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MiR-146a inhibits oxidized low-density lipoprotein-induced lipid accumulation and inflammatory response via targeting toll-like receptor 4

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ABSTRACT

Atherosclerosis is an inflammatory process due to oxidized low-density lipoprotein (oxLDL) accumulation in macrophages. We investigated the involvement of microRNAs in oxLDL accumulation and inflammatory response in macrophages. The expression of miR-146a decreases under oxLDL stimulation. MiR-146a significantly reduces intracellular LDL cholesterol content and secretion of interleukin 6, interleukin 8, chemokine (C–C motif) ligand 2 and matrix metallopeptidase 9. Toll-like receptor 4 (TLR4) is a relevant target of miR-146a, and miR-146a inhibits the activation of TLR4-dependent intracellular signaling pathways involved in cytoskeleton rearrangement, lipid uptake, and inflammatory cytokine secretion. These results indicate that miR-146a contributes to the regulation of both oxLDL accumulation and inflammatory response by negatively regulating TLR4 and thereby inhibiting the activation of TLR4-dependent signaling pathways. Over-expression of miR-146a may be useful in the prevention and treatment of atherosclerosis.

1. Introduction

Inhibition of lipid accumulation in macrophage-derived foam cells and reduction of consequent inflammatory response are crucial for preventing atherosclerotic plaque formation and coronary lesion progression [1]. Several scavenger receptors [e.g., CD36 molecule (CD36), scavenger receptor A (SRA)] possess ability to take up oxidized low-density lipoprotein (oxLDL) in macrophages, and have been linked to atherosclerotic process [2]. In animal experiments, activation of toll-like receptor 4 (TLR4) promotes vascular lipid accumulation [3], and TLR4-dependent signal pathways contribute to minimally modified low-density lipoprotein (mmLDL)-induced cytoskeleton rearrangement necessary for the process of lipoprotein uptake [4]. Similarly, oxLDL activates TLR4, triggering inflammatory response in macrophages through mitogen-activated protein kinase (MAPK) pathway [5].

MicroRNAs (miRNAs) inhibit transcription or translation by interacting with 3′ untranslated region (3′-UTR) of a target gene [6], and are involved in inflammatory process in patients with coronary artery disease [7,8]. MiR-146a interacts directly with interleukin-1 receptor-associated kinase 1 (IRAK1)/TNF receptor-associated factor 6 (TRAF6), suppressing secretion of inflammatory cytokines in macrophages [9,10]. Recently, miR-125-5p was considered to partly inhibit lipid uptake by down-modulation of oxysterol binding protein-like 9 (ORP9), and to decrease inflammatory cytokine expression in oxLDL-stimulated monocyte-derived macrophages [11]. However, the precise role of miRNAs in foam cell formation and inflammatory response remains unclear.

In this study, we sought to investigate the differential expression of miRNAs in oxLDL-stimulated macrophages, and to

Abbreviations: miRNAs, microRNAs; oxLDL, oxidized low-density lipoprotein; TLR2, toll-like receptor 2; TLR3, toll-like receptor 3; TLR4, toll-like receptor 4; TLRs, toll-like receptors; IL-6, interleukin 6; IL-8, interleukin 8; MCP-1, chemokine (C–C motif) ligand 2; MMP-9, matrix metallopeptidase 9; SRA, scavenger receptor A; CD36, CD36 molecule; mmLDL, minimally modified low-density lipoprotein; MAPK, mitogen-activated protein kinase; IRAK1, interleukin-1 receptor-associated kinase 1; TRAF6, TNF receptor-associated factor 6; ORP9, oxysterol binding protein-like 9; 3′-UTR, 3′ untranslated region; JNK, mitogen-activated protein kinase 8; NF-κB, nuclear factor of kappa light polypeptide gene enhancer in B-cells 1; ERK, mitogen-activated protein kinase 1; p38, mitogen-activated protein kinase 14; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction; LPS, lipopolysaccharide; poly I:C, polyinosinic:polycytidylic acid; MyD88, myeloid differentiation primary response gene (88); Pyk2, proline-rich tyrosine kinase 2; Syk, spleen tyrosine kinase; Src, v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian); AKT, v-akt murine thymoma viral oncogene homolog 1

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assess the effects of miR-146a on intracellular LDL cholesterol content and inflammatory cytokine secretion. The interaction of miRNA–mRNA was examined by luciferase assay, Western blot and rescue assay, to further elucidate the potential mechanisms.

2. Materials and methods

2.1. Cell culture and treatment

THP-1 cells (ATCC, VA, USA) were seeded on six-well plates at a density of 1.0×10^6 cells/well and differentiated into macrophages by administrating 100 ng/ml phorbol 12-myristate 13-acetate (CalBiochem, CA, USA) for 48 h. Low-density lipoprotein was purified to homogeneity by ultra-centrifugation, and oxidized using 20 μM cupric sulphate in PBS at 37 °C for 24 h. The level of oxidation was measured using TBAR determination with the malondialdehyde (MDA) standard, and the migration factor of oxLDL was 2.2. Macrophages were incubated with oxLDL (50 μg/ml) for 24 h to transform into foam cells. Hela cells (ATCC) were used in luciferase assay.

2.2. Analysis of differential expression of miRNAs

Total RNA (2–5 μg) was extracted using Trizol reagent (Invitrogen, CA, USA). Microarray analysis was performed using the μParaflo™ miRNA system (LC Sciences, USA). Pre-miRNAs (<300 nucleotides) were isolated and labeled with fluorescent dyes Cy3 and Cy5, and images were obtained using a laser scanner (GenePix 4000B, Molecular Devices). Microarray data were normalized with a LOWESS (locally weighted regression) filter, which was further verified by sequence-specific primer from TaqMan real-time polymerase chain reaction (PCR) (Applied Biosystems, CA, USA) for six times [12] in each candidate miRNA. TaqMan miRNA assay kits with a stemlooped primer for reverse transcription were used to accurately detect mature miRNAs.

2.3. Prediction of miR-146a target sites

A consensus approach employing miRanda, RNAhybrid, and TargetScan was applied to target prediction. In addition, RNAhybrid with options 3′-UTR_human for vertebrate sequences was utilized to calculate free energies of miR-146a: TLR4 mRNA and predicted target sites of miR-146a [13,14].

2.4. Luciferase assay

Human *tlr4* cDNA, containing putative and mutant target sites for miR-146a, was chemically synthesized and inserted into a pMIR-REPORT™ vector (Ambion, TX, USA). The pMIR-REPORT™ β-gal control vector (Ambion) was used as a reference. For luciferase assay, Hela cells were co-transfected with wild- (pMIR-tlr4-wt) or mutant-type (pMIR-tlr4-mt) reporter vector and miR-146a mimics using Lipofectamine 2000 (Invitrogen). Luciferase activity was measured 48 h after transfection using a dual-luciferase assay kit (Promega, WI, USA) [15].

2.5. Assessment of intracellular LDL cholesterol content

Cultured and transfected macrophages were washed with PBS (Gibco, NY, USA) and then fixed in 4% paraformaldehyde/PBS for 15 min. After rinsing with ddH_2O, macrophages were stained with 0.3% oil red O (Sigma–Aldrich, MO, USA) in 60% isopropanol for 10 min, and incubated with DiI-Ac-LDL (Invitrogen). Pictures were taken using confocal imaging fluorescence microscope (Leica Microsystems). The DiI fluorescence was analyzed using flow cytometry (BD, NJ, USA) ($n = 3$), and the oil red O staining was measured by OD 500 nm ($n = 3$). Intracellular LDL cholesterol content was quantified using a colorimetric method and expressed as relative value to total protein concentration ($n = 3$) (Biovision, CA, USA) [16].

2.6. Quantitative real-time PCR

For macrophages transfected with miR-146a mimics or inhibitors, the mRNA levels were determined after 24 h with a SYBR-Green reagent kit (Takara, Dalian, China) [17]. The gene specific primers used are shown in Supplementary Table 1.

2.7. Western blotting

Proteins were isolated and quantified using the BCA method (Pierce, Rockford, IL). For each sample, 30 μg protein was loaded onto a 10% SDS–polyacrylamide gel. After electrophoresis, protein was transferred onto a PVDF membrane (Millipore, MA, USA) by electro-elution. The membrane was incubated initially with TLR4, mitogen-activated protein kinase 14 (p38), phospho-p38, mitogen-activated protein kinase 1 (ERK), phospho-ERK, mitogen-activated protein kinase 8 (JNK) pan, phospho-JNK pan, nuclear factor of kappa light polypeptide gene enhancer in B-cells 1 (NF-κB), phospho-NF-κB, IRAK1 and TRAF6 (Cellsignaling, MA, USA) overnight at 4 °C, then with HRP-conjugated goat anti-Rabbit IgG (Cellsignaling) for 2 h at room temperature. After washing, the membrane was processed using Immobilon™ Western chemiluminescent HRP substrate (Millipore). β-Actin (Cellsignaling) was served as internal control.

2.8. Oligonucleotide transfection

Macrophages were transfected with 100 pmol MiRIDIAN mimics and 100 pmol inhibitors (Dharmacon, CO, USA), respectively, using Lipofectamine 2000 (Invitrogen). To confirm the efficiency

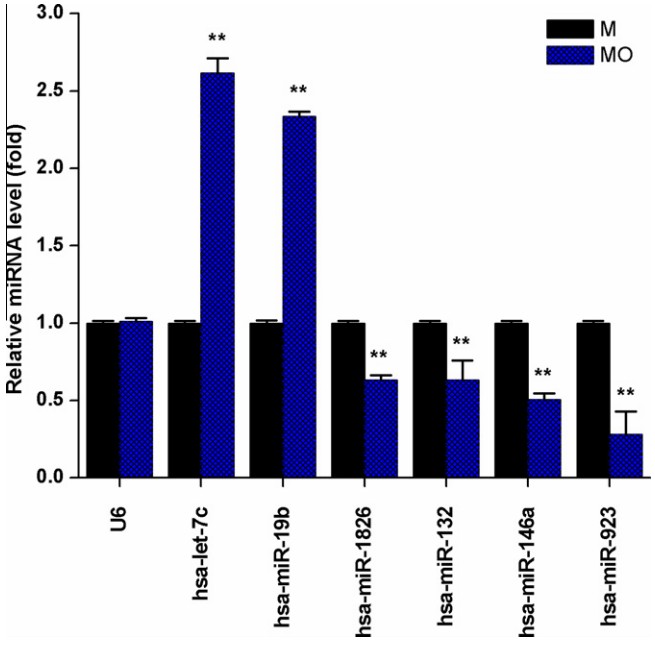

Fig. 1. Analysis of differentially expressed miRNAs in non-oxLDL-stimulated (M) and oxLDL-stimulated (MO) macrophages. The six most significant aberrantly expressed miRNAs were further identified by Taqman real-time PCR ($n = 6$). $^{**}P < 0.01$ vs. M.

of transfection, same amount of negative control (Dharmacon) labeled with FAM was also transfected. Small interference RNA (siRNA) was used for knockdown of *tlr4* (Dharmacon). Cells were transfected for 48 h and exposed to oxLDL (50 μg/ml) for 24 h.

2.9. Plasmid construction and transfection

Human *tlr4* cDNA was all chemically synthesized. The cDNA fragments were cloned into pVAX1 plasmid (Invitrogen), which was verified by sequencing. Macrophages were transfected with combination plasmid using Lipofectamine 2000 (Invitrogen). Cells were transfected for 48 h and exposed to oxLDL (50 μg/ml) for 24 h.

2.10. Human phosphokinase array

At 48 h after transfection with 100 pmol mimics and negative control, macrophages were treated with oxLDL for 1 h, and total protein was purified. Non-treated-macrophages were used as control. Cell lysates (250 mg total protein per array) were applied and incubated with Human Phospho-Kinase Array kit (R&D Systems, MN, USA). Gray values were quantified using NIH image software analysis [18].

2.11. Enzyme-linked immunosorbent assay (ELISA)

Levels of interleukin 6 (IL-6), interleukin 8 (IL-8), chemokine (C–C motif) ligand 2 (MCP-1) and matrix metallopeptidase 9 (MMP-9) in the supernatants of non-oxLDL-stimulated and oxLDL-stimulated macrophages transfected individually with native control, TLR4 cDNA recombination plasmid, TLR4 siRNA, miR-146a mimics and inhibitors were measured using ELISA kits (Invitrogen).

2.12. Statistical analysis

Data are presented as means ± S.E.M., and were compared using Student's *t*-test (for two group comparisons) or analysis of variance (>2 group comparisons) when appropriate. A *P*-value of <0.05 was considered statistically significant.

3. Results

3.1. Microarray analysis of miRNA expression

Compared with non-oxLDL-stimulated macrophages, let-7c and miR-19b were up-regulated, but miR-146a, miR-1826, miR-132 and miR-923 were down-regulated in oxLDL-stimulated macro-

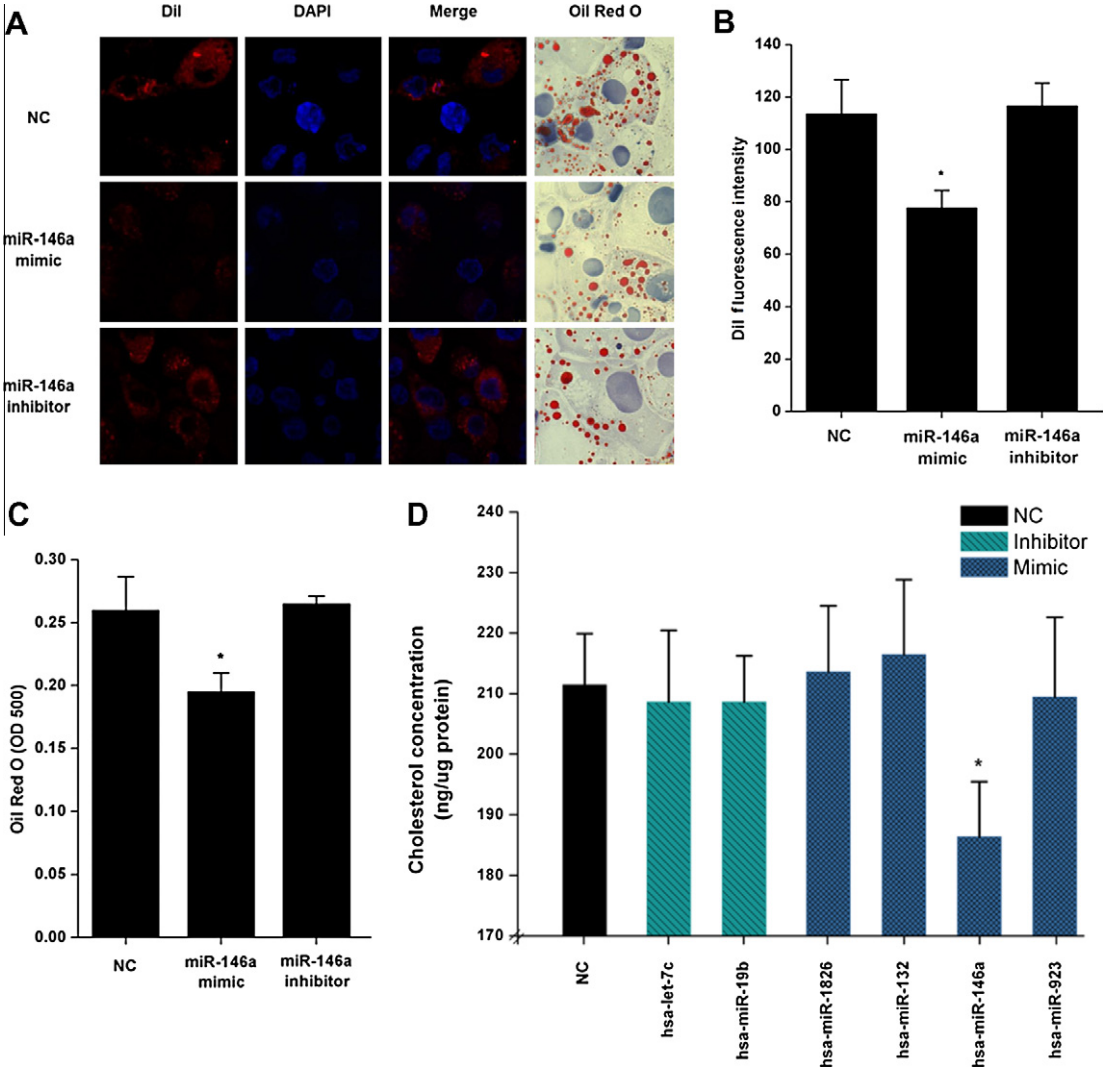

Fig. 2. Effect of miR-146a on LDL cholesterol content in macrophages. (A) Pictures acquired and processed at identical conditions are representative of three independent experiments (×1200). (B) Dil fluorescence intensity was analyzed using flow cytometry ($n = 3$; $^*P < 0.05$ vs. NC). (C) Oil red O was analyzed using OD 500 nm ($n = 3$; $^*P < 0.05$ vs. NC). (D) Total intracellular LDL cholesterol was determined using a colorimetric method ($n = 3$; $^*P < 0.05$ vs. NC). NC: Cells transfected with negative control miRNAs.

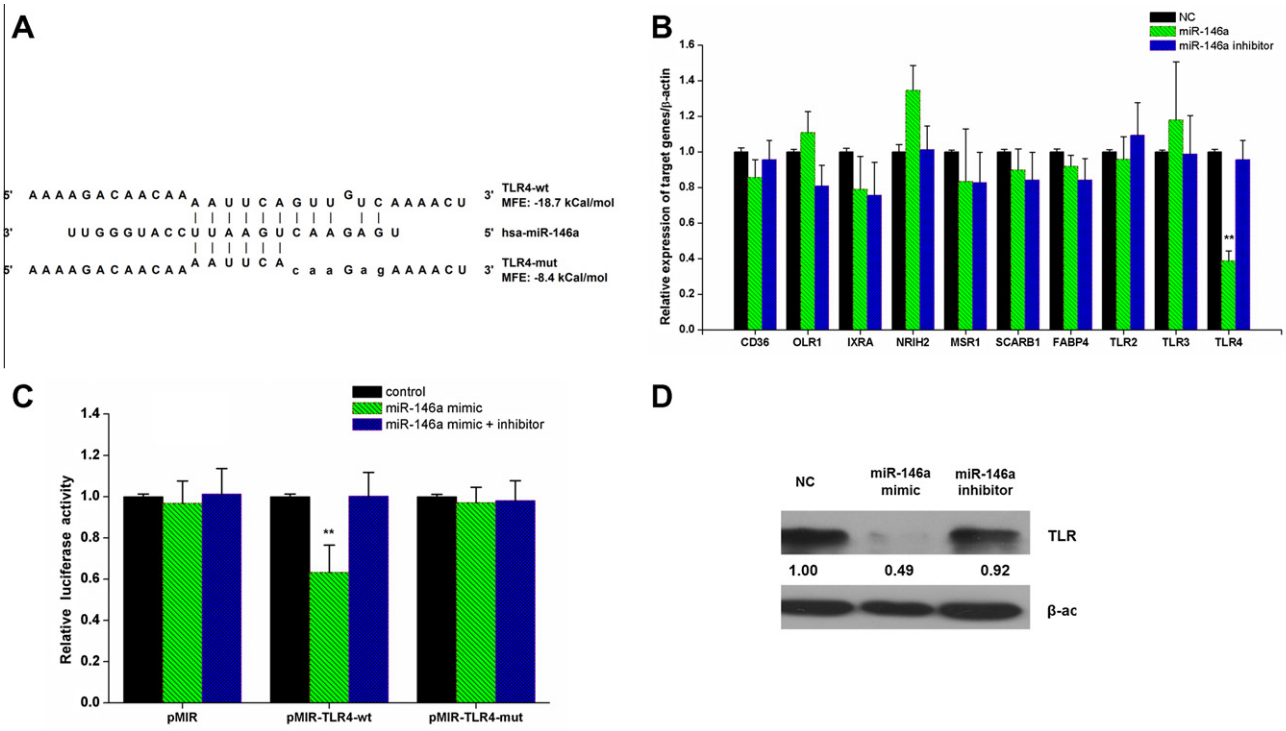

Fig. 3. Verification of target genes of miR-146a. (A) Sequence alignment and minimum free energy (MFE) of miR-146a and its targets in wild type (wt) or mutant type (mt) 3′-UTRs of TLR4. (B) MiR-146a mimics and inhibitors affected the gene expression patterns in macrophages stimulated with oxLDL ($n = 3$; $^{**}P < 0.01$ vs. NC). (C) Changes of luciferase activity by miR-146a mimic and inhibitor in 3′-UTR of TLR4 ($n = 3$; $^{**}P < 0.01$ vs. Hela cells not transfected with miRNAs [control]). (D) TLR4 protein levels were analyzed with Western blot and normalized by β-actin (TLR4/β-actin; $n = 3$). NC: As in Fig. 2.

phages (Fig. 1). Absolute values of individual miRNAs and normalization procedure that were significantly different between oxLDL-stimulated and non-oxLDL-stimulated macrophages are listed in Supplementary Table 2.

3.2. MiRNAs affected intracellular LDL cholesterol content

Compared with negative control, the DiI-Ac-LDL fluorescence, oil red O elution and intracellular LDL cholesterol content were decreased in the macrophages with over-expression of miR-146a (Fig. 2A–C), but was not altered in those transfected by let-7c and miR-19b inhibitors as well as miR-1826, miR-132 and miR-923 mimics (Fig. 2D). The expression of miR-146a mimics is shown in Supplementary Fig. 1D.

3.3. MiR-146a reduced TLR4 expression in oxLDL-stimulated macrophages

The minimum free energy of miR-146a and alignment sequence in TLR4 3′-UTR was −18.7 kcal/mol (Fig. 3A). In oxLDL-stimulated macrophages, the expression of TLR4 as target gene was decreased by miR-146a mimics, but the miR-146a inhibitors did not change TLR4 expression (Fig. 3B). After co-transfecting with miR-146a and pMIR-TLR4-wild vector, luciferase activity was decreased significantly. This effect was completely abolished by substituting this vector with its mutant version (Fig. 3C). In oxLDL-stimulated macrophages, TLR4 protein was significantly up-regulated (Supplementary Fig. 2A). TLR4 protein was significantly down-regulated by transfection with miR-146a mimics, but was only slightly reduced by miR-146a inhibitors in oxLDL-stimulated microphages (Fig. 3D). In non-treated-macrophages, miR-146a inhibitors attenuated miR-146a silencing TLR4 expression (Supplementary Fig. 2B). The efficiency of oligo and plasmid transfection and the expression of miR-146a mimics are shown in Supplementary Fig. 1A, B and D.

3.4. MiR-146a decreased inflammatory cytokine secretion

Levels of IL-6, IL-8, MCP-1, and MMP-9 were reduced by miR-146a with knock-down of TLR4, but increased by over-expression of TLR4 (Fig. 4). The efficiency of siRNA knock-down TLR4 expression and the expression of miR-146a mimics are shown in Supplementary Fig. 1C and D.

3.5. Signaling pathways regulated by miR-146a

OxLDL induced phosphorylation of v-src sarcoma (Schmidt-Ruppin A-2) viral oncogene homolog (avian) (Src), Yes, Fyn, Fgr, Lck, Hck, Lyn, FAK, JNK, proline-rich tyrosine kinase 2 (Pyk2), paxillin, p38 MAPK, ERK1/2, JNK pan and NF-κB. In contrast, miR-146a inhibited the activation of these kinases which were induced by ox-LDL (Fig. 5A–C) (Supplementary Tables 3 and 4). Similarly, over-expression of miR-146a decreased the expression of IRAK1 and TRAF6 (Fig. 5D). The expression of miR-146a mimics is shown in Supplementary Fig. 1D.

4. Discussion

This study demonstrated that miR-146a synchronously reduced LDL cholesterol level and inflammatory cytokine secretion in oxLDL-stimulated macrophages, by directly down-modulating TLR4 and inhibiting activation of TLR4-dependent signaling pathway.

Toll-like receptors (TLRs) play multiple roles in atherosclerosis [19], and are highly expressed in human atherosclerotic plaques [20]. Intracellular cholesterol content in macrophages was increased by approximately five-folds following stimulation with lipopolysaccharide (LPS) (a TLR4 ligand), which was greater than that with Zymosan [a toll-like receptor 2 (TLR2) ligand] or polyinosinic:polycytidylic acid (poly I:C) [a toll-like receptor 3 (TLR3)

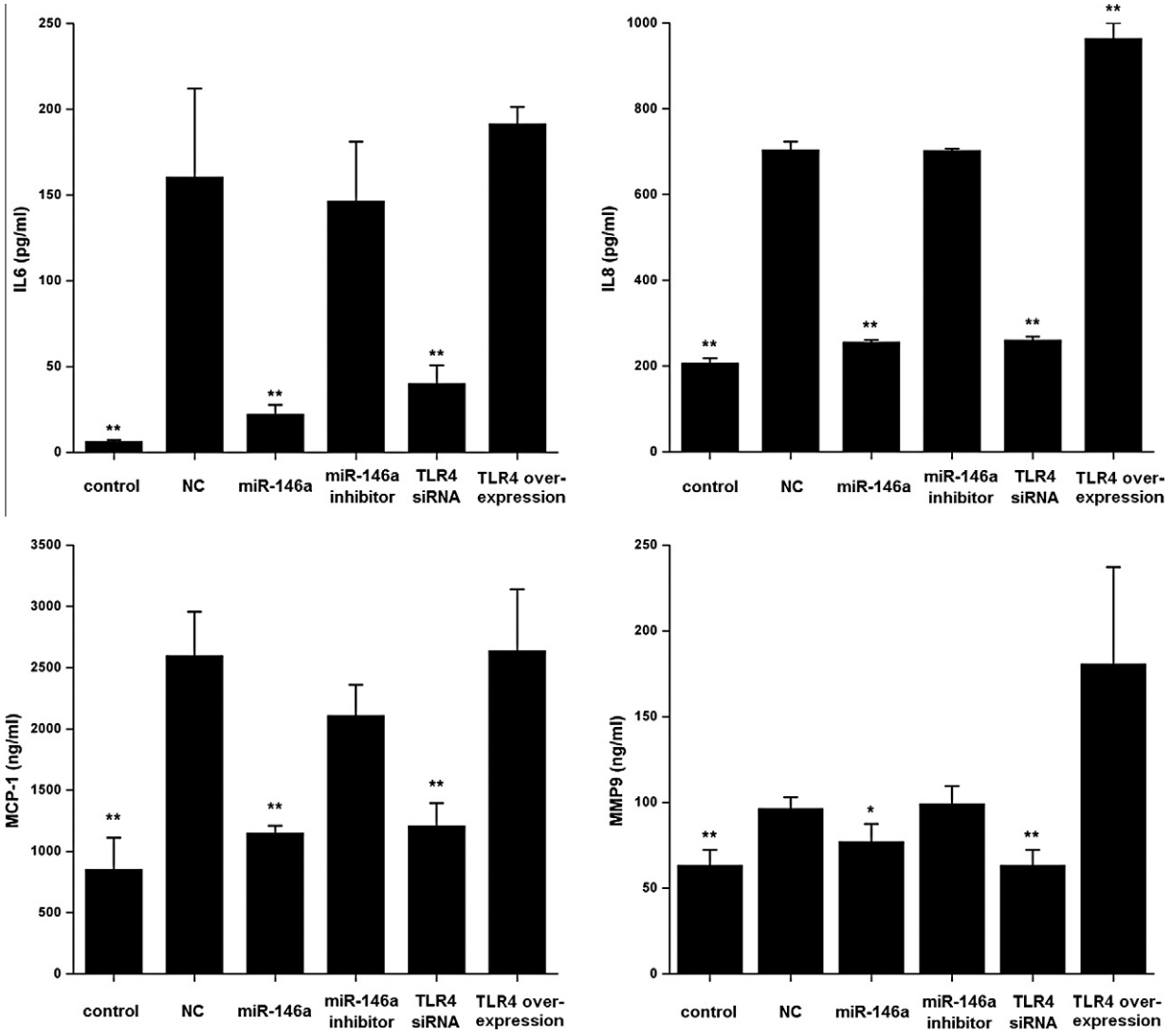

Fig. 4. Secretion of inflammatory cytokines in oxLDL-stimulated macrophages. Levels of IL-6, IL-8, MCP-1 and MMP-9 were significantly reduced by miR-146a ($n = 3$; $^{*}P < 0.05$, $^{**}P < 0.01$ vs. NC). NC: As in Fig. 2.

ligand] [16]. In contrast, in TLR4-deficient mice, lipid accumulation in circulating monocytes was significantly attenuated [21]. In this study, TLR4 was significantly up-regulated in oxLDL-stimulated microphages. Compared with over-expression of TLR4, knock-down of TLR4 resulted in a significant reduction in intracellular LDL cholesterol content and inflammatory cytokine secretion. Together with previous reports that TLR4 produces a vicious circle in oxLDL-induced pathological process in macrophages [22,23], our observations further substantiate the concept that TLR4 is an important mediator of foam cell formation.

In this study, with sustained oxLDL stimulation, TLR4 was up-regulated with a decrease in expression of miR-146a. In contrast, TLR4 was down-regulated by over-expression of miR-146a. Bioinformatics analysis demonstrated a possible interaction between miR-146a and TLR4, and measurement of luciferase activity further proved that miR-146a directly targeted TLR4. The results of rescue assay showed that intracellular LDL cholesterol content was decreased by co-transfection of miR-146a with $TLR4^{+3'UTR}$, and miR-146a significantly reduced IL-6, IL-8, MCP-1, and MMP-9 levels in oxLDL-stimulated macrophages. These findings suggest that the effects of miR-146a on lipid accumulation and inflammatory response may be attributed to its direct interaction with TLR4 in macrophages. In this study, TLR4 was only slightly decreased with transfection of miR-146a inhibitors in oxLDL-stimulated macrophages. This may be partly due to a less obvious effect at the presence of dominantly low level of miR-146a expression after oxLDL stimulation. In non-treated macrophages, the level of miR-146a was significantly reduced by miR-146a inhibitor, which attenuated TLR4 silencing.

The potential mechanism by which miR-146a inhibits lipid accumulation and inflammatory response via TLR4 remains unclear. Previous studies have shown that cytoskeleton rearrangement was induced by activation of TLR4-dependent spleen tyrosine kinase (Syk) signaling pathway, leading to enhanced lipid uptake [4]. Macrophage motility was increased by LPS (a TLR4 ligand) through Src and FAK [24], and macropinocytosis triggered by oxLDL was dependent largely on Src family kinases and JNK [25]. Fitzer-Attas et al. found that formation of actin cups and cytoskeleton rearrangement were delayed in $hck^{-/-}$ $fgr^{-/-}$ $lyn^{-/-}$ macrophages [26]. Hazeki et al. observed that LPS induced tyrosine phosphorylation of Pyk2 and its substrate paxillin (an integrin-associated focal adhesion adaptor protein), whereas PP2 (an inhibitor of Src family kinases) prevented TLR-induced phosphorylation of Pyk2 and paxillin [27]. In this study, oxLDL-induced phosphorylation of Src family kinases (Yes, Fyn, Fgr, Lck, Hck, and Lyn), FAK and JNK was significantly inhibited by miR-146a,

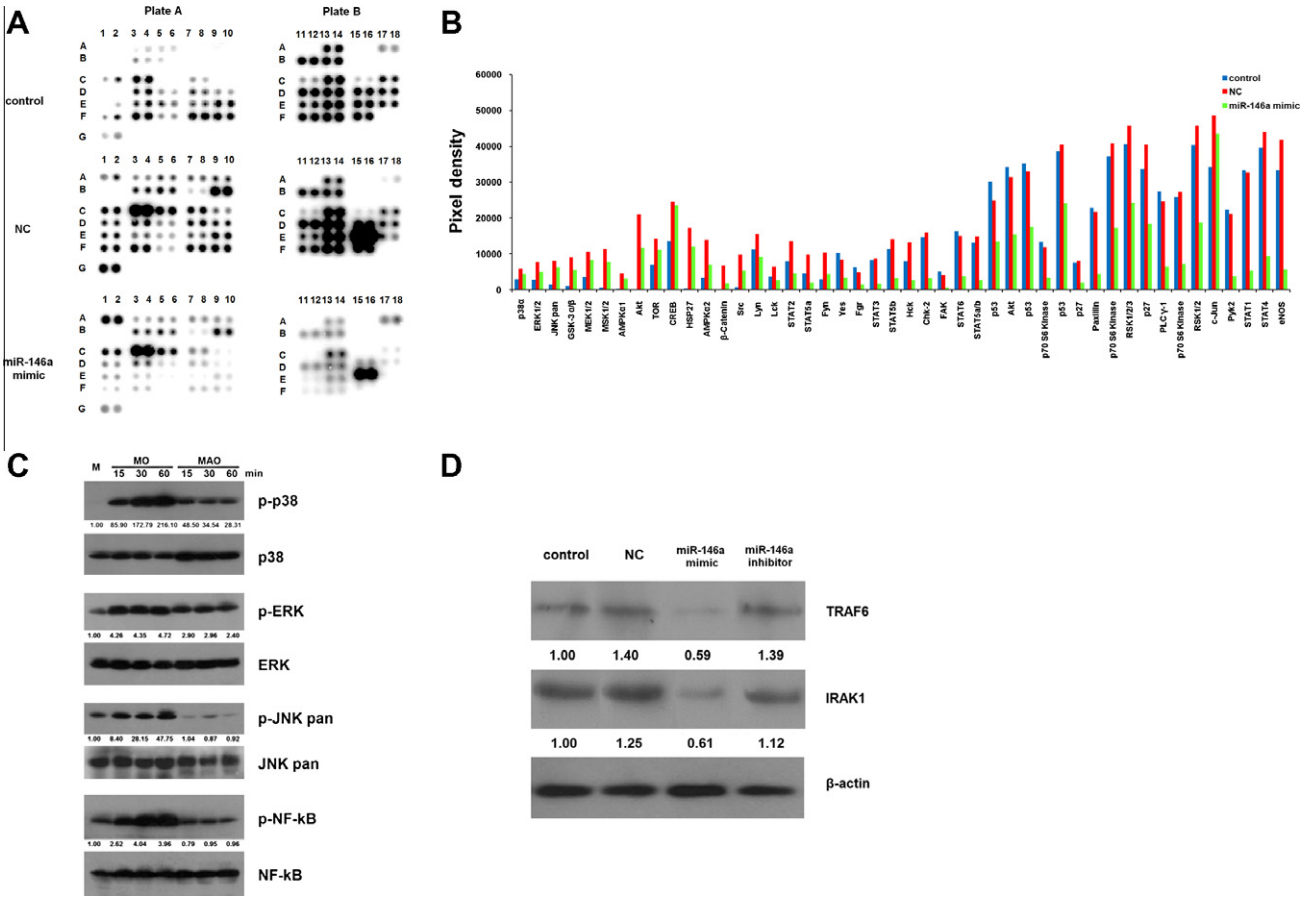

Fig. 5. Regulation of TLR4 signal pathway by miR-146a. (A) Phospho-kinase array for oxLDL-stimulated macrophages transfected with negative control miRNAs (NC) or miR-146a mimics. Non-oxLDL-stimulated macrophages were used as control (Plates A and B were incubated with 300 μg of cell lysate). (B) Protein profiling in the array was presented as pixel density. (C) Cell lysates were probed for phosphorylated p38, ERK, JNK pan and NF-κB, and the results were expressed in relative units (phosphorylated protein/total protein, $n = 3$). M: Non-oxLDL-stimulated macrophages; MO: oxLDL-stimulated macrophages; MAO: oxLDL-stimulated macrophages transfected with miR-146a. (D) TRAF6 and IRAK1 protein levels were analyzed with Western blot and normalized by β-actin (TRAF6 or IRAK1/β-actin; $n = 3$). Non-oxLDL-stimulated macrophages were used as control.

associated with a reduction in intracellular LDL cholesterol content in macrophages. These results highlight that miR-146a may inhibit cytoskeleton rearrangement and lipid uptake by blockade of TLR4–Src–FAK–JNK axis and inhibition of phosphorylation of Pyk2 and paxillin.

TLR4 plays a crucial role in inflammatory response in macrophages [10,28]. In this study, the secretion of inflammatory cytokines (IL-6, IL-8, MCP-1, and MMP-9) by oxLDL stimulation was dramatically inhibited with knock-down of TLR4 and over-expression of miR-146a, suggesting that miR-146a may regulate inflammatory response through interacting with TLR4. Lee et al. have shown that saturated and polyunsaturated fatty acids reciprocally modulate the activation of TLR4 and its downstream signaling pathways involving myeloid differentiation primary response gene (88) (MyD88)/IRAK/TRAF6 and PI3K/v-akt murine thymoma viral oncogene homolog 1 (AKT) [29]. Taganov et al. found that miR-146a interacts directly with IRAK1/TRAF6 in the TLR signaling pathway, which also suppresses the secretion of inflammatory cytokines [9]. In the present study, expression of TRAF6 and IRAK1 as well as TLR4 was down-regulated and phosphorylation of Akt was inhibited by miR-146a. Likewise, oxLDL-induced NF-κB activation was offset by over-expression of miR-146a. These results support a notion that miR-146a attenuates inflammatory response in macrophages by simultaneously blocking TLR4–TRAF6–IRAK1 and TLR4–Akt pathways [30,31]. Moreover, in agreement with previous reports that oxLDL could selectively activate p38 MAPK [32], we also observed that oxLDL stimulation resulted in a stronger activation of p38 MAPK relative to JNK and ERK1/2, whereas miR-146a dynamically inhibited the activation of p38 MAPK, JNK and ERK1/2, which have been involved in the inflammatory process following TLR4 activation [33]. Taken together, despite multiple mechanisms, TLR4 remains an important upstream target of miR-146a.

In conclusion, this study indicates that miR-146a synchronously inhibits oxLDL-induced foam cell formation and inflammatory response in macrophages by directly targeting TLR4 and inhibiting activation of TLR4-dependent signaling pathways. The beneficial clinical effects of over-expression of miR-146a in the prevention and treatment of atherosclerosis deserve further investigations.

Acknowledgement

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.02.009.

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