

# ALK7 Acts as a Positive Regulator of Macrophage Activation through Down-Regulation of PPAR $\gamma$ Expression

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**Aim:** Activin receptor-like kinase 7 (ALK7) acts as a key receptor for TGF- $\beta$  family members, which play important roles in regulating cardiovascular activity. However, ALK7's potential role, and underlying mechanism, in the macrophage activation involved in atherogenesis remain unexplored.

**Methods:** ALK7 expression in macrophages was tested by RT-PCR, western blot, and immunofluorescence co-staining. The loss-of-function strategy using AdshALK7 was performed for functional study. Oil Red O staining was used to observe the foam cell formation, while inflammatory mediators and genes related to cholesterol efflux and influx were determined by RT-PCR and western blot. A PPAR $\gamma$  inhibitor (G3335) was used to reveal whether PPAR $\gamma$  was required for ALK7 to affect macrophage activation.

**Results:** The results exhibited upregulated ALK7 expression in oxidized low-density lipoprotein (Ox-LDL) induced bone marrow derived macrophages (BMDMs) and mouse peritoneal macrophages (MPMs), isolated from ApoE-deficient mice, while ALK7's strong immunoreactivity in BMDMs was observed. ALK7 knockdown significantly attenuated pro-inflammatory, but promoted anti-inflammatory, macrophage markers expression. Additionally, ALK7 silencing decreased foam cell formation, accompanied by the up-regulation of ABCA1 and ABCG1 involved in cholesterol efflux but the down-regulation of CD36 and SR-A implicated in cholesterol influx. Mechanistically, ALK7 knockdown upregulated PPAR $\gamma$  expression, which was required for the ameliorated effect of ALK7 silencing macrophage activation.

**Conclusions:** Our study demonstrated that ALK7 was a positive regulator for macrophage activation, partially through down-regulation of PPAR $\gamma$  expression, which suggested that neutralizing ALK7 might be promising therapeutic strategy for treating atherosclerosis.

**Key words:** ALK7, Macrophage, PPAR $\gamma$ , Atherosclerosis

## Introduction

Cardiovascular diseases, associated with atherosclerosis, are recognized universally as the leading cause of mortality and morbidity worldwide<sup>1</sup>. Athero-

sclerosis is widely accepted as a complex, multi-factorial disease, and chronic inflammatory disease, which can ultimately lead to major adverse cardiovascular events, including arteriosclerotic occlusive diseases, arteriovenous thrombosis, aneurysms, myocardial

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infarction, stroke, and sudden cardiac death<sup>2</sup>). The endothelial dysfunction initiates the atherosclerosis process, characterized by secretion of chemokines and adhesion molecules to fasten the circulating monocytes to the endothelium<sup>3</sup>. Monocytes, and the subsequently differentiated macrophages, play determinate roles in multiple aspects of atherogenesis<sup>4</sup>. Macrophages are responsible for the inflammatory response in atherogenesis by regulating the balance between activation of classically pro-inflammatory M1 macrophages (analogous to the T-cell nomenclature T-helper cell 1 [Th1]), which is universally induced by a combination of interferon- $\gamma$  (IFN- $\gamma$ ) with lipopolysaccharide, and, alternatively, anti-inflammatory M2 macrophages (analogous to Th2 T cells), which can be induced by IL-4 or IL-13<sup>5-8</sup>). Notably, the macrophages' uptake of modified low-density lipoprotein (LDL) and subsequent transformation into foam cells continually contribute to development of atherosclerotic lesions and vulnerable plaques<sup>9</sup>. Therefore, it is urgent to explore the key regulator of macrophage inflammation and foam cell transformation implicated in atherosclerosis.

The activation of transforming growth factor type- $\beta$  (TGF- $\beta$ ) axis, from ligands, receptors, and binding proteins to ligand traps, plays an important role in regulating cardiovascular diseases<sup>10,11</sup>). As a key type I receptor for TGF- $\beta$  family members, activin receptor-like kinase 7 (ALK7) has also emerged as the determinate receptor for nodal and activin, both of which are implicated in various diseases<sup>12</sup>). In past decades, ALK7 signaling acted as a novel suppressor of tumorigenesis and metastasis, characterized by the formation of a homeostatic tissue barrier, which is identified as an effective approach for attenuating the attack from cancer cells and inhibiting the tumor's capability for metastatic seeding<sup>13, 14</sup>). Besides the effect on tumor development, ALK7 is specifically expressed during the late phase of adipocyte differentiation<sup>15</sup>). The adipocytes' specific ALK7 dysfunction causes increased lipolysis, which leads to decreased fat accumulation<sup>16</sup>), while ALK7 is also associated with multiple factors implicated in metabolic disease<sup>17</sup>). ALK7 decreases inflammatory adipocytokines secretion and improves glucose tolerance and insulin sensitivity<sup>18</sup>). It also protects against the development of pathological cardiac hypertrophy<sup>19</sup>). Notably, ALK7 activation initiates multiple downstream signaling pathways, including Smad family members, PI3K-AKT, Ras-Erk, and mitogen-activated protein kinases (MAPK)<sup>19-21</sup>), which are involved in atherogenesis. However, ALK7's specific effect on the development of macrophage activation implicated in atherogenesis is unexplored.

In the present study, upregulated ALK7 expres-

sion was induced in macrophages upon oxidized low-density lipoprotein (Ox-LDL) treatment. The loss-of-function study demonstrated that ALK7 knockdown significantly alleviated pro-inflammatory, but promoted anti-inflammatory, macrophage polarization, accompanied by decreased foam cell formation. It enhanced ABCA1 and ABCG1 related to cholesterol efflux but reduced CD36 and SR-A involved in cholesterol influx. Mechanistically, we verified that PPAR $\gamma$  was required for the attenuated effect of macrophage activation, regulated by ALK7 knockdown.

## Materials and Methods

### Cell Culture and siRNA Transfection

Bone marrow derived macrophages (BMDMs) were isolated and harvested from the femurs and tibiae of ApoE-deficient mice under sterile conditions. The nucleated bone marrow cells were collected from each mouse and then cultured in 10 ml of RPMI with 10% fetal bovine serum and MCSF (50 ng/ml). Mouse peritoneal macrophages (MPMs) were collected from ApoE deficiency mice via peritoneal lavage four days after the intraperitoneal injection of 4% thioglycolate (1 ml). Cells were cultured in RPMI containing 10% fetal bovine serum and 1% penicillin-streptomycin. The collected macrophages were seeded at  $2 \times 10^6/35$ -mm well in six-well plates. To knockdown ALK7 expression, ALK7 specific short hairpin RNA (shRNA)-expressing (shALK7) constructs, using a pENTR/U6-shRNA vector, were used to generate AdshALK7 recombinant adenoviral vectors, and short hairpin RNA (AdshRNA) served as controls. The BMDMs were transfected with the above adenovirus according to the manufacturer's protocol. The cells were then stimulated with 15 ng/ml Ox-LDL, which was purchased from sigma (O0625-25g), for 24 hours after serum starvation for one day.

### Quantitative Real-Time PCR

Total RNA from macrophages was isolated with TRIzol reagent and was held constant at 20  $\mu$ l per tube in each experiment. Then the cDNA obtained via reverse-transcription of RNA was synthesized using a Transcriptor First Stand cDNA Synthesis Kit. Quantitative real-time PCR was performed using a Light-Cycler 480 Real-time PCR System (Roche) in accordance with the manufacturer's instructions. The relative expression levels of the target genes were normalized against GAPDH gene expression, and the primers are shown in **Table 1**.

### Western Blotting Analysis

Cellular proteins were lysed in RIPA assay buffer.

**Table 1.** The primers for real-time PCR

Primer	Sequence(5' to 3')
<i>ALK7-F</i> <i>ALK7-R</i>	GACATGAAAACATCCTTGGT ACTTCTGGTCACAAACAACC
<i>TNF-<math>\alpha</math>-F</i> <i>TNF-<math>\alpha</math>-R</i>	CAGCCTCTTCTCATTCCCTGCT GGGTCTGGGCCATAGAACTG
<i>iNOS-F</i> <i>iNOS-R</i>	CATTCAGATCCCGAAAACGCT TGTAGGACAATCCACAACCTCGC
<i>COX2-F</i> <i>COX2-R</i>	AGAGGTGTATCCCCCACAG TGTCGCACACTCTGTGTGTC
<i>IL-6-F</i> <i>IL-6-R</i>	TTGCCTTCTTGGGACTGATG TCATTTCCACGATTTCCAG
<i>Arg-1-F</i> <i>Arg-1-R</i>	AAAGGCCGATTACCTGAGC AGGTAGTCAGTCCCTGGCTT
<i>chi3I3-F</i> <i>chi3I3-R</i>	TGAAGGAGCCACTGAGGTCT TGAAGGAGCCACTGAGGTCT
<i>TGF-<math>\beta</math>-F</i> <i>TGF-<math>\beta</math>-R</i>	AGAGCCCTGGATACCAACTATTG TGCGACCCACGTAGTAGACG
<i>Mrc-1-F</i> <i>Mrc-1-R</i>	CGTTTCGGTGGACTGTGGA GTTGTGGGCTCTGGTGGG
<i>KLF4-F</i> <i>KLF4-R</i>	ACTAACCGTTGGCGTGAGGA TTACTGCTGCAAGCTGCACC
<i>SR-A-F</i> <i>SR-A-R</i>	TGGAGGAGAGAATCGAAAGCA CTGGACTGACGAAATCAAGGAA
<i>CD36-F</i> <i>CD36-R</i>	GACTGGGACCATTTGGTGATGA AAGGCCATCTCTACCATGCC
<i>ABCA1-F</i> <i>ABCA1-R</i>	AGGCACTCAAGCCACTGCTTGT TGCCTCTGCTGTCTAACAGCGT
<i>ABCG1-F</i> <i>ABCG1-R</i>	GGTTGCGACATTTGTGGGTC TTCTCGGTCCAAGCCGTAGA
<i>PPAR<math>\gamma</math>-F</i> <i>PPAR<math>\gamma</math>-R</i>	GCTTGTGAAGGATGCAAGGG GATATCACTGGAGATCTCCGCC
<i>PPAR<math>\delta</math>-F</i> <i>PPAR<math>\delta</math>-R</i>	CGAGTTCTTGCGAAGTCTCC CCGTCTTCTTTAGCCACTGC
<i>IRF4-F</i> <i>IRF4-R</i>	GGTGAGGAGTTTCCAGACCC GGTGAGGAGTTTCCAGACCC
<i>NR4A-F</i> <i>NR4A-R</i>	GCTGCAGAATGACTCCACC ACAGCAGCACTGGGCTTA
<i>Nrf2-F</i> <i>Nrf2-R</i>	TCTTGAGTAAGTCGAGAAGTGT GTTGAAACTGAGCGAAAAAGGC
<i>STAT6-F</i> <i>STAT6-R</i>	CCTGGTCCGGTTCAGATGCTTT GTGCGGCAAGATGCTGTTTC
<i>STAT3-F</i> <i>STAT3-R</i>	CAAGGGCTTCTCCTTCTGGG CCTGGGTCAGCTTCAGGATG
<i>GAPDH-F</i> <i>GAPDH-R</i>	TGAAGGGTGGAGCCAAAAG AGTCTTCTGGGTGGCAGTGAT

**Table 2.** Antibody for Immunoblot

Primary Antibody	Cat No	Manufacturer	Sources of species
ALK7	sc-374538	Santa Cruz Biotechnology	Mouse
iNOS	ab3523	Abcam	Rabbit
IL-6	AF-406-NA	R&D Systems	Goat
Arg-1	610708	BD Biosciences	Mouse
KLF4	ab151733	Abcam	Rabbit
CD36	sc-7309	Santa Cruz Biotechnology	Mouse
SR-A	sc-166139	Santa Cruz Biotechnology	Mouse
ABCA1	ab7360	Abcam	Rabbit
ABCG1	ab244442	Abcam	Rabbit
PPAR $\gamma$	16643-1-AP	Proteintech	Rabbit
GAPDH	2118	Cell Signaling Technology	Rabbit

**Table 3.** Antibody for Immunofluorescence

Primary Antibody	Cat No	Manufacturer	Sources of species
ALK7	sc-374538	Santa Cruz Biotechnology	Mouse
PPAR $\gamma$	16643-1-AP	Proteintech	Rabbit
CD68	Ab125212	Abcam	Rabbit

Protein concentrations were then quantified with a BCA protein assay kit. The 40 micrograms of protein were separated by via SDS-PAGE on a 5% or 10% acrylamide gel and transferred to a PVDF membrane (Millipore), which was subsequently blocked with milk and incubated with the appropriate primary antibody overnight at 4°C. After incubation with the appropriate secondary antibody for one hour at room temperature, relative expression was visualized using a Fluor Chem E imager (Protein Simple, Fluor Chem E). The specific protein expression was normalized against GAPDH expression, and the antibodies are shown in [Table 2](#).

### Immunofluorescence Staining

We performed immunofluorescent staining of BMDMs as described<sup>19</sup>. After incubating with primary antibodies at 4°C overnight, the slices went through rewarming at 37°C for half an hour and were washed with PBS before incubation with appropriate fluorescence-labeled secondary antibody for one hour. Cell nuclei were stained with DAPI, and images were captured using a confocal laser scanning microscope. The antibodies are shown in [Table 3](#).

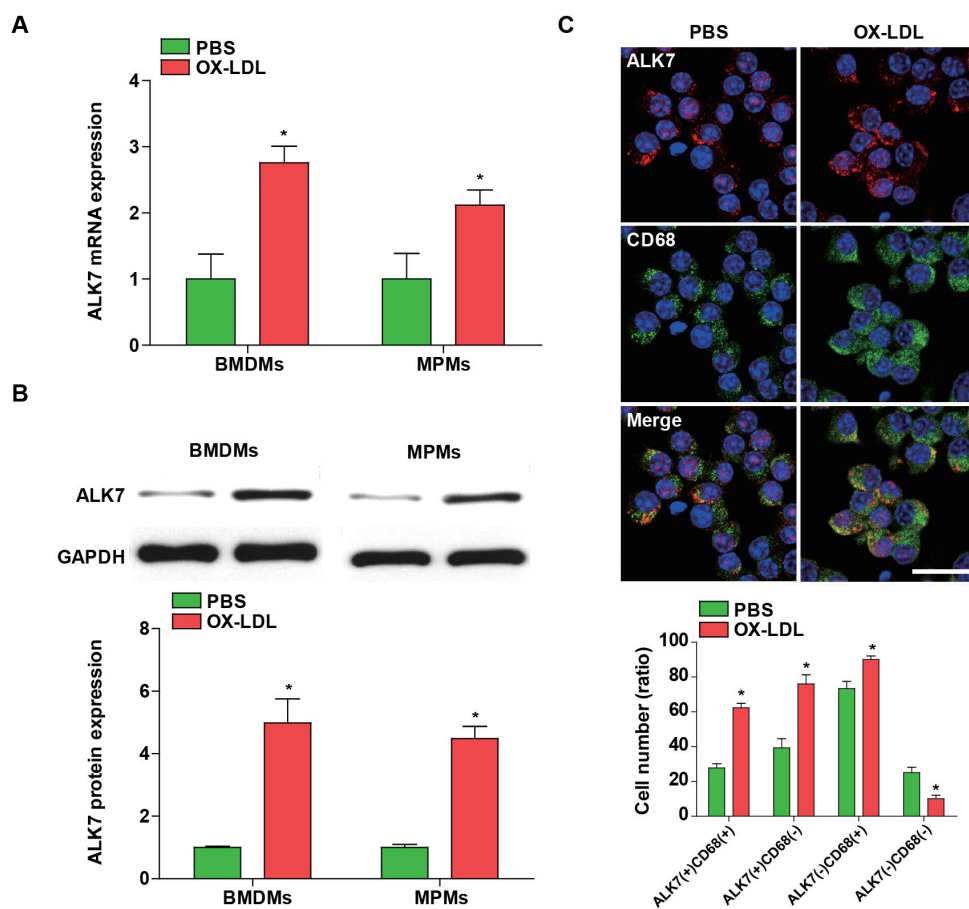
### Statistical Analysis

All data were represented as the means  $\pm$  SD. Comparisons between groups were evaluated using a two-tailed Student t test or one-way ANOVAs. All statistical analyses were completed using SPSS, version 22.0. A value of  $P < 0.05$  were considered statistically significant.

## Result

### The Up-Regulation of ALK7 in Activated Macrophages

To determine ALK7's potential role in macrophage activation in atherogenesis, isolated BMDMs and MPMs were treated with Ox-LDL. Notably, ALK7 mRNA was markedly upregulated upon Ox-LDL stimulation, as determined by RT-PCR ([Fig. 1A](#)), whereas minimal baseline ALK7 expression was observed with PBS treatment. The differential ALK7 mRNA expression was further confirmed at the protein level by western blot analysis ([Fig. 1B](#)). Moreover, double immunofluorescence staining revealed ALK7's strong immunoreactivity in activated BMDMs, as evaluated by the increased ratio of ALK7(+)/CD68(+) to ALK7(+)/CD68(-) with Ox-LDL



**Fig. 1.** Increased ALK7 expression in BMDMs and MPMs with Ox-LDL treatment

A. RT-PCR analysis of ALK7 mRNA in BMDMs and MPMs after 24h of stimulation with Ox-LDL. B. Western blot analysis of ALK7 protein levels in BMDMs and MPMs, administrated with Ox-LDL. C. Double immunofluorescence staining of BMDMs, with an anti-ALK7 antibody (red) and a CD68 antibody as a macrophage marker (green), treated with PBS or Ox-LDL. The bottom panel showed the statistical analysis of the ratio of the indicated group in the cell slices.  $n=3$ . Scale bar = 25  $\mu\text{m}$ . \* $P < 0.05$  compared with PBS treated group.

treatment compared to that of PBS administration (**Fig. 1C**). Collectively, these findings indicate that increased ALK7 expression was positively correlated with macrophage activation.

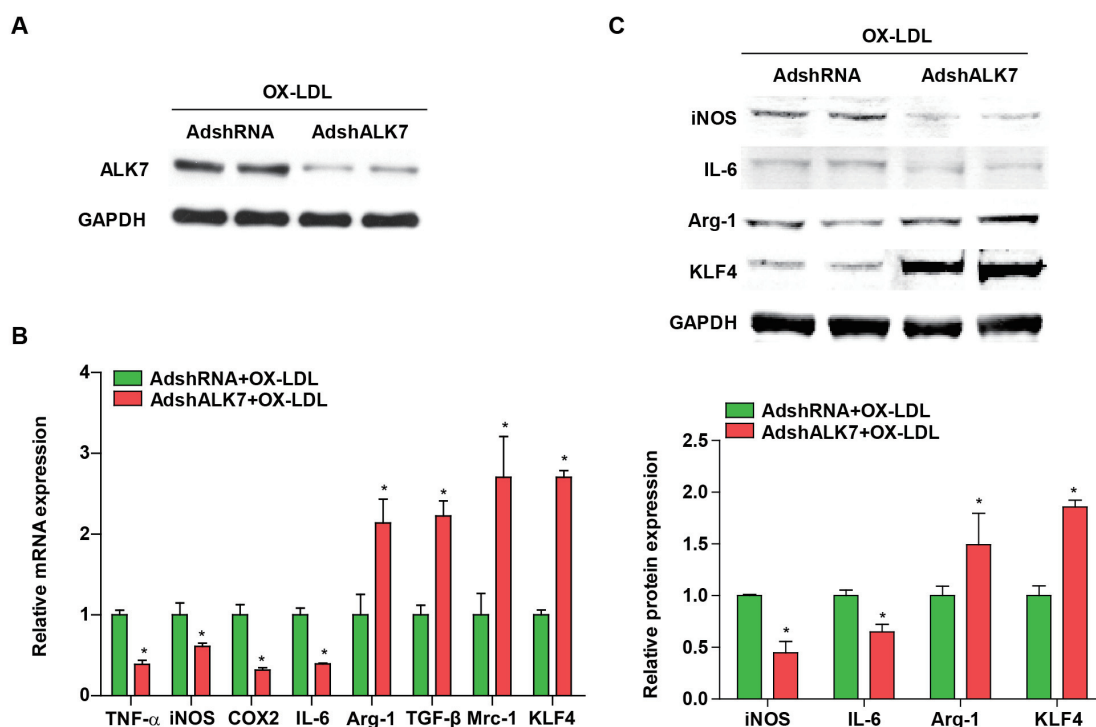
### ALK7 Knockdown Attenuates Pro-Inflammatory Macrophage Polarization

Macrophages are responsible for the inflammatory response in atherogenesis, exhibit remarkable plasticity, and switch between classical activation (M1) and alternative activation (M2) phenotypes<sup>7, 8</sup>. To explore upregulated ALK7's contribution to macrophage activation, BMDMs were transfected with adenovirus harboring ALK7 short hairpin RNA (AdshALK7). The results showed that ALK7 expression decreased significantly in BMDMs after transfection with AdshALK7 (**Fig. 2A**). After administration of Ox-LDL, RT-PCR analysis revealed that the

mRNA expression levels of marker genes related to the pro-inflammatory M1 macrophage phenotype (including TNF- $\alpha$ , iNOS, Cox-2, and IL-6) had decreased dramatically, and the mRNA expression levels of genes related to the anti-inflammatory M2 macrophage phenotype (including Arg-1, TGF- $\beta$ , Mrc-1, and KLF4) had increased because of the ALK7 knockdown (**Fig. 2B**). Western blot analysis confirmed the changes in iNOS and IL-6 expression, as well as Arg-1 and KLF4 expression (**Fig. 2C**). Collectively, these data confirmed that ALK7 knockdown promoted the alternative activation of macrophages.

### ALK7 Silencing Decreases Foam Cell Formation

Accumulation of cholesterol in macrophages, and subsequent foam cell formation, is a critical initial step in macrophage activation and is implicated in atherogenesis<sup>22</sup>. Neutral lipid staining with oil red O



**Fig. 2.** ALK7 is essential for pro-inflammatory macrophage activation

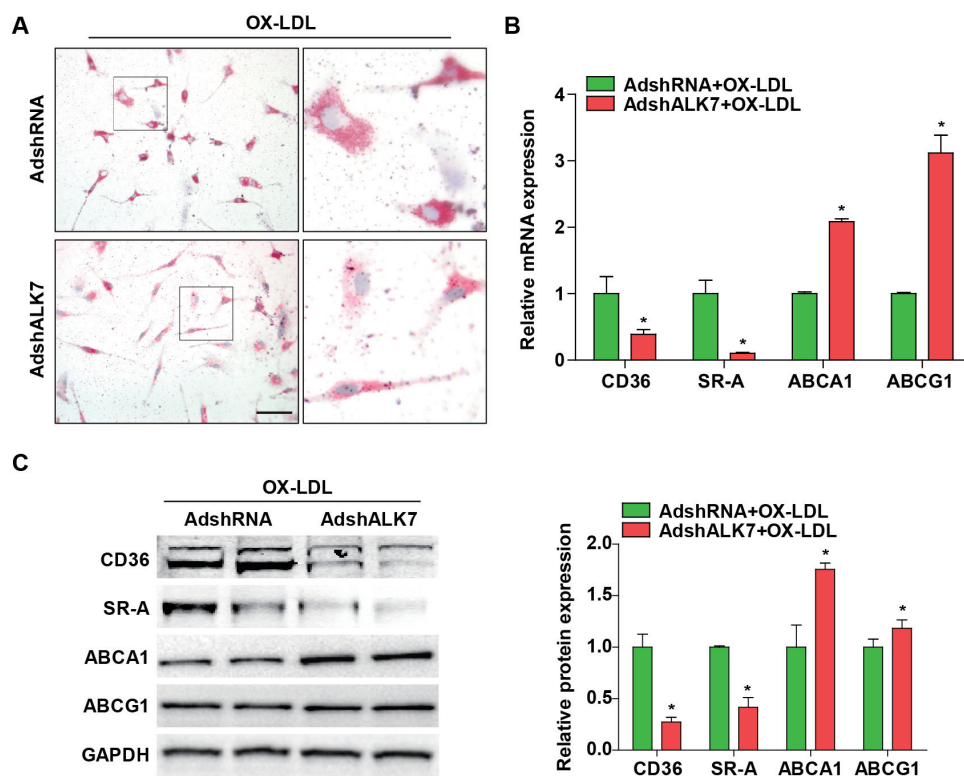
A. Western blot analysis of ALK7 protein expression in BMDMs transfected with AdshALK7. B. Impairment of M1 markers gene expression and enhanced M2 markers gene expression in BMDMs by ALK7 knockdown. C. Western blot analysis of iNOS and IL-6, as well as Arg-1 and KLF4 protein level in BMDMs by ALK7 knockdown. \* $P < 0.05$  compared with control group.

showed significantly decreased foam cell formation in BMDMs transfected with AdshALK7 (Fig. 3A). Taking into account that variations in cholesterol uptake and efflux can contribute to cholesterol accumulation and foam cell formation, the related markers for each process were tested<sup>23</sup>. We observed that the mRNA levels of genes involved in cholesterol influx (CD36 and SR-A) decreased, whereas the mRNA levels of genes implicated in cholesterol efflux (ABCA1 and ABCG1) increased in BMDMs transfected with AdshALK7 (Fig. 3B). Additionally, the above results were further verified by Western blotting (Fig. 3C).

### PPAR $\gamma$ is Required for the Effect of ALK7 Knockdown on Macrophage Inactivation

Next, ALK7's evident macrophage activation prompted us to investigate the underlying mechanism. Previous studies have suggested that multiple targets are implicated in regulating macrophage polarization and atherogenesis, including PPAR $\gamma$ , PPAR $\delta$ , KLF4, IRF4, NR4A, Nr1h2, STAT6 and STAT3<sup>24, 25</sup>. Among the genes tested in our study, PPAR $\gamma$  showed the greatest increase in mRNA in response to ALK7 silencing under stimulation with Ox-LDL (Fig. 4A). Western blot analysis further confirmed the up-regula-

tion of PPAR $\gamma$  expression in BMDMs transfected with AdshALK7 (Fig. 4B). Using confocal microscopy, we further noticed dramatically increased PPAR $\gamma$  expression in the nuclei of macrophages with ALK7 knockdown, which was indicated by the increase in PPAR $\gamma$  positive cells [PPAR $\gamma$ (+)CD68(+) and PPAR $\gamma$ (+)CD68(-)] compared with those of the non-silenced groups (Fig. 4C). Additionally, a previous study indicated that PPAR $\gamma$  is responsible for the ALK7 silencing-mediated decrease in inflammatory adipocytokine secretion, which improved glucose tolerance and insulin sensitivity<sup>16, 18</sup>. Subsequently, we examined whether PPAR $\gamma$  also mediated ALK7's effect on macrophage activation *in vitro*. PPAR $\gamma$  expression was dramatically down-regulated in macrophages upon administration of a PPAR $\gamma$ -specific antagonist (G3335) (Fig. 4D). Functionally, PPAR $\gamma$  inactivation blunted ALK7 knockdown-mediated down-regulation of pro-inflammatory M1 phenotype cytokine expression (TNF- $\alpha$  and iNOs) and upregulation of anti-inflammatory M2 phenotype cytokine expression (chi313 and TGF- $\beta$ ) (Fig. 4E). Moreover, the decrease in expression of genes involved in cholesterol influx (CD36 and SR-A) and increase in expression of genes implicated in cholesterol efflux (ABCA1



**Fig. 3.** Suppression of foam cell formation by ALK7 silencing

A. Oil red staining of BMDMs after ALK7 silencing under stimulation with Ox-LDL. Scale bar = 100  $\mu$ m. B-C. ALK7 silencing alters the expression of the genes involved in cholesterol influx (CD36 and SR-A) and cholesterol efflux (ABCA1 and ABCG1) in BMDMs under stimulation with Ox-LDL at mRNA (B) and protein (C) levels, which were examined by RT-PCR and Western blot, respectively. \* $P < 0.05$  compared with control group.

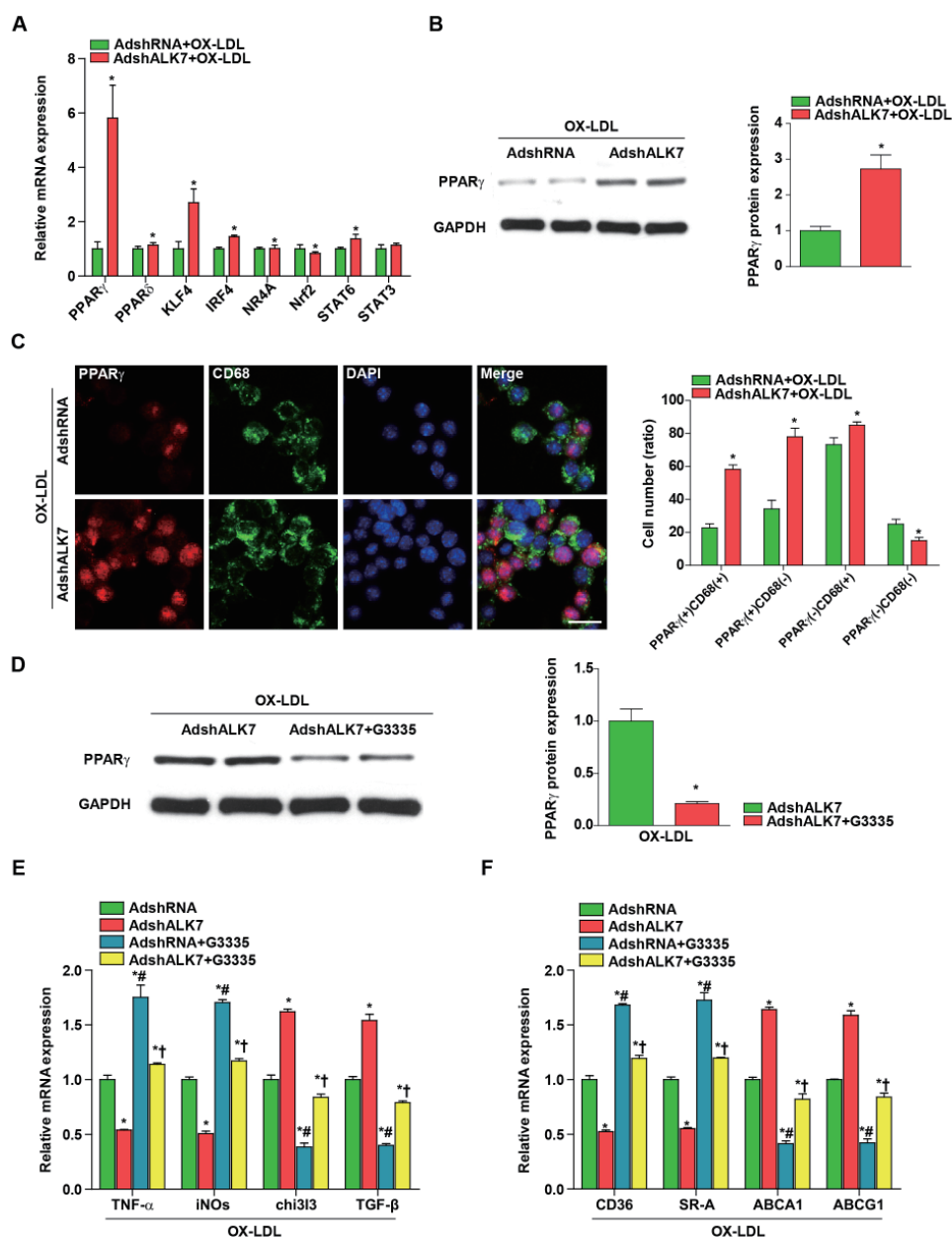
and ABCG1) were largely reversed by PPAR $\gamma$  inactivation in the presence of Ox-LDL (Fig. 4F). Based on these findings, we concluded that PPAR $\gamma$  inactivation was required for ALK7-mediated macrophage activation.

## Discussion

Macrophage activation is responsible for the development of atherosclerosis, which is the determinate inflammatory process underlying severe cardiovascular disease<sup>6</sup>. Our current study first demonstrated that ALK7 was a novel independent indicator of atherogenesis, as exhibited by upregulated macrophages' expression upon Ox-LDL administration. By performing loss-of-function analysis of ALK7 *in vitro*, we observed that ALK7 knockdown promoted M2 phenotype macrophages but attenuated M1 macrophage switching. Furthermore, ALK7 knockdown significantly ameliorated foam cell formation by up-regulating ABCA1 and ABCG1, which are involved in cholesterol efflux, and down-regulating CD36 and SR-A, which are implicated in cholesterol influx.

Mechanistically, silencing ALK7 dramatically promoted PPAR $\gamma$  expression, and the ALK7 knockdown-mediated inhibition of macrophage activation was largely reversed by treatment with G3335, a PPAR $\gamma$  inhibitor. Based on the present study's results, we propose ALK7 has an unexplored role in regulating macrophage activation during atherosclerosis development, at least partially through regulating PPAR $\gamma$  expression.

In recent decades, tremendous efforts have been made to elucidate the underlying mechanisms of atherosclerosis that effectively suppress development of atherogenesis-related cardiovascular diseases. Recently, TGF- $\beta$  family members have been reported to be upregulated in mouse and human atherosclerotic lesions<sup>26</sup>. More importantly, many lines of evidence have demonstrated that TGF- $\beta$  and activin A play pivotal and protective roles against the development of atherosclerotic lesions by modulating key cellular signals<sup>27-29</sup>. Notably, ALK7 is well recognized as an important receptor of the TGF- $\beta$  family members, nodal, and activin<sup>11</sup>. Consistent with the above findings, ALK7 may act as a novel target for atherosclerosis manage-



**Fig. 4.** PPAR $\gamma$  accounts for the effect of ALK7 silencing on macrophage activation

A. RT-PCR analysis of the representative macrophage polarization markers in BMDMs after ALK7 silencing under stimulation with Ox-LDL. B. Western blot analysis of PPAR $\gamma$  in BMDMs after ALK7 silencing under stimulation with Ox-LDL. C. Representative images, showing double immunofluorescence staining of BMDMs, for PPAR $\gamma$  (red) and macrophages (CD68, green). The right panel showed the statistical analysis of the ratio of the indicated group in the cell slices.  $n=3$ . Scale bar = 25  $\mu$ m. D. PPAR $\gamma$  protein levels in BMDMs treated with a PPAR $\gamma$  antagonist (G3335). E. RT-PCR analysis of M1 and M2 marker genes expression in macrophage transfected with AdshRNA or AdshALK7 which was pretreated with G3335 or control. F. RT-PCR analysis of CD36 and SR-A, as well as ABCA1 and ABCG1 in macrophage transfected with AdshRNA or AdshALK7 which was pretreated with G3335 or control. \* $P < 0.05$  compared with AdshRNA group; # $P < 0.05$  vs. AdshALK7 group; † $P < 0.05$  vs. AdshRNA with G3335 group.

ment. In agreement with our expectation, we found that ALK7 expression increased, primarily in macrophages upon Ox-LDL administration using co-immunofluorescence staining of macrophage-specific mark-

ers. Previous studies indicated that activin A affected macrophage polarization through binding to the type 1 receptors ALK4 or ALK7, while M2-related macrophages can be induced by IL-4/13, IL-10, and TGF-



$\beta$ , which promote inflammation resolution under physiological conditions<sup>30</sup>). As expected, by knocking down ALK7 in macrophages, we observed promotion of M2 polarization of macrophages but attenuation of M1 macrophage switching. Additionally, ALK7 silencing also affected macrophage-derived foam cell formation through increased expression of cholesterol efflux-associated ABCA1 and ABCG1 and decreased expression of cholesterol uptake-associated CD36 and SR-A.

There has been an impressive increase in the appreciation of inflammation's importance in atherogenesis in recent decades. The most attention has been focused on the role of recruited macrophages in regulating the inflammatory response and foam cell formation within atherosclerotic lesions<sup>9</sup>). Within this heterogeneous group of macrophages, there are classically activated, pro-inflammatory, M1 macrophages, and reparative M2 macrophages, which are derived by different pathways and have different targets<sup>6, 25</sup>). Our study results demonstrate that PPAR $\gamma$  was the most significantly upregulated gene in macrophages, and this observation was verified by western blotting and co-immunofluorescence staining. Recent work has identified the nuclear hormone receptor PPAR $\gamma$  as a critical signaling molecule in determining macrophage phenotype *in vitro* and in atherosclerotic plaques<sup>31</sup>). Specifically, PPAR $\gamma$  activation polarizes monocytes to become M2 macrophages, and M2 macrophages induced by PPAR $\gamma$  inhibit the secretion of pro-inflammatory mediators in M1 macrophages<sup>32</sup>). Moreover, PPAR $\gamma$  is responsible for suppressing lipolysis that leads to fat accumulation in obesity<sup>16</sup>). PPAR $\gamma$  is also known to decrease the secretion of inflammatory adipocytokines, thereby improving glucose tolerance and insulin sensitivity through ALK7 inactivation<sup>18</sup>). Based on these observations, we hypothesized that PPAR $\gamma$  in macrophages is required for macrophage inactivation by ALK7 silencing. In the current study, we noticed that ALK7 knockdown significantly promoted M2 polarized macrophages but attenuated M1 polarized macrophages, while these effects were reversed by PPAR $\gamma$  inactivation.

Macrophages' uptake of modified LDL in the subendothelium, and the subsequent transformation into foam cells, has been suggested to play a critical role in atherosclerosis development, which is regulated by an imbalance in cholesterol efflux, influx, and synthesis<sup>9</sup>). PPAR $\gamma$  has emerged as an important regulator of foam cell formation via multiple signaling pathways<sup>23</sup>). PPAR $\gamma$  activation represses SR-A and inhibits triglyceride accumulation via down-regulated apoB-48 receptor expression in human macrophages<sup>33</sup>). PPAR $\gamma$  reduces ACAT1 mRNA levels to decrease the cholesterol esterification rate<sup>34</sup>). Moreover, the positive regu-

lation of cholesterol efflux in macrophages by PPAR $\gamma$  is highlighted by enhanced SR-B1, ABCA1, ABCG1, and apoE expression<sup>35</sup>). In our study, we demonstrated that PPAR $\gamma$  activation was responsible for the ALK7 knockdown's suppressive effect on foam cell formation through upregulation of ABCA1 and ABCG1 and down-regulation of CD36 and SR-A.

In conclusion, our findings demonstrated that silencing ALK7 expression in macrophages attenuated macrophage activation, as characterized by an increased ratio of M2 to M1 macrophages, as well as upregulation of the genes involved in cholesterol efflux and down-regulation of the genes involved in cholesterol influx. The protective effects of ALK7 knockdown against macrophage activation were partially mediated by up-regulating PPAR $\gamma$  expression. Our work highlights the important role of neutralizing ALK7 as a potential therapeutic approach for atherosclerosis management.

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## Conflict of Interest

None.

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