been previously described (McLaren et al., 2010a; Li et al., 2010). A similar approach was used to prepare adenovirus-encoding shRNA against p38α, PI3K-γ and -δ (McLaren et al., 2010a; Li et al., 2010; Michael et al., 2012b) (see Supplementary Table 2 for the oligonucleotide sequences used for the preparation of these recombinant viruses). The recombinant virus was added to the cells at a multiplicity of infection of 100, as optimized in our previous studies (McLaren et al., 2010a; Li et al., 2010; Michael et al., 2012b). The infection was left for 2.5 h at 37 °C, 5% (v/v) CO_2, and then the differentiation performed with 0.16 μM PMA. The cells were then incubated for 48 h at 37 °C, 5% (v/v) CO_2, followed by cytokine stimulation and harvesting for RNA extraction.

2.7. IHC

Serial 5 μm paraffin sections were de-waxed, rehydrated and the endogenous peroxidase activity inhibited as previously described (Scholtes et al., 2012). After rinsing, sections were heated in 10 mM citrate buffer (pH 6.0) for 2 periods of 6 min. Following blocking with 20% (v/v) goat serum in PBS, sections were incubated overnight at 4 °C with antibodies against human ADAMTS-1 (Santa Cruz, sc-25581), ADAMTS-4 (Thermo Fisher Scientific, PA1-1749) and ADAMTS-5 (Santa Cruz, sc-134952), or an antibody against human macrophage CD68 (Dako, Cambridgeshire, UK, PGM1) or human α-smooth muscle actin (Dako, 1A4) in 1% (w/v) BSA in PBS. After overnight incubation, sections were washed and incubated with an appropriate biotinylated secondary antibody, followed by incubation with extravidin horseradish peroxidase (Sigma–Aldrich, St Louis, MO) and stained with 3,3-diaminobenzidine (DAB) (Sigma–Aldrich). The sections were then counterstained with hematoxylin, mounted in DPX mountant and visualized on a light microscope (Leica).

2.8. Statistical analysis

All data are presented as mean (±SD) on the assigned number of independent experiments where, in experiments involving HMDM, this refers to the number of independent experiments

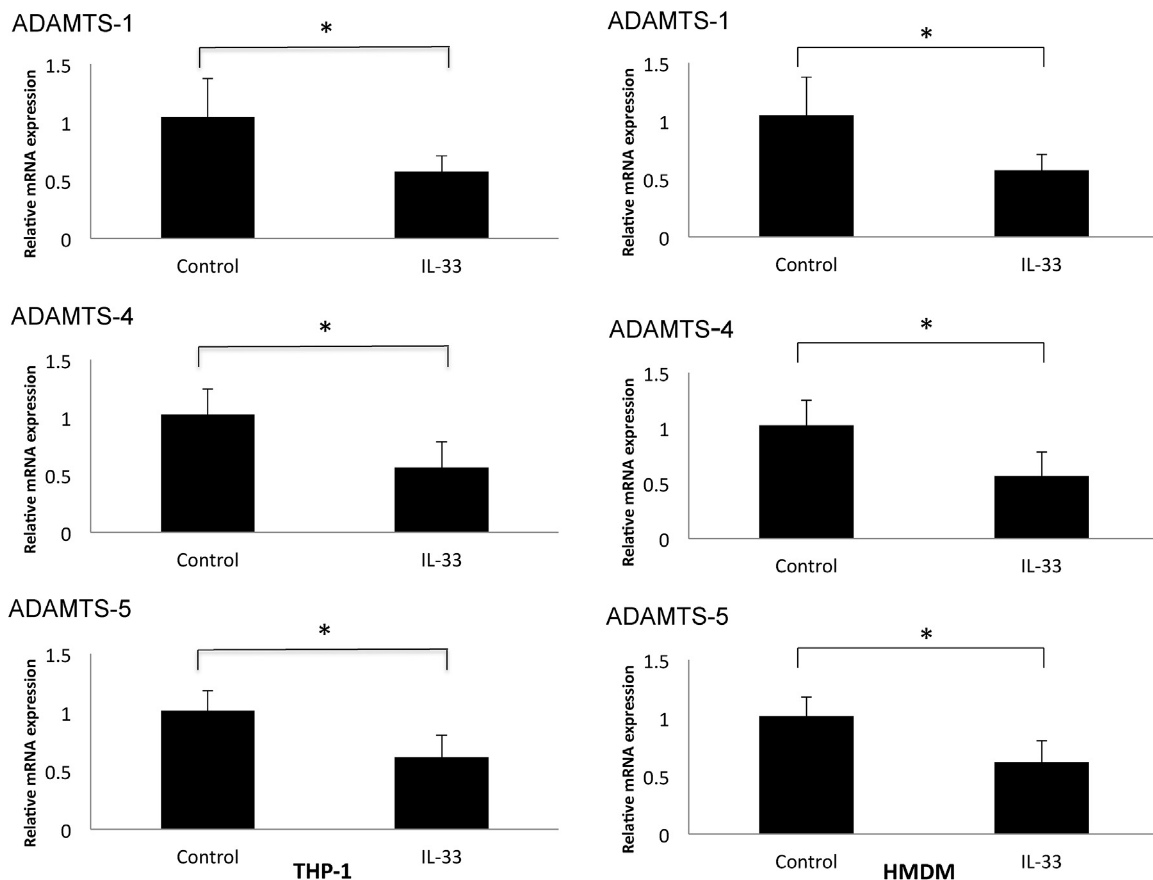

Fig. 1. IL-33 inhibits the expression of ADAMTS-1, -4 and -5 in human macrophages.
THP-1 macrophages or HMDM were treated for 24 h with vehicle (Control) or 10 ng/ml IL-33. Total cellular RNA was isolated and subjected to RT-qPCR using primers against ADAMTS-1, -4, -5 or GAPDH. The mRNA expression levels were calculated using the comparative Ct method and normalized to GAPDH with those from control, vehicle-treated cells given an arbitrary value of 1. Data represent the mean ± SD of 3 independent experiments. Statistical analysis was performed using Student's *t* test or Wilcoxon *t*-test (*$P < 0.05$).

performed using samples from different donors. Data sets were tested for normality using the Shapiro–Wilk test. Single comparisons of data were carried out using a Student's *t*-test (two-tailed, paired) or Wilcoxen *t* test. For multiple comparisons one-way ANOVA was mainly used along with Tukey's *post hoc* test, where homogeneity of variance was met, or Welch's test of equality of means with Games–Howell *post hoc* analysis. Results were regarded as significant if $P \leq 0.05$.

3. Results

3.1. ADAMTS-1, -4 and -5 were present in human atherosclerotic lesions and their expression in macrophages was inhibited by IL-33

In order to evaluate whether ADAMTS-1, -4 and -5 were expressed in human coronary artery atherosclerotic lesions, IHC was carried out using antisera against these proteases along with CD68 and α-smooth muscle actin (Supplementary Fig. 1). The use of a negative isotype control antisera showed no background staining produced by any non-specific interactions. For α-smooth muscle actin, the DAB staining was concentrated in the fibrous cap region of the plaque and in the media layer of the coronary artery. The CD68 staining indicated that macrophages and macrophage foam cells were present in the shoulder regions of the plaque and also in and around the lipid-rich core. ADAMTS-1, -4 and -5 were all expressed within the human atherosclerotic lesions. These proteases were mainly expressed by macrophages and, to a lesser extent, by smooth muscle cells and endothelial cells.

We next investigated the action of IL-33 on ADAMTS-1, -4 and -5 expression in human macrophages using the extensively employed THP-1 cell line with key findings confirmed in primary cultures of HMDM. Our previous studies showed that 10 ng/ml IL-33 inhibited macrophage foam cell formation *in vitro* (McLaren et al., 2010b) and this concentration was therefore used for all experiments. In addition, previous studies have shown that marked IL-33 dependent modulation of foam cell formation and associated changes in gene expression occurred at 24 h (McLaren et al., 2010b) and this time point was therefore used in the current study. We have previously shown that IL-33 induces apoE mRNA expression and this response was thus used as a positive control for the action of this cytokine (data not shown). IL-33 produced a significant reduction in the expression of ADAMTS-1, -4 and -5 mRNA in both THP-1 macrophages and primary cultures of HMDM (Fig. 1). This inhibitory effect of IL-33 was specific to ADAMTS members as the expression of MMP-9 was induced by this cytokine (Supplementary Figure 2). Further studies on the mechanisms underlying the actions of IL-33 focused on ADAMTS-1 and -4 as model genes.

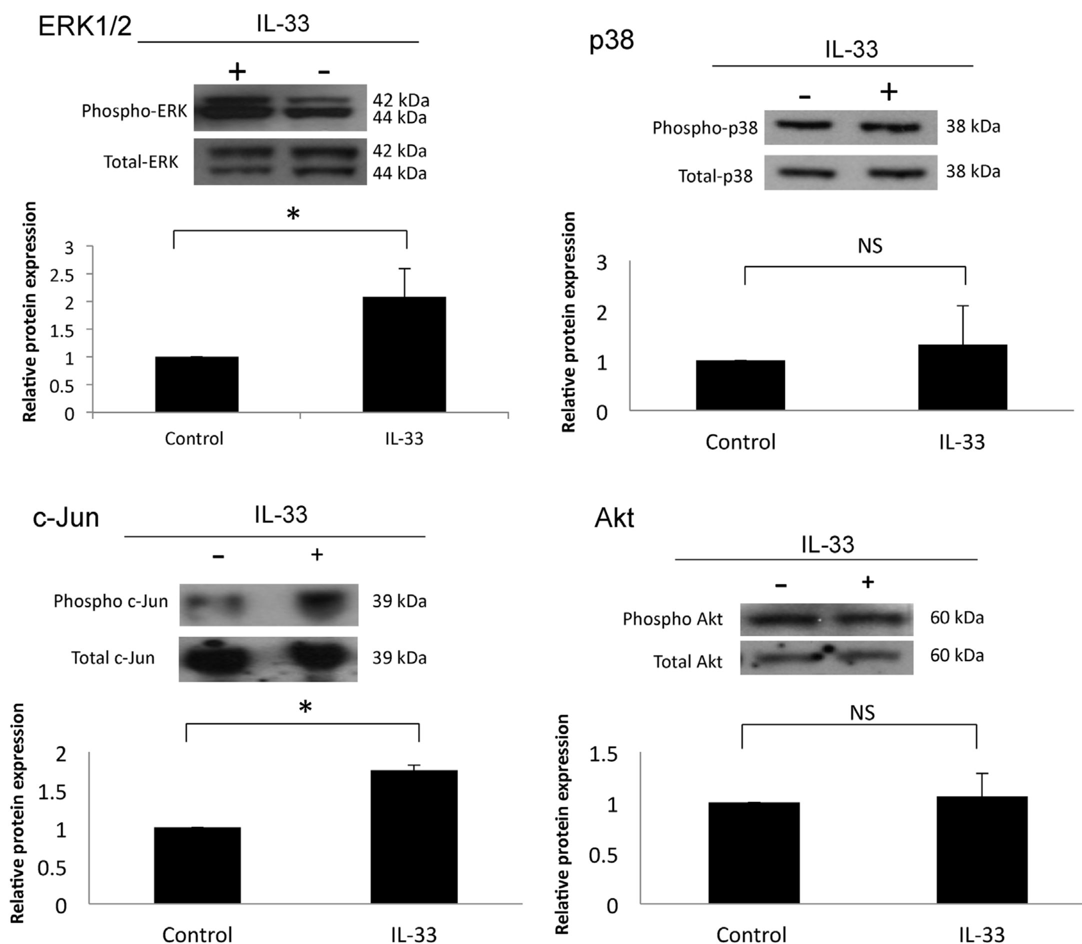

Fig. 2. IL-33 activates ERK1/2 and JNK/c-Jun pathways in human macrophages.
THP-1 macrophages were treated for 24 h with vehicle (Control; −) or 10 ng/ml IL-33 (+). Equal amounts of total cellular protein were subjected to Western blot analysis with antisera against phospho-ERK, -c-Jun, -p38, -Akt; or total-ERK, -c-Jun, -p38 and -Akt, as indicated. The levels of phospho protein were normalized to the corresponding total protein and are displayed as fold change with respect to the control (arbitrarily assigned as 1). A representative image is shown with the histograms indicating the mean ± SD from 3 (ERK, c-Jun and Akt) or 5 (p38) independent experiments. Statistical analysis was performed using Student's *t* test (*$P < 0.05$; NS, not significant).

3.2. The IL-33-mediated attenuation of ADAMTS-1 and -4 expression was dependent on the ST2 receptor

Using RNA purified from BMDM of wild type and ST2 deficient mice, we have previously shown that the effect of IL-33 on the expression of key genes implicated in the control of macrophage cholesterol homeostasis requires the ST2 receptor (McLaren et al., 2010b). In order to investigate if the ST2 receptor was also involved in the IL-33-mediated inhibition of ADAMTS-1 and -4 expression, the same RNA was used for RT-qPCR. As shown in Supplementary Figure 3, mIL-33 attenuated mADAMTS-1 and -4 expression in BMDM from wild type mice but not those that were deficient in ST2, thereby indicating a requirement for the ST2 receptor in the response. These data also show that the inhibitory action of IL-33 on the expression of ADAMTS-1 and -4 was conserved between human and mouse macrophages.

3.3. IL-33 stimulation of human macrophages activated ERK-1/2, JNK-1/2 and c-Jun but not p38 MAPK or Akt

Previous studies have shown the involvement of a number of signaling pathways in the action of IL-33, including NF-κB, MAPK and PI3K, though gene- and cell type-specific differences often exist (Brint et al., 2002; Funakoshi-Tago et al., 2008, 2011; Choi et al., 2009; Tare et al., 2010; Yagami et al., 2010; Kamekura et al., 2012). Initial transient transfection assays with a luciferase reporter vector containing four NFκB consensus binding site (pNFκB-Luc) showed no activation by IL-33 (data not shown). We then determined the action of IL-33 on MAPK and PI3K pathways by monitoring the effect of this cytokine on phosphorylation-dependent activation of either the kinase itself (ERK and p38) or key downstream targets (c-Jun for JNK and Akt for PI3K). As shown in Fig. 2, IL-33 produced a significant increase in the levels of phospho-ERK1/2 and -c-Jun without having an effect on the levels of phospho-p38 and -Akt. In contrast, the total levels of all these proteins were not affected by the cytokine. Overall, therefore, these results suggest activation of the ERK1/2 and JNK/c-Jun signaling pathways by IL-33 in human macrophages.

3.4. The IL-33-mediated inhibition of ADAMTS-1 and -4 expression was dependent on ERK-1/2, JNK-1/2, c-Jun, PI3Kγ and PI3Kδ but not p38α

The role of the various pathways was investigated with RNA interference assays using either adenoviral encoding shRNA or validated siRNA. In the case of adenovirus encoding ERK-1 or -2 shRNA,

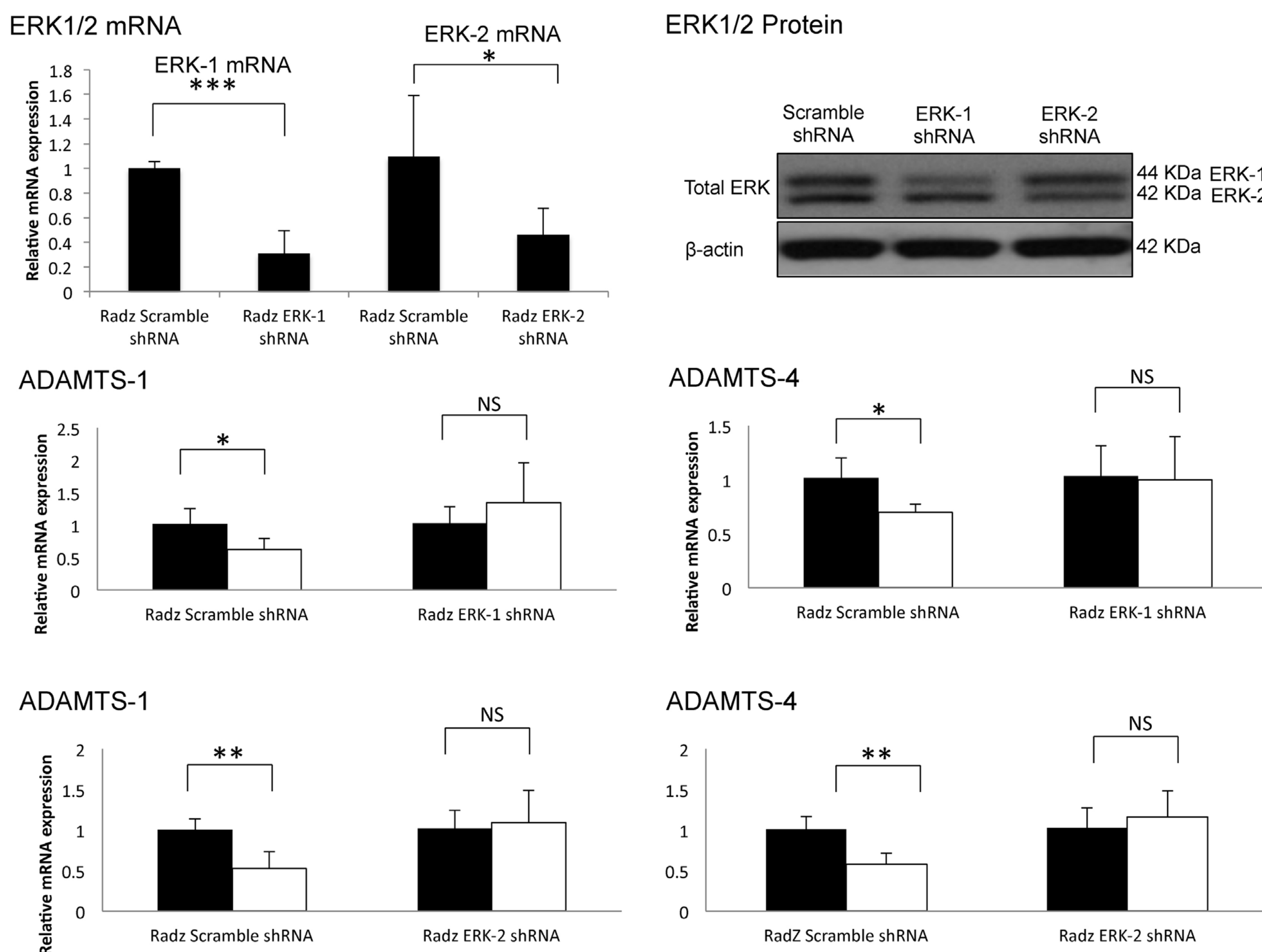

Fig. 3. ERK1/2 was required for the IL-33-inhibited expression of ADAMTS-1 and -4.
Knockdown in THP-1 macrophages was carried out using adenoviral vectors encoding either a scramble shRNA or ERK-1 shRNA or ERK-2 shRNA. The cells were either treated for 24 h with vehicle (filled bars) or 10 ng/ml IL-33 (open bars). For mRNA expression, total cellular RNA was isolated and subjected to RT-qPCR using primers against ERK-1, -2, ADAMTS-1, -4 or GAPDH. The mRNA expression levels were calculated using the comparative Ct method and normalized to GAPDH with those from control, vehicle-treated cells given an arbitrary value of 1. Data represent the mean ± SD of 3 independent experiments. Statistical analysis was performed using Student's *t* test (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; NS, not significant). For protein expression, whole cell lysates from vehicle-treated cells were subjected to SDS-PAGE and Western blotting using antibodies against total-ERK and β-actin as indicated. The image shown is representative of 2 independent experiments.

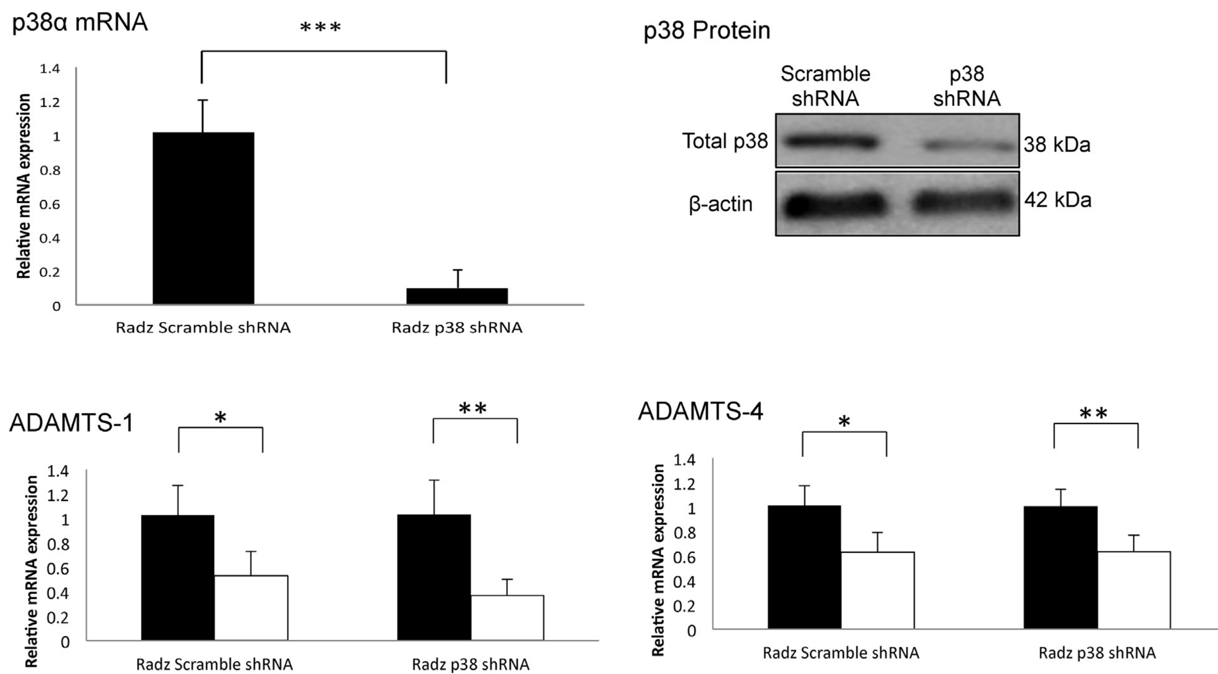

Fig. 4. p38α was not required for the IL-33-inhibited expression of ADAMTS-1 and -4.
Knockdown in THP-1 macrophages was carried out using adenoviral vectors encoding either scramble shRNA or p38α. The cells were either treated for 24 h with vehicle (filled bars) or 10 ng/ml IL-33 (open bars). For mRNA expression, total cellular RNA was isolated and subjected to RT-qPCR using primers against p38α, ADAMTS-1, -4 or GAPDH. The mRNA expression levels were calculated using the comparative Ct method and normalized to GAPDH with those from control, vehicle-treated cells given an arbitrary value of 1. Data represent the mean ± SD of 3 independent experiments. Statistical analysis was performed using Student's *t* test (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$). For protein expression, whole cell lysates from vehicle-treated cells were subjected to SDS-PAGE and Western blotting using antibodies against p38 and β-actin as indicated. The image shown is representative of 2 independent experiments.

RT-qPCR showed knockdown of the corresponding RNA by 69% and 54% respectively (Fig. 3). The knockdown was specific as ERK-2 expression was not changed by the ERK-1 shRNA, and *vice versa*, ERK-1 expression was not affected by ERK-2 shRNA (Supplementary Fig. 4). Consistent with our previous studies (*e.g.* Michael et al., 2012b), the specific knockdown of ERK-1 and -2 mRNA expression was also seen at the level of the corresponding proteins (Fig. 3). The IL-33-mediated reduction in ADAMTS-1 and -4 expression in cells infected with a scramble shRNA sequence was attenuated when the expression of ERK-1 or -2 was knocked down (Fig. 3). Similar results were obtained when knockdown experiments were carried out using ERK-2 siRNA (Supplementary Fig. 5).

p38α is the most abundant isoform of p38 kinases within inflammatory cells and has been widely studied in relation to cytokine actions (Chung, 2011). As shown in Fig. 4, adenovirus-encoding p38α shRNA produced a significant knockdown of the corresponding mRNA by about 90% in THP-1 macrophages. The knockdown was also confirmed at the level of the protein (Fig. 4). The IL-33-mediated reduction in ADAMTS-1 and -4 expression in control cells was not affected following knockdown of p38α, thereby indicating that this kinase was not required for the response (Fig. 4).

JNK-1 and -2 are key components of the JNK MAPK signaling pathway with c-Jun a major downstream target (Kim and Choi, 2010). The roles of JNK-1/2 and c-Jun were investigated by siRNA-mediated knockdown assays. The expression of JNK-1, -2 and c-Jun mRNA was significantly reduced by siRNA transfection by 54%, 45% and 38% respectively (Fig. 5). The knockdown of c-Jun was also confirmed at the level of protein expression (Fig. 5). Consequently, the IL-33-mediated reduction in ADAMTS-1 and -4 expression was attenuated when the cells were transfected with siRNA against JNK-1, -2 or c-Jun siRNA (Fig. 5).

The catalytic p110-γ and -δ subunits of PI3K are expressed by haematopoietic cells and are activated downstream of G-protein-coupled receptors, receptor tyrosine kinases and certain cytokine receptors (Williams et al., 2010). Adenoviral encoding shRNA against PI3Kγ and PI3Kδ were used to study their roles in the IL-33-mediated inhibition of ADAMTS-1 and -4 expression. The knockdown obtained was about 91% for PI3Kγ and 86% for PI3Kδ and this inhibited the IL-33-mediated reduction in ADAMTS-1 and -4 expression (Fig. 6).

3.5. Confirmation of findings in primary HMDM

In order to rule out the possibility that the results from RNA interference assays were peculiar to THP-1 macrophages, key findings were confirmed in primary cultures of HMDM. Because of low transfection efficiency associated with such cultures, adenoviral-mediated RNA interference assays were performed. The studies were therefore restricted to ERK1/2, p38α and p110-δ/γ because of availability of adenoviral shRNA encoding virus. The knockdown produced was about 74% for ERK-1, 71% for ERK-2, 66% for p38α, 81% for PI3Kγ and 77% for PI3Kδ (Supplementary Fig. 6). The specificity of knockdown was confirmed by representative experiments with ERK-1 and -2 (Supplementary Fig. 6). Similar to THP-1 macrophages, the IL-33-mediated inhibition of ADAMTS-1 and -4 expression in control HMDM was diminished with adenoviral encoding shRNA for ERK-1, ERK-2, PI3Kγ and PI3Kδ but not p38α (Fig. 7).

4. Discussion

Recent studies have suggested a potentially important role for the ADAMTS family in atherosclerosis (Salter et al., 2010). We show here that ADAMTS-1, -4 and -5 were expressed in human coronary artery lesions and their expression in human macrophages was inhibited by the anti-atherogenic cytokine IL-33. In addition, we identify important roles for the ERK, JNK and PI3K signaling pathways in the response.

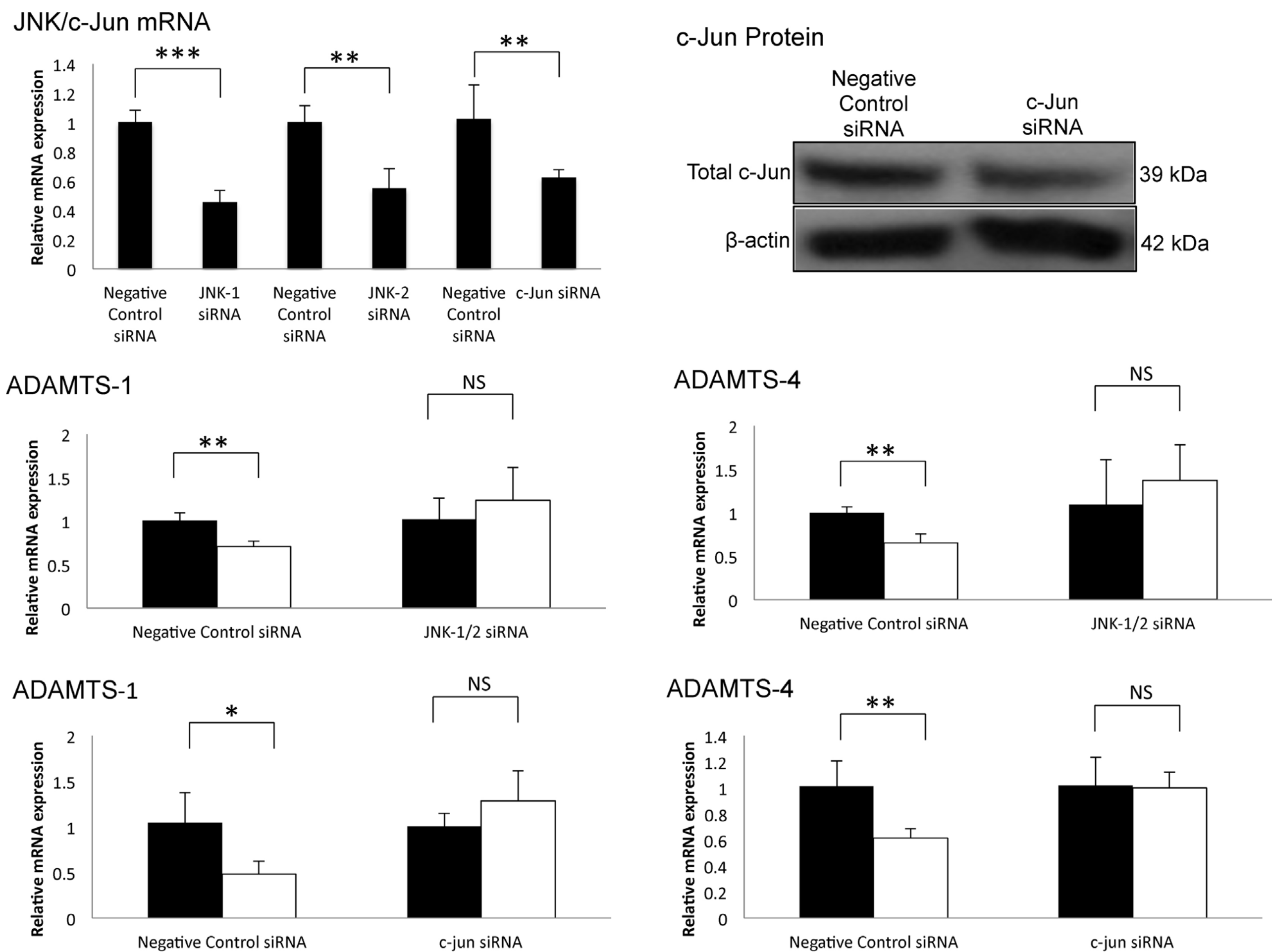

Fig. 5. JNK1/2 and c-Jun were required for the IL-33-inhibited expression of ADAMTS-1 and -4.
siRNA-mediated knockdown was carried out using either a scramble siRNA sequence or that against JNK1/2 or c-Jun. The cells were then either treated for 24 h with vehicle (filled bars) or 10 ng/ml IL-33 (open bars). For mRNA expression, total cellular RNA was isolated and subjected to RT-qPCR using primers against JNK-1, -2, c-Jun, ADAMTS-1, -4, or GAPDH. The mRNA expression levels were calculated using the comparative Ct method and normalized to GAPDH mRNA with those from control, vehicle-treated cells given an arbitrary value of 1. Data represent the mean ± SD of 3 independent experiments. Statistical analysis was performed using Student's *t* test (***$P<0.001$; **$P<0.01$ *$P<0.05$; NS, not significant). For protein expression, whole cell lysates from vehicle-treated cells were subjected to SDS-PAGE and western blotting using antibodies against c-Jun and β-actin as indicated. The image shown is representative of 2 independent experiments.

The expression of ADAMTS-1, -4 and -5 was at the highest level in macrophages and to a lesser extent in vascular smooth muscle cells and endothelial cells of human atherosclerotic plaques (Supplementary Fig. 1). Interestingly, a recent study has shown elevated ADAMTS-1 expression within plaques from patients with acute myocardial infarction compared to stable angina (Lee et al., 2011). In separate studies serum levels of ADAMTS-4 have also shown a significant correlation with the severity of coronary artery disease (Zha et al., 2010; Chen et al., 2011). These findings, taken together, outline the potential regulatory role that ADAMTS proteases could have over the stability of the atherosclerotic plaque.

We have recently investigated the regulation of expression of ADAMTS-1, -4 and -5 during macrophage differentiation and in response to several other cytokines (Ashlin et al., 2013). The expression of ADAMTS-1, -4 and -5 was induced during differentiation of monocytes into macrophages. IL-17 and TL1A alone had little effect on the expression of these proteases but induced them synergistically when present together. In addition, a differential action was seen with the pro-atherogenic cytokine IFN-γ and the anti-atherogenic cytokine TGF-β. IFN-γ suppressed the expression of ADAMTS-1 without affecting ADAMTS-4 and -5 whereas TGF-β induced the expression of ADAMTS-1 and -5 and inhibited that of ADAMTS-4. In contrast to such differential actions, IL-33 attenuated the expression of all three ADAMTS members (Fig. 1).

IL-33 has been found to have a protective role during atherosclerosis development (Miller, 2011). The cytokine promotes T_h2 responses, thereby slowing inflammation and stabilizing the developing plaque (Miller and Liew, 2011). In addition, IL-33 reduces atherosclerosis in apo$E^{-/-}$ mice fed a high fat diet (Miller et al., 2008). Our data demonstrate that IL-33 reduces the expression of ADAMTS proteases which have previously been implicated in ECM remodeling and destabilization of the atherosclerotic plaque (Salter et al., 2010). We also found that IL-33 increases the expression of MMP-9 (Supplementary Fig. 2). Normal physiological levels of MMP-9 expression have previously been implicated with a plaque stabilizing role during atherosclerosis progression (Newby, 2012). These data taken together suggest gene specific regulatory patterns for IL-33 that results in an anti-atherogenic plaque phenotype.

Of the ADAMTS family of proteases, the promoter regions of only ADAMTS-4 and -5 have been characterized (Mizui et al., 2000; Thirunavukkarasu et al., 2006, 2007). We found that the activity of the available ADAMTS-4 promoter (−383 to +406) was suppressed by IL-33 (data not shown), thereby indicating that the cytokine inhibited the expression of this gene, at least in part, at the transcriptional level. Mizui and colleagues (2000) previously showed that the −383 to +10 region of the ADAMTS-4 promoter was required for full promoter activity. This region of the promoter contains one site for Sp1 and three AP-2 sites (Mizui et al., 2000). The

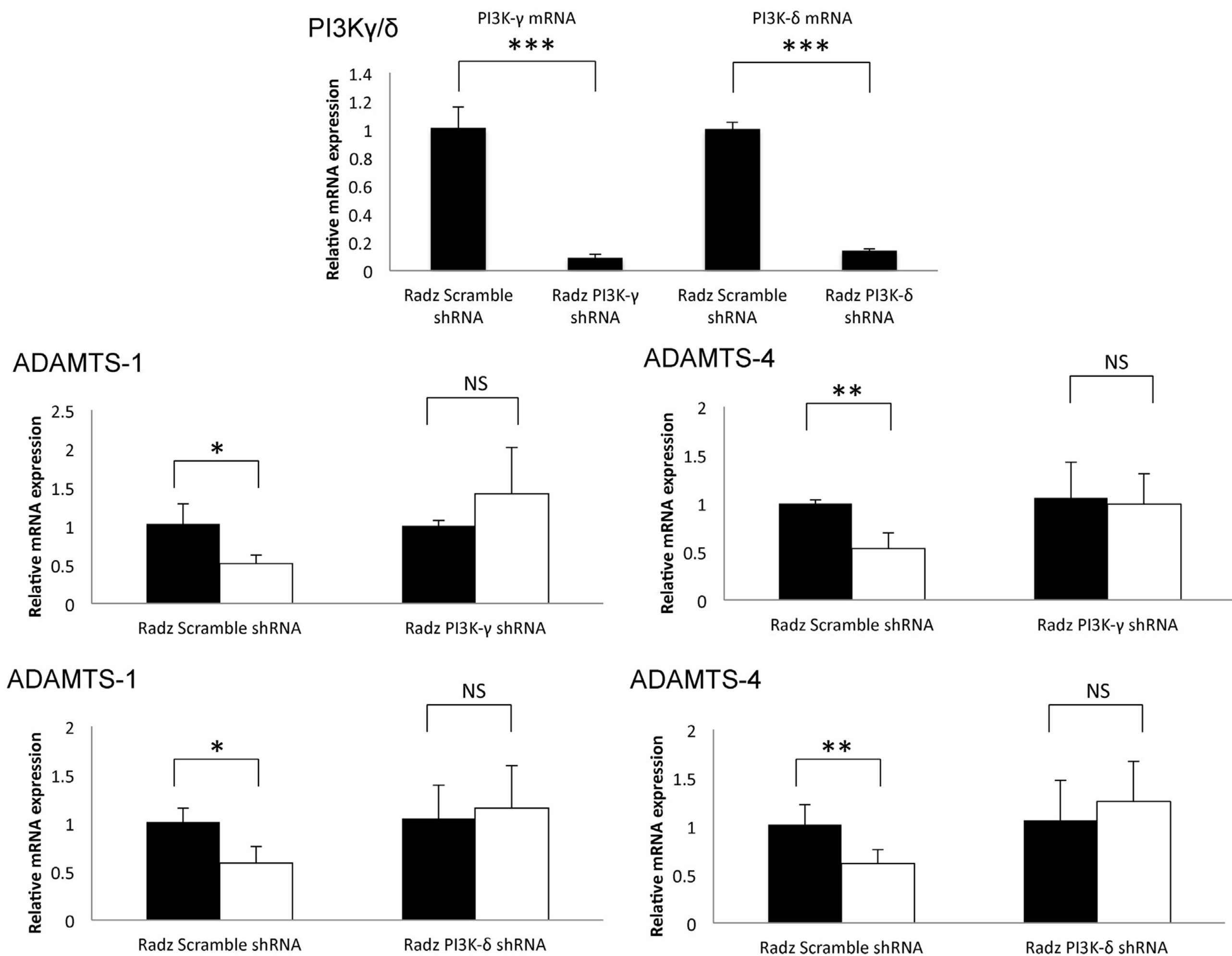

Fig. 6. p110-γ and -δ were required for the IL-33-inhibited expression of ADAMTS-1 and -4.
Knockdown in THP-1 macrophages was carried out using adenoviral vectors encoding either a scramble shRNA or that for p110-γ or -δ. The cells were then either treated for 24 h with vehicle (filled bars) or 10 ng/ml IL-33 (open bars). Total cellular RNA was isolated and subjected to RT-qPCR using primers against p110-γ, -δ, ADAMTS-1, -4 or GAPDH. The mRNA expression levels were calculated using the comparative Ct method and normalized to GAPDH mRNA levels with those from control, vehicle-treated cells given an arbitrary value of 1. Data represent the mean ± SD of 3 independent experiments. Statistical analysis was performed using Student's *t* test (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; NS, not significant).

roles of these sites in transcriptional repression by IL-33 remains to be determined.

IL-33 has been shown to promote inflammation by inducing lymphocytic infiltration in a ST2-independent manner, *in vivo* (Luzina et al., 2012). It was therefore of interest to determine if the ST2 receptor was involved in the transduction of the cytokine-mediated regulation of ADAMTS-1 and -4 gene expression. This was indeed found to be the case by RT-qPCR analysis of RNA from BMDM of wild type and ST2 receptor-deficient mice (Supplementary Fig. 3). This finding is consistent with many other studies that have shown that the ST2 receptor is involved in regulation of gene expression by IL-33 (Kakkar and Lee, 2008; Miller and Liew, 2011; Luzina et al., 2012).

Several studies have investigated the actions of IL-33 in different cell types with some cell- and gene-specific responses being identified (Brint et al., 2002; Funakoshi-Tago et al., 2008, 2011; Choi et al., 2009; Tare et al., 2010; Yagami et al., 2010; Kamekura et al., 2012). For example, p38 MAPK was activated in lung endothelial cells but not epithelial cells (Yagami et al., 2010). In addition, ERK was not activated in the basophil-like chronic myelogenous leukemia cell line, KU812 (Tare et al., 2010). A previous study in mouse peritoneal macrophages found IL-33-mediated activation of ERK, JNK, p38 and NFκB (Funakoshi-Tago et al., 2011). It was therefore of interest to investigate which signaling cascades were activated by IL-33 in human macrophages. The use of western blots and transfection-based assays showed, for the first time, IL-33-mediated activation of ERK and c-Jun/JNK but not Akt and p38 MAPK in human macrophages (Fig. 2). These results differ slightly from previous studies on mouse peritoneal macrophages where p38 MAPK was found to be activated in addition to ERK and JNK (Funakoshi-Tago et al., 2011). This might potentially reflect species-specific differences. Indeed, species-specific responses have been observed previously during the regulation of macrophage functions by the peroxisome proliferator-activated receptor and liver X receptor families of transcription factors (Rigamonti et al., 2008). Also the concentration of IL-33 used on peritoneal macrophages (100 ng/ml) (Funakoshi-Tago et al., 2011) was 10 fold higher than that used within this study, which was based on previous dose response experiments (McLaren et al., 2010b). Indeed, the concentration of 10 ng/ml lies within the physiological range of human IL-33 levels (can reach up to 40 ng/ml) and has been employed widely in other *in vitro* studies (McLaren et al., 2010b).

RNA interference assays showed that ERK-1/2, PI3Kγ/δ and JNK/c-Jun were involved in transducing the reduction of ADAMTS-1 and -4 expression by IL-33, but p38α was not required (Figs. 3–7). It is worth noting that the findings in the majority of previous

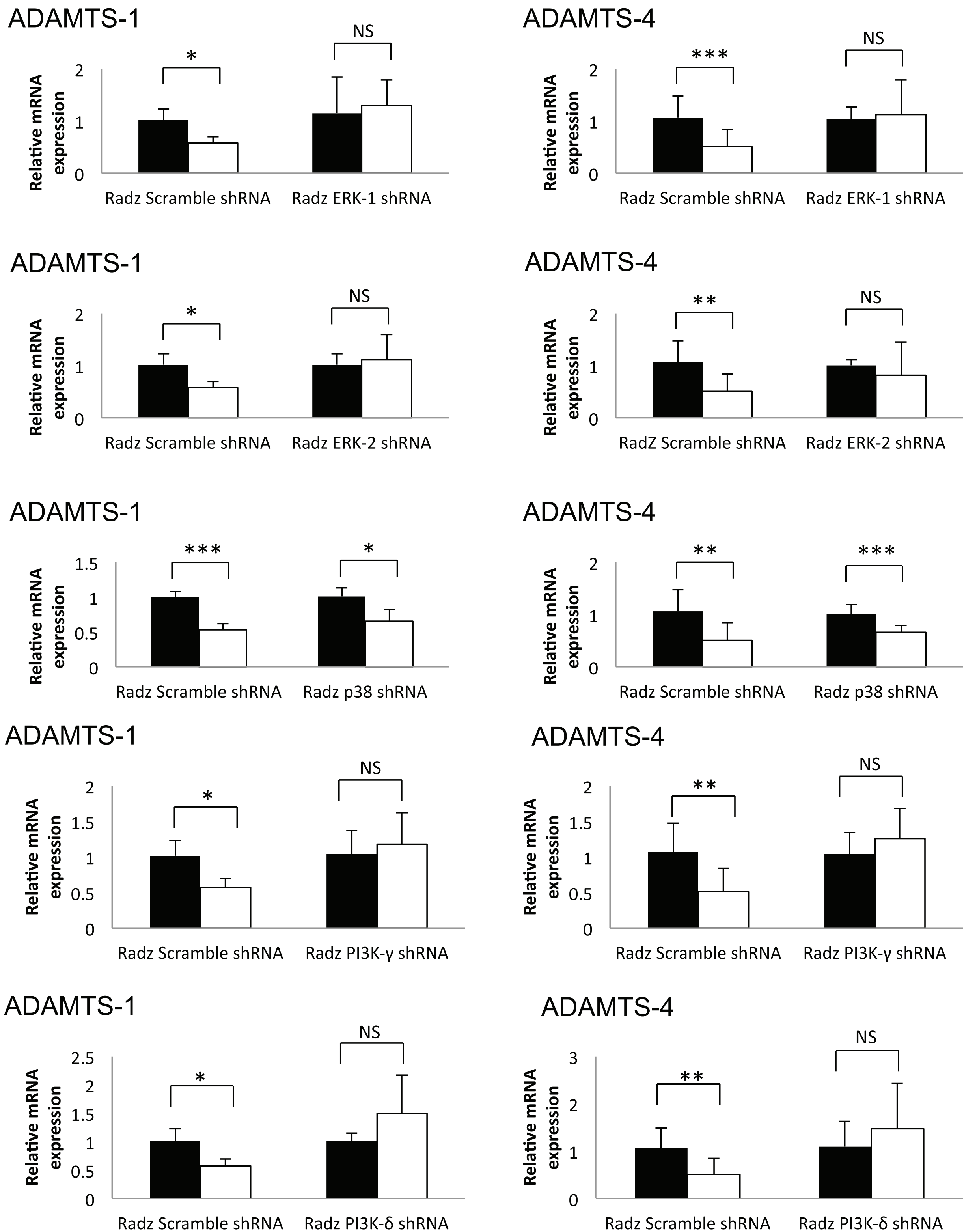

Fig. 7. ERK1/2 and p110-γ/δ but not p38α were required for the IL-33-inhibited expression of ADAMTS-1 and -4 in HMDM.
Knockdown in HMDM was carried out using adenoviral vectors encoding a scramble shRNA or that against ERK-1/-2, p38α or p110-γ/δ. The cells were then either treated for 24 h with vehicle (filled bars) or 10 ng/ml IL-33 (open bars). Total cellular RNA was isolated and subjected to RT-qPCR using primers against ADAMTS-1, -4 or GAPDH. The mRNA expression levels were calculated using the comparative Ct method and normalized to GAPDH mRNA levels with those from control, vehicle-treated cells given an arbitrary value of 1. Data represent the mean ± SD of 3 independent experiments. Statistical analysis was performed using Student's *t* test (***$P < 0.001$; **$P < 0.01$; *$P < 0.05$; NS, not significant).

studies that investigated IL-33 signaling in other cell types was based mainly on the use of pharmacological inhibitors of the various signaling cascades (Brint et al., 2002; Funakoshi-Tago et al., 2008, 2011; Choi et al., 2009; Tare et al., 2010; Yagami et al., 2010; Kamekura et al., 2012). Although pharmacological inhibitors are usually the first method of investigation in cell signaling pathways, it is important that the findings are confirmed by knockdown/knockout approaches due to the associated non-specific actions of chemical inhibitors (Cohen P, 2010). The use of RNA interference enables very specific knockdown of selected isoforms of proteins with limited off-target effects (Eggert et al., 2006), as demonstrated here in the case of ERK-1 and -2 (Fig. 3). The requirement of ERK-1/2 and c-Jun/JNK was associated with the activation of the corresponding kinase/protein (Fig. 2). However, the requirement of PI3K-γ and -δ was independent of the activation of one of its key downstream targets, Akt (Fig. 2). This suggests that targets other than Akt were potentially involved in the response.

5. Conclusion

The data presented here demonstrate for the first time that the anti-atherogenic cytokine IL-33 attenuates the expression of ADAMTS-1, -4 and -5 in human macrophages. The mechanism of signal transduction was dependent on ST2 binding, ERK-1, ERK-2, c-Jun, JNK-1, JNK-2, PI3Kγ and PI3Kδ. Further studies, which are beyond the scope of the present manuscript, would be to delineate how these signaling pathways modulate the action of key transcription factors involved in inhibiting the expression of these ADAMTS members. In addition, the therapeutic implications of this study extend to IL-33 being identified as a potential therapeutic target for modulating the expression of ADAMTS-1, -4 and -5 during atherosclerotic plaque development.

Acknowledgements

This work was supported by BHF (PG/10/55/28467 to DPR and FS/07/053/24069 to JLJ). We thank Prof. Foo Liew (University of Glasgow) for femur and tibia from wild type and ST2 deficient mice. Tim Ashlin and Melanie Buckley were recipients of studentships from the BBSRC and MRC respectively.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biocel.2013.11.008.

References

Arning A, Hiersche M, Witten A, Kurlemann G, Kurnik K, Manner D, et al. A genome wide association study identifies a gene network of ADAMTS genes in the predisposition of pediatric stroke. Blood 2012;120:5231–6.

Ashlin TG, Kwan APL, Ramji DP. Regulation of ADAMTS-1, -4 and -5 expression in human macrophages: Differential regulation by key cytokines implicated in atherosclerosis and novel synergism between TL1A and IL-17. Cytokine 2013;64:234–42.

Brint EK, Fitzgerald KA, Smith P, Coyle AJ, Gutierrez-Ramos JC, Fallon PG, et al. Characterization of signaling pathways activated by the interleukin 1 (IL-1) receptor homologue T1/ST2. A role for Jun N-terminal kinase in IL-4 induction. J Biol Chem 2002;277:49205–11.

Chen L, Yang L, Zha Y, Cui L. Association of serum a disintegrin and metalloproteinase with thrombospodin motif 4 levels with the presence and severity of coronary artery disease. Coron Artery Dis 2011;22:570–6.

Choi YS, Choi HJ, Min JK, Pyun BJ, Maeng YS, Park H, et al. Interleukin-33 induces angiogenesis and vascular permeability through ST2/TRAF6-mediated endothelial nitric oxide production. Blood 2009;114:3117–26.

Chung KF. p38 mitogen-activated protein kinase pathways in asthma and COPD. Chest 2011;139:1470–9.

Cohen P. Guidelines for the effective use of chemical inhibitors of protein function to understand their roles in cell regulation. Biochem J 2010;425:53–4.

Didangelos A, Mayr U, Monaco C, Mayr M. Novel role of ADAMTS-5 protein in proteoglycan turnover and lipoprotein retention in atherosclerosis. J Biol Chem 2012;287:19341–5.

Eggert US, Field CM, Mitchison TJ. Small molecules in an RNAi world. Mol Biosyst 2006;2:93–6.

Funakoshi-Tago M, Tago K, Hayakawa M, Tominaga S, Ohshio T, Sonoda Y, et al. TRAF6 is a critical signal transducer in IL-33 signaling pathway. Cell Signal 2008;20:1679–86.

Funakoshi-Tago M, Tago K, Sato Y, Tominaga S, Kasahara T. JAK2 is an important signal transducer in IL-33-induced NF-κB activation. Cell Signal 2011;23:363–70.

Huang WC, Sala-Newby GB, Susana A, Johnson JL, Newby AC. Classical macrophage activation up-regulates several matrix metalloproteinases through mitogen activated protein kinases and nuclear factor-κB. PLoS One 2012;7:e42507.

Jonsson-Rylander AC, Nilsson T, Fritsche-Danielson R, Hammarstrom A, Behrendt M, Andersson JO, et al. Role of ADAMTS-1 in atherosclerosis: remodeling of carotid artery, immunohistochemistry, and proteolysis of versican. Arterioscler Thromb Vasc Biol 2005;25:180–5.

Kakkar R, Lee RT. The IL33/ST2 pathway: therapeutic target and novel biomarker. Nature Rev Drug Discovery 2008;7:827–40.

Kamekura R, Kojima T, Takano K, Go M, Sawada N, Himi T. The role of IL-33 and its receptor ST2 in human nasal epithelium with allergic rhinitis. Clin Exp Allergy 2012;42:218–28.

Kim EK, Choi EJ. Pathological roles of MAPK signaling pathways in human diseases. Biochim Biophys Acta 2010;1802:396–405.

Lee CW, Hwang I, Park CS, Lee H, Park DW, Kang SJ, et al. Comparison of ADAMTS-1, -4 and -5 expression in culprit plaques between acute myocardial infarction and stable angina. J Clin Pathol 2011;64:399–404.

Lee CW, Hwang I, Park CS, Lee H, Park DW, Kang SJ, et al. Expression of ADAMTS-2, -3, -13, and -14 in culprit coronary lesions in patients with acute myocardial infarction or stable angina. J Thromb Thrombolysis 2012;33:362–70.

Li N, McLaren JE, Michael DR, Clement M, Fielding CA, Ramji DP. ERK is integral to the IFN-γ-mediated activation of STAT1, the expression of key genes implicated in atherosclerosis, and the uptake of modified lipoproteins by human macrophages. J Immunol 2010;185:3041–8.

Luzina IG, Pickering EM, Kopach P, Kang PH, Lockatell V, Todd N, et al. Full-length IL-33 promotes inflammation but not Th2 response *in vivo* in an ST2-independent fashion. J Immunol 2012;189:403–10.

McLaren JE, Calder CJ, McSharry BP, Sexton K, Salter RC, Singh NN, et al. The TNF-like protein 1A-death receptor 3 pathway promotes macrophage foam cell formation *in vitro*. J Immunol 2010a;184:5827–34.

McLaren JE, Michael DR, Salter RC, Ashlin TG, Calder CJ, Miller AM, et al. IL-33 reduces macrophage foam cell formation. J Immunol 2010b;185:1222–9.

McLaren JE, Michael DR, Ashlin TG, Ramji DP. Cytokines, macrophage lipid metabolism and foam cells: implications for cardiovascular disease therapy. Prog Lipid Res 2011;50:331–47.

Michael DR, Ashlin TG, Buckley ML, Ramji DP. Macrophages, lipid metabolism and gene expression in atherogenesis: a therapeutic target of the future? Clin Lipidol 2012a;7:37–48.

Michael DR, Salter RC, Ramji DP. TGF-β inhibits the uptake of modified low density lipoprotein by human macrophages through a Smad-dependent pathway: a dominant role for Smad-2. Biochim Biophys Acta 2012b;1822:1608–16.

Miller AM, Xu D, Asquith D, Denby L, Li Y, Sattar N, et al. IL-33 reduces the development of atherosclerosis. J Exp Med 2008;205:339–46.

Miller AM, Liew FY. The IL-33/ST2 pathway—a new therapeutic target in cardiovascular disease. Pharmacol Ther 2011;131:179–86.

Miller AM. Role of IL-33 in inflammation and disease. J Inflamm (Lond) 2011;8:22.

Mizui Y, Yamazaki K, Kuboi Y, Sagane K, Tanaka I. Characterization of 5′-flanking region of human aggrecanase-1 (ADAMTS4) gene. Mol Biol Rep 2000;27:167–73.

Newby AC. Matrix metalloproteinase inhibition therapy for vascular diseases. Vascul Pharmacol 2012;56:232–44.

Reilly MP, Li M, Jing H, et al. Identification of ADAMTS7 as a novel locus for coronary atherosclerosis and association of ABO with myocardial infarction in the presence of coronary atherosclerosis: two genome-wide association studies. Lancet 2011;377:383–92.

Rigamonti E, Chinetti-Gbaguidi G, Staels B. Regulation of macrophage functions by PPAR-alpha, PPAR-gamma, and LXRs in mice and men. Arterioscler Thromb Vasc Biol 2008;28:1050–9.

Salter RC, Ashlin TG, Kwan APL, Ramji DP. ADAMTS proteases: key roles in atherosclerosis? J Mol Med (Berl) 2010;88:1203–11.

Salter RC, Arnaoutakis K, Michael DR, Singh NN, Ashlin TG, Buckley ML, et al. The expression of a disintegrin and metalloproteinase with thrombospondin motifs 4 in human macrophages is inhibited by the anti-atherogenic cytokine transforming growth factor-β and requires Smads, p38 mitogen-activated protein kinase and c-Jun. Int J Biochem Cell Biol 2011;43:805–11.

Scholtes VP, Johnson JL, Jenkins N, Sala-Newby GB, de Vries JP, de Borst GJ, et al. Carotid atherosclerotic plaque matrix metalloproteinase-12-positive macrophage subpopulation predicts adverse outcome after endarterectomy. J Am Heart Assoc 2012;1:e001040.

Tare N, Li H, Morschauser A, Cote-Sierra J, Ju G, Renzetti L, et al. KU812 cells provide a novel *in vitro* model of the human IL-33/ST2L axis: functional responses and identification of signaling pathways. Exp Cell Res 2010;316:2527–37.

Thirunavukkarasu K, Pei Y, Moore TL, Wang H, Yu X, Geiser AG, et al. Regulation of the human ADAMTS-4 promoter by transcription factors and cytokines. Biochem Biophys Res Commun 2006;345:197–204.

Thirunavukkarasu K, Pei Y, Wei T. Characterization of the human ADAMTS-5 (aggrecanase-2) gene promoter. Mol Biol Rep 2007;34:225–31.

Wagsater D, Bjork H, Zhu C, Bjorkegren J, Valen G, Hamsten A, et al. ADAMTS-4 and -8 are inflammatory regulated enzymes expressed in macrophage-rich areas of human atherosclerotic plaques. Atherosclerosis 2008;196:514–22.

Williams O, Houseman BT, Kunkel EJ, Aizenstein B, Hoffman R, Knight ZA, et al. Discovery of dual inhibitors of the immune cell PI3Ks p110delta and p110gamma: a prototype for new anti-inflammatory drugs. Chem Biol 2010;17:123–34.

Yagami A, Orihara K, Morita H, Futamura K, Hashimoto N, Matsumoto K, et al. IL-33 mediates inflammatory responses in human lung tissue cells. J Immunol 2010;185:5743–50.

Zha Y, Chen Y, Xu F, Zhang J, Li T, Zhao C, et al. Elevated level of ADAMTS4 in plasma and peripheral monocytes from patients with acute coronary syndrome. Clin Res Cardiol 2010;99:781–6.