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Oxidized LDL-mediated upregulation of ADAMTS-4 in monocytes/macrophages involves ROS-NF-κB-SIRT-1 pathway

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ABSTRACT

Background and aims: ADAMTS-4 is a protease enzyme involved in vascular remodeling and atherosclerosis. It was found to be upregulated in macrophages seen in atherosclerotic lesions. This study aimed to investigate the expression and regulation of ADAMTS-4 in oxidized LDL-induced human monocytes/macrophages system. *Methods:* Peripheral blood mononuclear cells (PBMCs) isolated from human blood, and treated with oxidized LDL (50 μg mL^{-1}) were used as the model system for the study. mRNA and protein expressions were studied by PCR, ELISA, and western blot analysis. ROS production and cell viability were determined by DCFDA staining and MTT assay, respectively. *Results:* In the presence of oxidized LDL, monocytes get differentiated into macrophages, which were confirmed by the increased expression of macrophage differentiation markers and pro-inflammatory cytokine TNF-α. Oxidized LDL increased the mRNA and protein expression of ADAMTS-4 in monocytes/macrophages. N- Acetyl cysteine, ROS scavenger, downregulate the protein expression of ADAMTS-4. The expression of ADAMTS-4 was decreased significantly in the presence of NF-κB inhibitors. SIRT-1 activity was significantly downregulated in the macrophages and was reversed in the presence of the SIRT-1 agonist, resveratrol. Acetylation of NF-κB and hence the expression of ADAMTS-4 were significantly

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downregulated in the presence of SIRT-1 activator, resveratrol. *Conclusions:* Our study suggests that oxidized LDL significantly upregulated the expression of ADAMTS-4 in the monocytes/macrophages through ROS- NF-κB- SIRT-1 pathway.

KEYWORDS

atherosclerosis, oxidized LDL, monocytes /macrophages, TNF-α, NF-κB, ROS, ADAMTS-4, SIRT-1

INTRODUCTION

Cardiovascular diseases are the leading cause of death all over the world. Atherosclerosis is characterized by the complete or partial blockage of medium and large-sized arteries by plaque formation [1]. The major risk factors include obesity, sedentary lifestyle, alcohol consumption, smoking, and high-fat diet [2]. These risk factors lead to inflammation, LDL and activated immune cells like monocytes and T-Lymphocytes leading to endothelial dysfunction [3]. Activated monocytes enter the arterial intima by the process called extravasation and LDL gets oxidized in the arterial extracellular matrix (ECM) [4, 5]. In presence of OxLDL, the monocytes get differentiated into macrophages and produce several pro-inflammatory cytokines, chemokines, etc., and thus initiate an inflammation cascade [6]. Macrophages express several lipid scavenging receptors like CD-36 and CD-68 [3]. Oxidized LDL binds to the LDL scavenging receptors and is engulfed by the macrophages. The macrophages gradually become lipid-laden foam cells and they form the core of the lipid-rich plaque [7]. The inflammatory molecules formed during the process lead to the activation and migration of smooth muscle cells from the media layer to the intima of the arterial wall. In intima, smooth muscle cells (SMCs) get proliferated and produce many matrix components like aggrecan, versican, collagen, elastin, etc, which form the outer cap of the plaque. The activated cells can produce several proteases like A Disintegrin-like and Metalloproteinase with Thrombospondin motifs (ADAMTS) family of proteinases. They can cleave ECM components like aggrecan, collagen, elastin, versican, etc. [8]. The progress of atherosclerosis is associated with an increase in the expression of these matrix-degrading proteinases, which play a key role in the development and rupture of the plaque [9].

ADAMTS family of matrix-degrading enzymes belongs to the M12 sub-family of matrix proteinases and consists of 19 members with various substrate specificities [10]. Among ADAMTS members, ADAMTS-1, 4, 5, 8, and 9 were reported to have a role in atherosclerosis [11]. ADAMTS-4 is the major aggrecanase, also known as aggrecanase I. ADAMTS-4 cleaves versican, one of the major structural components of vascular ECM [12]. ADAMTS-4 is expressed in macrophage-rich areas in atherosclerotic plaques [13]. ADAMTS-4 is reported to enhance atherosclerosis by promoting monocyte migration, differentiation, proliferation, and survival via promoting the production of pro-inflammatory cytokines such as GM-CSF, CCL2, and CXCL12 [14]. Wagsater et al. reported that cytokines like TNF-α and IFN-γ induce ADAMTS-4 production in PMA-differentiated THP-1 macrophages [13]. TIMP-3 is the potent endogenous tissue inhibitor of ADAMTS-4 that binds to the C-terminal thrombospondin domain other than the catalytic domain and thus blocks their binding with the substrate [15].

TNF-α induced ADAMTS-4 expression was regulated through the NF-κB pathway in nucleus pulposus cells [16]. Phosphorylation and acetylation are the main post-translational

modifications that regulate the NF-κB activity [17]. NF-κB activation involves the IKK complex which phosphorylates IκB and releases NF-κB [18]. Phosphorylation and acetylation of NF-κB at specific sites facilitate its binding on DNA and enhance transcriptional activity. SIRT-1 is a class III NAD^+ dependant histone deacetylase that inhibits the transcriptional activity of NF-κB by the deacetylation of lysine 310 residue [19]. The study on the role of SIRT-1 in the regulation of ADAMTS-4 expression during mo-mφ differentiation helps to develop a new strategy for the treatment of atherosclerosis.

The mechanism involved in the regulation of ADAMTS-4 during OxLDL-mediated monocyte-macrophage differentiation is not studied in detail. LDL undergoes modification in the vessel wall and oxidized LDL uptake by the macrophages results in foam cell formation, and it is thought to be one of the earliest events in the nascent plaque formation [20]. ROS formed during mo-mφ differentiation act as a signaling molecule and it can induce NF-κB activation [21]. Hence we investigated the transcriptional level regulation of ADAMTS-4 expression using oxLDL-treated human PBMCs as the model system.

MATERIALS AND METHODS

Preparation of oxLDL

Serum LDL was precipitated using Heparin-$MnCl_2$ solution. Isolated LDL was treated with 10 μM $CuSO_4$ for 24 h at 37 °C for oxidation [22]. After oxidation, it was dialyzed against 1X PBS containing 0.05M EDTA for 48 h. The degree of oxidation was measured in terms of thiobarbituric acid reactive substances (TBARS) using malondialdehyde as the standard. The level of TBARS was compared to native LDL [22]. Data is available in the supplementary file.

PBMCs isolation and treatment

PBMCs isolated from voluntary donors were used for the study with approval from the human ethical committee, University of Kerala (ULECRIHS/UOK/2018/25). The cells were isolated by density gradient centrifugation using Histopaque-1077, washed with 1X PBS, and were resuspended in RPMI-1640, and seeded on collagen- I coated culture plates [23]. After 4 h of incubation, the unattached cells were removed and the attached cells were treated with oxLDL. The cells and culture supernatant were used for mRNA and protein expression studies.

MTT assay

The effect of oxLDL on the cell viability was determined by using 3- (4, 5 Dimethylthiazol-2- yl)–2,5 diphenyltetrazolium bromide (MTT) assay [24]. The cells were seeded in 96 well plates and were treated with different concentrations of oxLDL for 48 h. MTT solution (5 mg mL^{-1}) was added and incubated for 4 h at 37 °C. Then MTT solvent (Isopropanol) was added and read the absorbance at 570 nm.

β-D- glucuronidase assay

The cells were harvested and lysed in citrate buffer (0.1M, pH 4.6) containing 0.2% Triton X-100. The cell lysate was used to assay the β-D- glucuronidase enzyme using para nitrophenyl

β-D glucuronide (N1627, Sigma Aldrich) as substrate at 37°C. The p-nitrophenol formed was determined by measuring absorbance at 405 nm [25].

ELISA

ELISA was performed by the method of Engvall and Perlmann [26]. Samples were coated in a 96-well plate and kept overnight at 37 °C. Then washed the wells with 1X PBS and incubated with primary antibodies of ADAMTS-4 (AV41556, Sigma Aldrich), TNF-α (NBP1-19532, Novus Biologicals), and NF-κB p65 (#8242, Cell Signaling Technology) at room temperature for 2h. Unbound antibodies were washed with 1X PBS- Tween20 and incubated with a secondary antibody. The color was developed using O- Phenylenediamine (P8787, Sigma Aldrich) in citrate phosphate buffer (pH 5) and H_2O_2, and absorbance was measured at 490 nm.

PCR

Total RNA was isolated using Trizol reagent (1596018; Invitrogen) and converted into cDNA with Superscript first-strand cDNA synthesis Kit (1708891; Bio-rad) according to manufacturer's instruction. qPCR was performed using Bio-Rad Syber green master mix (1725121; Bio-Rad) with Eppendorf realplex2 master cycler. The semiquantitative PCR was performed using Thermo Scientific DyNAzymeII Polymerase (#F-501L; Thermo Scientific) and dNTP mix (R0191; Thermo scientific). The samples were analyzed for the expression of ADAMTS-4, CD-36, CD-68, TNF-α, and GAPDH with specific primers. The samples were tested in triplicates. The real-time PCR results were expressed as fold changes to control. Primer sequences are given as follows: ADAMTS-4 Forward, CAAGGTCCCATGTGCAACGT., ADAMTS-4 Reverse, CATCTGCCACCACCAGTGTCT., TNF-α Forward, TGCACTTTGGAGTGATCGGC., TNF-α Reverse, ACTCGGGGTTCGAGAAGATG., CD-36 Forward, TCACTGCGACATGATTAATGGTACA., CD-36 Reverse, ACGTCGGATTCAAATACAGCATAGAT., CD-68 Forward, GCTACATGGCGGTGGAGTACAA., CD-68 Reverse, ATGATGAGAGGCAGCAAGATGG., GAPDH Forward, ACAACTTTGGCATTGTGGAA., GAPDH Reverse, GATGCAGGGATGATGTTCTG.

Detection of cellular ROS

Cellular ROS production was detected using DCFDA (6883, Sigma Aldrich), a fluorescent dye, and images were taken by fluorescent microscopy. The isolated PBMCs were treated with oxLDL and NAC for 48 h. The cells were incubated with DCFDA in 1X PBS for 45min, washed with 1X PBS, and observed under a fluorescent microscope (Zeiss Axio Vert.A1). ROS generation was detected by the oxidation of DCFDA to green fluorescence [5].

Immunoblot analysis

Immunoblot was performed to determine the level of NF-κB- p65, SIRT-1, Acetyl NF-κB- p65, and ADAMTS-4. The cell lysate was mixed with reducing sample buffer and kept at 95 °C for 10 min. Then samples were run on 10% PAGE gel [27]. After electrophoresis, the proteins were transferred to a nitrocellulose membrane using the wet transfer technique. The membrane was incubated with primary antibodies of NF-κB p65 (#8242, Cell Signaling Technology), SIRT-1 (HPA006295, Sigma Aldrich), ADAMTS-4 (AV41556, Sigma Aldrich), Acetyl NF-κB p65

(E-AB-20211, Elabscience) and β Actin (#4970, Cell Signaling Technology) at 4 °C overnight and was incubated with Anti-rabbit secondary antibody (#7074, Cell Signaling Technology) for 2 h at room temperature [28]. After washing, the membrane was incubated with enhanced chemiluminescence (ECL) reagent (1705060, Bio-Rad). The positive immunobands were documented with ChemiDoc XRS + Imager (Bio-Rad) and quantified using Image Lab software 6.1.

SIRT-1 activity assay

Cell lysate were prepared in lysis buffer, which contains 50 mM Tris-HCl (pH 8), 125 mM NaCl, 1 mM DTT, 5 mM $MgCl_2$, 1 mM EDTA, 10% glycerol, 0.1% NP-40, 1 mM PMSF and protease inhibitor [29]. Cell lysate (20 μg/well) was used for the assay. The assay was carried out according to the manufacturer's protocol (K325; Biovision). The fluorescence intensity was measured using Varioskan LUX (Thermo Scientific) using an excitation wavelength of 400 nm and emission wavelength of 505 nm.

Statistical analysis

Each experiment was performed at least thrice. Data were expressed in Mean ± SEM (standard error of the mean) and were analyzed by Graph Pad Prism version 9. The significance of the difference between the two groups was determined by Student's *t*-test, and multiple groups were determined by one-way analysis of variance (ANOVA).

RESULTS

Effect of oxLDL on cell viability

MTT assay was conducted to check the viability of cells. Isolated PBMCs were maintained in culture in the presence of different concentrations of oxLDL (10, 20, 50, 100, 150 μg mL^{-1}) for 48 h. The cells were incubated with MTT reagent and the formation of formazan crystals was measured at 540 nm. The cells showed ≥80% viability to all tested concentrations of oxLDL (Fig. 1).

Confirmation of monocyte-macrophage differentiation in the presence of oxLDL

In order to confirm the monocyte-macrophage differentiation, we studied the expression of monocyte-macrophage differentiation markers. Here we used β- D glucuronidase, CD-36, and CD-68 as the markers for monocyte-macrophage differentiation. β- D glucuronidase is an enzyme expressed by macrophages during the differentiation [25]. CD-36 and CD-68 are the scavenger receptors expressed by the macrophages and are able to bind with modified LDL [4]. For the study, PBMCs were incubated with oxidized LDL (50 μg mL^{-1}) for 48 h. The cells were harvested and analyzed for the β- D glucuronidase enzyme activity. The results showed that there was a significant increase in the β- D glucuronidase enzyme activity in oxLDL treated cells compared to untreated control cells (Fig. 2A).

For the mRNA expression studies, PBMCs were incubated with oxidized LDL (50 μg mL^{-1}) for 24 h. The mRNA expression of CD-36 and CD-68 was significantly increased in oxLDL treated cells compared to control cells (Fig. 2B–D).

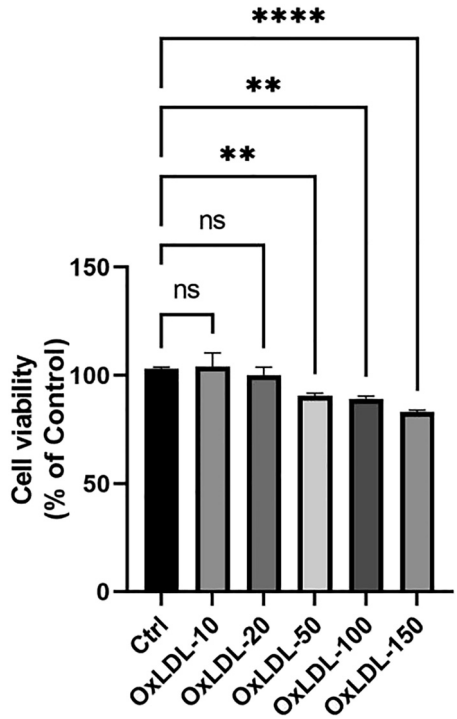

Fig. 1. MTT assay: PBMCs were maintained in culture with oxLDL for 48 h. The cells were incubated with MTT reagent (5 mg mL^{-1}) for 3 h. Formazan crystals were dissolved in DMSO and the absorbance was measured at 540 nm. Results given are the average of triplicate experiments and expressed as a percentage of control± SEM, analyzed in one-way ANOVA. $^{****}P < 0.0001$ and $^{**}P < 0.01$ compared to control

Expression of ADAMTS-4 during monocyte-macrophage differentiation

Since ADAMTS-4 is localized in macrophage-rich regions of atherosclerotic plaque, we have studied the production of ADAMTS-4 during mo-mφ differentiation. To study the expression of ADAMTS-4, PBMCs were incubated in the presence of oxLDL (50 μg mL^{-1}) for 48 h. The cells were analyzed for ADAMTS-4 expression by western blot and the secretion of ADAMTS-4 in culture supernatants by ELISA. The result showed that ADAMTS-4 expression and secretion were increased during the mo-mφ differentiation compared to untreated control cells (Fig. 3A and B).

In order to study the mRNA expression of ADAMTS-4, the cells were treated with oxLDL (50 μg mL^{-1}) for 24 h. The mRNA expression of ADAMTS-4 was increased in oxLDL treated cells compared to untreated control cells (Fig. 3C).

Effect of oxLDL on the production of TNF-α

Previous reports indicated that TNF-α induces the production of ADAMTS-4 in human osteoarthritic chondrocytes and THP-1 macrophages [13, 30]. In our study, oxLDL significantly

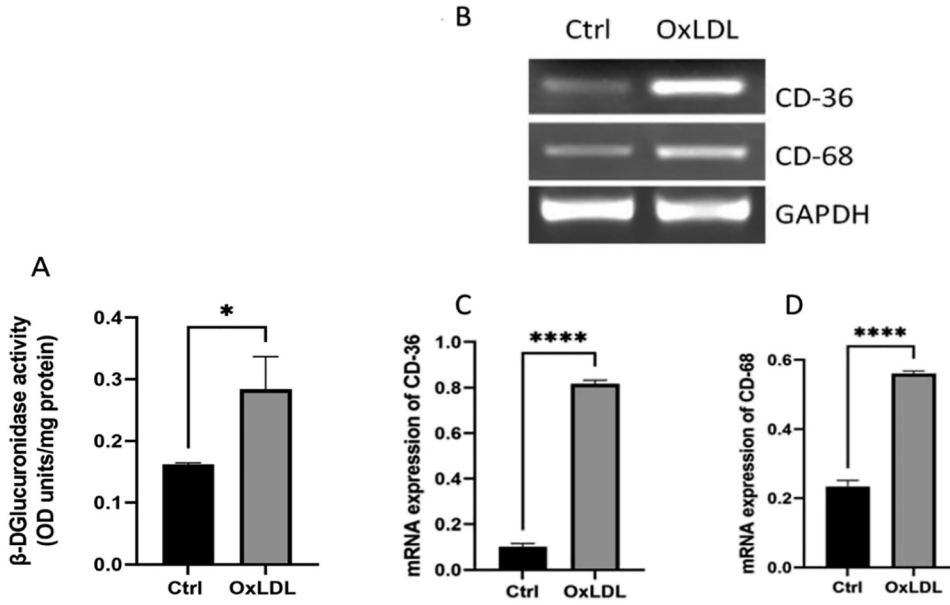

Fig. 2. Effect of oxLDL on mo-mφ differentiation: PBMCs were maintained in culture with oxidized LDL (50 μg mL^{-1}) for 48 h in the presence of 5% CO_2 at 37 °C. The cell lysate was analyzed for β-D glucuronidase activity **(A).** PBMCs were maintained in culture with oxidized LDL (50 μg mL^{-1}) for 24 h in the presence of 5% CO_2 at 37 °C. The mRNA isolated was analyzed for CD-36 and CD-68 expression. Representative images of PCR **(B)** and summary data **(C, D)**. The mean values of three experiments, presented as mean and SEM and analyzed in Student's *t*-test, $^{****}P < 0.0001$, $^{*}P < 0.05$

upregulated the production of ADAMTS-4 in the mo-mφ system. Further to check whether the upregulation of ADAMTS-4 by oxLDL is mediated through TNF-α, we examined the effect of oxLDL on the production of TNF-α in PBMCs. The cells were treated with oxLDL (50 μg mL^{-1}) for 48 h and the results showed that the production of TNF-α was significantly increased in OxLDL-treated cells (Fig. 4A). The mRNA expression of TNF-α also showed a significant increase in oxLDL treated cells compared to untreated control (Fig. 4B). These results indicate that oxLDL induced the upregulation of TNF-α in monocytes, which may, in turn, induce the production of ADAMTS-4.

ROS-mediated up-regulation of ADAMTS-4 in mo-mφ

OxLDL induces the production of ROS in macrophages. This was confirmed by DCFDA staining and our result showed that in the presence of oxLDL, the cells produce more ROS which was indicated by the enhanced green fluorescence and NAC treatment reduced the ROS production significantly compared to OxLDL-treated cells (Fig. 5A–D). To study the role of ROS on OxLDL-mediated production of ADAMTS-4 during monocyte-macrophage differentiation, PBMCs were pre-treated with 2 mM N- Acetylcysteine (NAC) and maintained in the presence of oxLDL for 48 h. The cell lysate was analyzed for the production of ADAMTS-4 by western

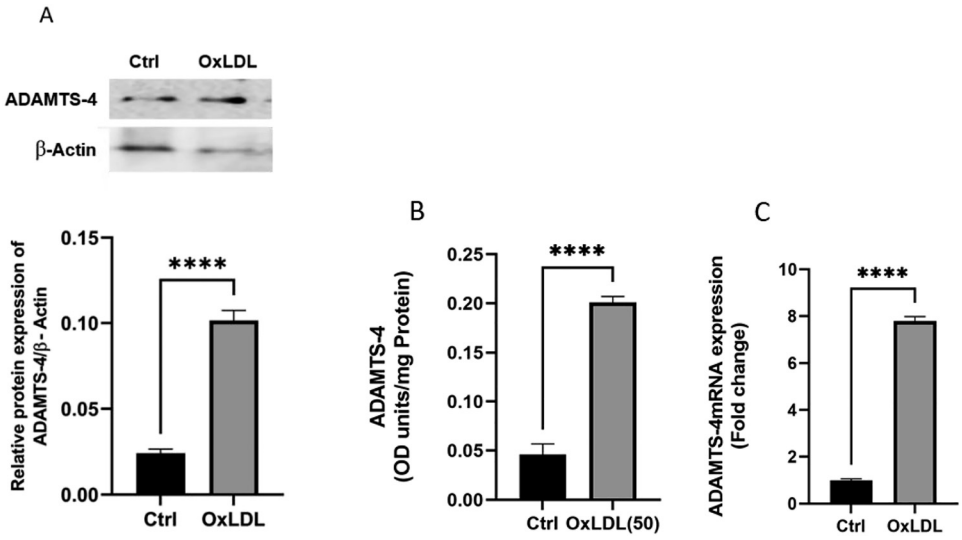

Fig. 3. ADAMTS-4 expression during mo-mφ differentiation: Isolated PBMCs were maintained in culture with oxidized LDL (50 μg mL^{-1}) for 48 h in the presence of 5% CO_2 at 37 °C. Cells and culture supernatants were collected and analyzed for ADAMTS-4 by western blot (**A**) and ELISA(**B**). The mRNA expression of ADAMTS-4 in 24 h was analyzed by qRT-PCR(**C**). The results are the mean values of three experiments, presented as mean and SEM and analyzed by Student's *t*-test, $^{****}P < 0.0001$

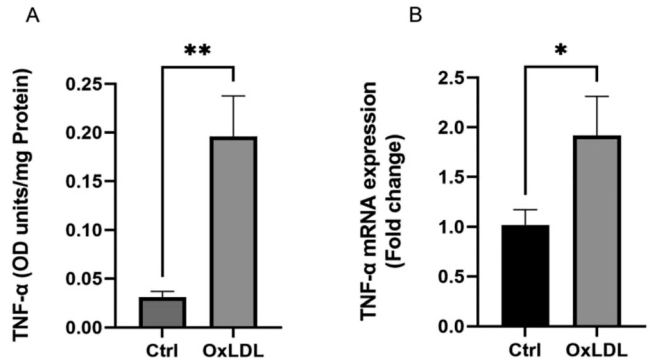

Fig. 4. Effect of oxLDL on the production of TNF-α: Isolated PBMCs were maintained in culture with oxidized LDL (50 μg mL^{-1}) for 48 h in the presence of 5% CO_2 at 37 °C. The culture supernatant was analyzed for TNF-α by ELISA **(A)**. Isolated PBMCs were maintained in culture with oxidized LDL (50 μg mL^{-1}) for 4 h in the presence of 5% CO_2 at 37 °C. mRNA isolated from the cells was analyzed by qPCR **(B)**. The results are the mean values of three experiments, presented as mean ± SEM and analyzed in Student's *t*-test. $^{**}P < 0.01$ and $^{*}P < 0.05$ compared to control

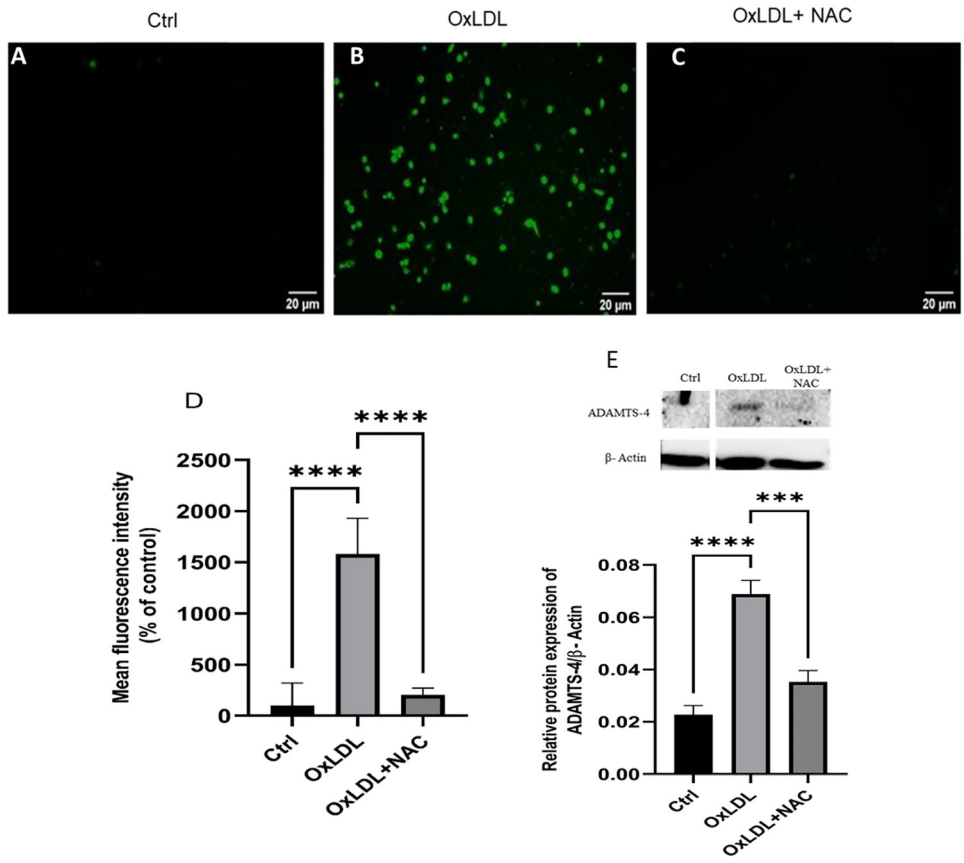

Fig. 5. Effect of N- Acetylcysteine on the production of ADAMTS-4: Isolated PBMCs were maintained in culture in the presence of oxLDL (50 µg mL^{-1}) and NAC (2 mM) for 48 h. Untreated cells served as control. The cells were treated with DCFDA and ROS-producing cells showed green fluorescence **(A, B, C, and D)**. The cells were analyzed for ADAMTS-4 by western blot **(E)**. The results given are the average of triplicate experiments ±SEM and analyzed in one-way ANOVA. $^{****}P < 0.0001$, $^{***}P < 0.001$

blot. Results showed that ADAMTS-4 expression by PBMCs increased on treatment with oxLDL compared to untreated control cells. Pre-treatment with NAC significantly downregulated the expression of ADAMTS-4 by OxLDL-treated cells (Fig. 5E).

Role of NF-κB on the regulation of ADAMTS-4 in mo-mφ

Since, the expression of ADAMTS-4 was regulated through NF-κB pathway, we have studied the effect of ROS on the expression and activation of NF-κB in oxLDL treated PBMCs. The effect of NAC on the expression and nuclear translocation of NF-κB in OxLDL-treated PBMCs was studied by western blot and ELISA respectively. The results indicate that the expression and nuclear translocation of NF-κB were increased on treatment with oxLDL and which were

significantly downregulated on NAC pre-treatment (Fig. 6A and B). We confirmed the role of NF-κB signaling pathway in ADAMTS-4 regulation by studying the expression of ADAMTS-4 in the presence of NF-κB inhibitor BAY11-7082 (10 μM). The expression of ADAMTS-4 was significantly downregulated in the presence of BAY11-7082 compared to OxLDL-treated cells (Fig. 6C).

The role of SIRT-1 on the regulation of ADAMTS-4

SIRT-1 is an NAD^+-dependent histone deacetylase, which regulates the transcriptional activity of NF-κB by removing K310 acetylation [19]. The acetylation of NF-κB- p65 at K310 residue enhances its transcriptional activity by enhancing its binding with DNA and other transcriptional factors [19]. Here, we studied the expression of SIRT-1 in oxLDL induced mo-mφ system. The result showed that the expression of SIRT-1 significantly downregulated in oxLDL treated cells compared to untreated control cells (Fig. 7A). Next, we confirmed the role of SIRT-1 on the expression of ADAMTS-4 by using SIRT-1 agonists, resveratrol, and rutin. ADAMTS-4 expression was significantly downregulated in the presence of SIRT-1 agonists (Fig. 7B and C). The decreased SIRT-1 activity during mo-mϕ was significantly reversed in the presence of resveratrol (Fig. 7D). SIRT-1 mediated NF-κB regulation was confirmed by studying the deacetylation of NF-κB in the presence of resveratrol by immunoblot analysis. Our result indicated that SIRT-1 may be down-regulating the expression of ADAMTS-4 through the deacetylation of NF-κB (Fig. 7E).

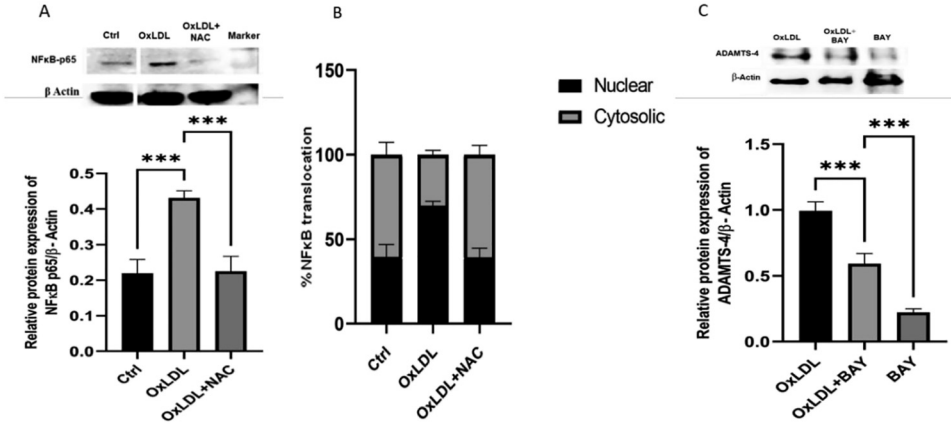

Fig. 6. NF-κB activation regulates the expression of ADAMTS-4. Isolated PBMCs were maintained in culture in the presence of oxLDL (50 μg mL^{-1}) and NAC (2 mM) for 48 h. Untreated cells served as control. The cells were analyzed for NF-κB expression and nuclear translocation by western blot and ELISA respectively **(A, B)**. The expression of ADAMTS-4 in the presence of NF-κB pathway inhibitor BAY11-7082 was studied by pre-treating the PBMCs with 10 μM BAY11-7082 for 1 h and incubating with oxLDL for 48 h. The cells were analyzed for the expression of ADAMTS-4 by western blot **(C)**. The results given are the average of triplicate experiments ±SEM and analyzed in one-way ANOVA. $^{***}P < 0.001$

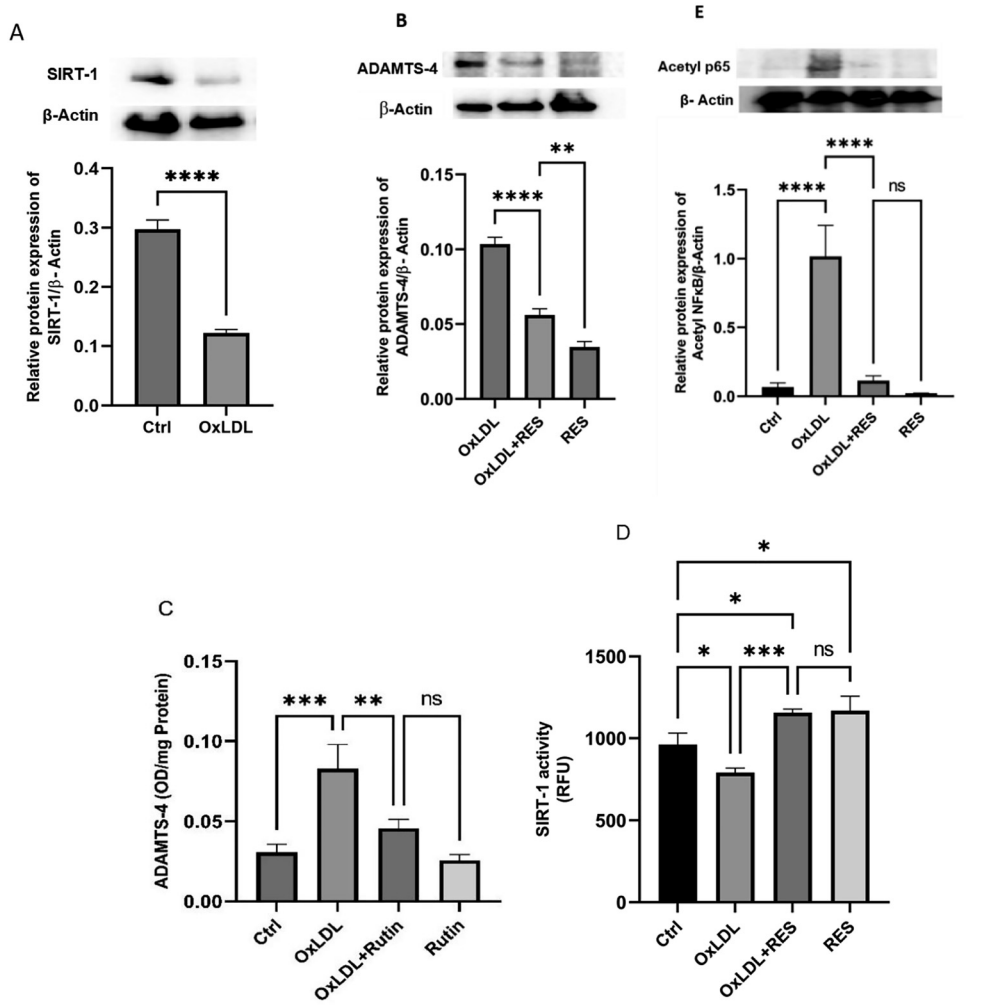

Fig. 7. SIRT-1 downregulate the expression of ADAMTS-4. SIRT-1 expression during mo-mφ differentiation was studied by incubating the PBMCs with oxLDL (50 μg mL^{-1}) for 48 h. The cells were analyzed for SIRT-1 expression (**A**). To study the role of SIRT-1 in ADAMTS-4 regulation, PBMCs were pre-treated with resveratrol (10 μM) for 16 h and maintained in culture in the presence of oxLDL (50 μg mL^{-1}) for 48 h and ADAMTS-4 expression was analyzed by western blot (**B**). PBMCs were pre-treated with rutin (100 μM) for 4 h and maintained in culture in the presence of oxLDL (50 μg mL^{-1}) for 48 h and ADAMTS-4 expression was analyzed by ELISA (**C**). The SIRT-1 activity was studied in the presence of resveratrol by fluorimetric method (**D**). ADAMTS-4 expression was also studied by pre-treating the monocytes with 100 μM rutin for 4 h and incubated in the presence of oxLDL for 48 h. For studying the acetylation of NF-κB, monocytes were pre-treated with resveratrol for 16 h and maintained in culture in the presence of oxLDL (50 μg mL^{-1}) for 30 min and the expression was analyzed by western blot (**E**). The results given are the average of triplicate experiments ±SEM and analyzed in one-way ANOVA. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ $^{****}P < 0.0001$

DISCUSSION

Hyperlipidemia is one of the major risk factors for the development of atherosclerosis. Oxidized forms of lipoproteins initiate plaque formation by the activation of monocytes leading to endothelial dysfunction and foam cell formation [31]. Macrophages and foam cells express a large number of vascular remodelling proteinases which are involved in the different stages of atherosclerosis progression [8]. ADAMTS, a group of matrix-degrading enzymes secreted by the inflammatory cells are associated with plaque and are reported to be involved in vascular remodelling and atherosclerosis [32]. ADAMTS-4 and ADAMTS-8 were seen in macrophage–rich areas of human atherosclerotic plaques [13] and ADAMTS-4 was found to be upregulated during the development of atherosclerotic plaque in mice [13]. ADAMTS-4 plays a major role in the breakdown of aggrecan and versican in the arterial wall [33]. Versican, the primary proteoglycan present in all three layers of the arterial wall, binds with a number of cell receptors, cytokines, lipoproteins, and ECM components [33], breakdown product versikine, enhances plaque formation by facilitating the migration of cells like macrophage and VSMCs, and LDL retention [33]. Macrophages are the major producers of proteases enzymes and ADAMTS-4 expression was found co-localized with CD-68 expressing cells in the atherosclerotic plaques [13]. But little is known about the expression of ADAMTS-4 in macrophages. Our study opens the molecular mechanism involved in the regulation of ADAMTS-4 expression during OxLDL-mediated monocyte-macrophage differentiation.

In our study, human monocytes were differentiated into macrophages in the presence of oxidized LDL. Monocyte-macrophage differentiation was confirmed by studying the expression of differentiation markers. An increase in the expression of CD-36 and CD-68, the scavenger receptors, and the increased activity of β- D glucuronidase enzyme in our system indicated monocyte-macrophage differentiation. Our present study focused on the molecular mechanism involved in the upregulation of ADAMTS-4 during monocyte-macrophage differentiation. Our results suggest that the upregulation of ADAMTS-4 in OxLDL-mediated macrophage formation is possibly mediated through the ROS-NF-κB-SIRT-1 pathway. Evidence in support of this is the following: (a) oxLDL significantly upregulated the expression of ADAMTS-4 mRNA, protein, and its secretion; (b) ROS production was significantly upregulated on treatment with oxLDL and was reversed in the presence of N-acetyl cysteine, ROS scavenger; (c) OxLDL mediated upregulation of ADAMTS-4 in macrophages was reversed by NAC; (d) Increased NF-κB, expression and activation during macrophage formation was also reversed in the presence of NAC; (e) OxLDL mediated upregulation of ADAMTS-4 in macrophages was reversed in the presence of NF-κB inhibitors; (f) ADAMTS-4 expression was down-regulated in the presence of SIRT-1 agonists, resveratrol and rutin (g) SIRT-1 activity and NF-κB deacetylation were significantly upregulated in the presence of resveratrol in macrophages.

The data presented in this study showed that the expression of ADAMTS-4 was upregulated in human monocyte-derived macrophages in the presence of oxLDL. A previous study showed that ADAMTS-4 expression was increased in PMA-differentiated THP-1 macrophages and THP-1 cells maintained in the presence of a conditioned medium of differentiated THP-1 macrophages [13]. TNF-α has previously been shown to enhance the production of ADAMTS-4 in PMA-differentiated THP-1 cells [13]. In our study also, a significant increase in TNF-α expression was found during the OxLDL-mediated macrophage formation, which can also lead to the upregulation of ADAMTS-4.

Macrophages are the major ROS producers and it plays an important role in maintaining pro-inflammatory phenotype [34]. ROS released during the differentiation of monocytes enhances the process by inducing the inflammation signalling pathways [34]. A previous study has shown that oxLDL induces ROS production in THP-1 macrophages [35] and in our study also, the production of ROS during oxLDL-induced mo-mφ differentiation was confirmed by DCFDA staining. Several studies reported that ROS activates pro-inflammatory gene expression in macrophages through the NF-κB pathway [34]. ROS is the major signalling molecule that activates NF-κB pathway through IKK activation [34]. Here we studied the role of ROS in NF-κB activation in the presence of NAC, the ROS inhibitor. Both the expression and activation of NF-κB were significantly downregulated in the presence of NAC. These results indicated that ROS-mediated NF-κB activation is taking place during OxLDL-mediated macrophage formation. It has been reported that NAC suppresses TNF- α induced NF-κB activation by suppressing both IKK-α and IKK- β in HeLa cells [36], indicating that NAC can also act as an inhibitor of NF-κB pathway. Here, our study indicated the involvement of ROS- NF-κB pathway in the regulation of ADAMTS-4 in macrophages. The ADAMTS-4 expression in macrophages was significantly downregulated in the presence of NAC and BAY11-7082.

NF-κB, the key transcription factor of many inflammatory genes involved in atherosclerosis is regulated by post-translational modifications like phosphorylation and acetylation. Among the acetylation sites, the K310 acetylation of p65 subunit is considered to be the major acetylation that regulates the transcriptional activity of NF-κB. Phosphorylation of NF-κB-p65 at serine 276 and serine 536 facilitates the acetylation of NF-κB-p65 at K310 residue [24]. Suppressing the phosphorylation and acetylation of NF-κB could be an effective therapeutic strategy for regulating inflammatory genes. Previous reports indicated that gallic acid downregulated TNF-α induced ADAMTS-4 expression by suppressing the phosphorylation and acetylation of NF-κB-p65 in nucleus pulposus cells [16]. SIRT-1 is a class III histone deacetylase involved in the deacetylation of non-histone proteins like NF-κB [19]. Previous studies reported that SIRT-1 a key player in inflammation, downregulated NF-κB activity by deacetylating the K310 residue of p65 subunit [37]. Bai et al. reported that the knockdown of SIRT-1 significantly upregulated the expression of ADAMTS-4 in chondrosarcoma cells [38]. Studies in coronary artery disease (CAD) patients also showed decreased expression of SIRT-1 in monocytes compared to healthy individuals [39]. In our study also, the expression of SIRT-1 was significantly downregulated in human monocyte-differentiated macrophages. SIRT-1 overexpression leads to significant downregulation of ADAMTS-4 in human osteoarthritis chondrocytes [40]. Our study showed that ADAMTS-4 expression was significantly downregulated in the presence of SIRT-1 agonists, resveratrol, and rutin. This indicates that the expression of ADAMTS-4 during mo-mϕ differentiation is regulated through SIRT-1. ROS could inhibit SIRT-1 activity by directly oxidizing its cysteine residues [37]. In our study also the SIRT-1 activity significantly downregulated during mo-mφ differentiation and was reversed in the presence of resveratrol. The increased production of ROS in our system may lead to the inhibition of SIRT-1 activity and thereby upregulating the expression of ADAMTS-4 in macrophages.

SIRT-1 mediates its effects on inflammation mainly through the deacetylation of NF-κB in inflammation [37]. Our results also showed that increased acetylation of NF-κB-p65 at K310 in macrophages was reversed in the presence of the SIRT-1 agonist, resveratrol. SIRT-1 mediated deacetylation of NF-κB may be partly responsible for the downregulation of ADAMTS-4. Taken

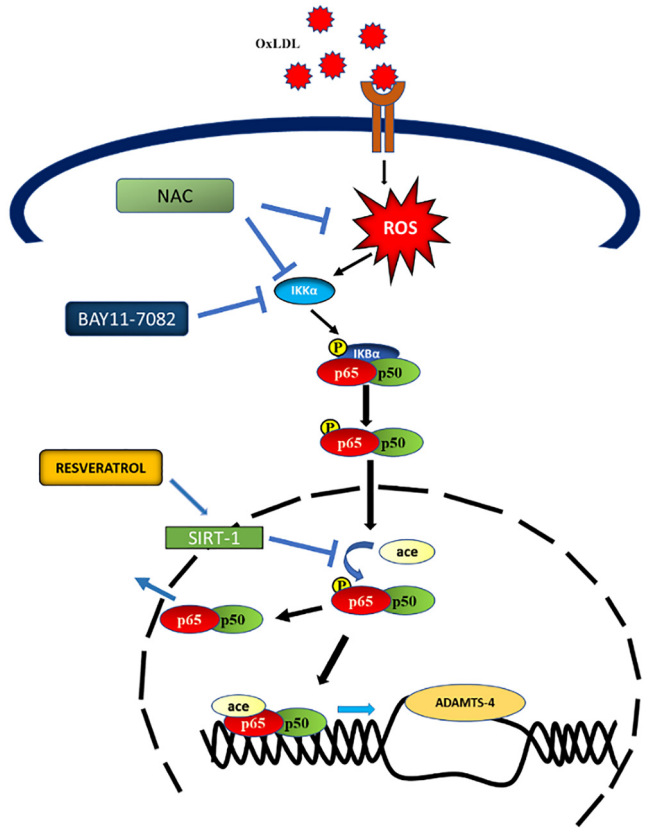

Fig. 8. OxLDL induces ROS production during mo-mϕ differentiation, which activates IKK and results in the activation and nuclear translocation of NF-κB. ROS- NF-κB pathway leads to the expression of ADAMTS-4 in macrophages, which was confirmed by using BAY11-7082 and NAC. SIRT-1 agonists down-regulate the expression of ADAMTS-4 in macrophages. It indicates the involvement of SIRT-1 in the regulation of ADAMTS-4 by down-regulating the transcriptional activity of NF-κB through its deacetylation

together, our study showed that the OxLDL-induced upregulation of ADAMTS-4 expression in macrophages is mediated through ROS- NF-κB- SIRT-1 pathway (Fig. 8).

In conclusion, the expression of ECM remodeling enzymes like ADAMTS-4 is significantly upregulated by OxLDL-mediated monocyte-macrophage differentiation. This OxLDL-mediated upregulation of ADAMTS-4 is mediated through ROS- NF-κB- SIRT-1 pathway. Therapeutic strategies targeting this pathway would be helpful for the management of inflammatory diseases like atherosclerosis.

Conflict of interests: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Author contributions: Aswani. S.S and Saja. K conceptualized the study and designed the experiments, analyzed the data, and interpreted the results of the experiments. Aswani. S .S prepared the figures and drafted the manuscript. Aswani.S. S, Mithra. S. Mohan and Aparna N.S performed the experiments. Saja. K and P. T. Boban reviewed and edited the manuscript. All authors read and approved the manuscript.

Ethics approval: All the procedures carried out in the research with the participation of humans complied with the ethical standards of the Human ethical committee, University of Kerala (ULECRIHS/UOK/2018/25). Informed voluntary consent was obtained from every participant in the study.

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SUPPLEMENTARY MATERIAL

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