Original Article
Toll-like receptor 9 inactivation ameliorates ox-LDL injured HUVEC activity *in vitro*

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Abstract: Background: Inflammatory responses that are induced by oxidized low density lipoprotein (ox-LDL) play substantial functions in atherogenesis, and could be elevated in diabetic patients. Toll-like receptor 9 (TLR9) is an innate inflammatory receptor, and is enhanced in human umbilical vein endothelial cells (HUVECs) under high glucose conditions. Ox-LDL-TLR9 pathway activation and further inflammation mediated by monocytes are involved in the atherosclerosis formation. Objective: The objective was to determine whether TLR9 plays a role on ox-LDL-induced inflammation in HUVECs. The function and impact of the ox-LDL-TLR9 pathway on the inflammatory responses in HUVECs was examined. Methods: HUVECs were treated with ox-LDL and then subsequently treated with IRS869, a special antagonist of TLR9, and ODN1826, a type B CpG oligodeoxynucleotide (ODN) which can inhibit the expression of TLR9 and activate TLR9, respectively. HUVEC viability was analyzed by CCK8, changes of mitochondrial membrane potential (MTP), and LOX-1, IκBα, pIκBα, NF-κBp65, pNF-κBp65 expression. Results: Compared with the control group, ox-LDL significantly reduced the viability of HUVECs. TLR9 inactivation improved the viability of HUVECs and reduced the decrease of MTP injured by ox-LDL. TLR9 inactivation could inhibit LOX-1's expression and results in inactivation of NF-κB signaling pathway. Conclusion: TLR9 is involved in the inflammatory responses induced by ox-LDL in HUVECs. Inhibition of TLR9 could improve HUVECs survival activity and improve the status of growth damaged by ox-LDL, according to a preliminary protection.

Keywords: TLR9, IRS869, ODN1826, HUVECs, ox-LDL

Introduction

Atherosclerosis, a chronic inflammatory disease, is characterized by monocyte adherence to intrinsic endothelial cells and ox-LDL accumulation in the arterial wall [1]. Between the blood vessel wall and blood, blood endothelial cells maintain the integrity and function of the vascular structure. Elevated degrees of serum low-density lipoproteins (LDL) and endothelial disorder are regarded as a predilection for atherogenesis [2]. Induction of endothelial cell activation, dysfunction, and subsequent monocyte adhesion and endothelial leakage is a crucial step for atherosclerotic plaque constitution that is induced via ox-LDL [3]. Endothelial cells (ECs) are in fact activated by ox-LDL via inducing the expression of oxidized low density lipoprotein receptor 1 (lectin-like oxidized low density lipoprotein receptor 1, LOX-1) and release inordinate reactive oxygen species [4]. In addition, ox-LDL significantly triggers activation of

the nuclear factor kappa B predominate (nuclear factor kappa B, NF-κB) signaling pathway, and promotes its nuclear transformation, leading to oxidation of vascular endothelial cells and inflammatory lesions [5, 6]. Increased damage of vascular endothelial cells and secretion of a large number of inflammatory factors, promote monocyte accumulation and foam cell formation, formation of fatty streaks, and development of atheromatous plaques.

TLRs, pathogen pattern recognition receptors, are featured by the release and expression of cytokines and chemokines implicated in the progression and development of atherosclerosis [7]. TLR9 is an endosomal protein recognizing both autologous DNA and bacterial DNA. ODN1826 is a type B CpG ODN which can activate mouse TLR9 [8], while IRS869, a special antagonist of TLR9, can inhibit the expression of TLR9 and related cytokine. TLR9 detects unmethylated CpG DNAs or CpG ODN. Upon

ligation with its cognate ligands, the MyD88 adapter protein is recruited by TLR9. Nuclear factor κB (NF-κB) dependent pro-inflammatory cytokines pathway are then initiated [9]. To date, TLR9 continues to be the only known receptor for immunostimulatory DNA [8, 10]. TLR9 was foremost identified in antigen presenting cells [10] consistent with their role in immune surveillance. TLR9 is likewise expressed in non-immune cells, including HUVECs [11], dermal microvascular [12], lymphatic endothelial cells [13], and endothelial cells of human atherosclerotic plaques [14]. The balance of TFPI and TF is altered by CpG DNA signaling via TLR9 in HCAECs, as reported earlier. Most studies hitherto have focused on implications of TLR4 or TLR2 on atherosclerosis, while data on the role of TLR9 in atherosclerosis is scarce. TLRs have been viewed as major culprits in the development of atherosclerosis, contributing to both clinical complications and its progression.

However, direct evidence for an effect of blocking TLR9 on accelerated atherosclerosis has been lacking. In this research, we investigated the role of TLR9 in the inflammatory response and induction via ox-LDL in HUVECs.

Materials and methods

Cell culture, treatment, and transfection

HUVECs were cultivated in the endothelial growth medium (EGM) (Promocell, USA) comprising 2% fetal calf serum (FCS), growth factors (epidermal growth factor, vascular endothelial growth factor, basic fibroblast growth factor, and insulin-like growth factor). Cells were maintained in endothelial cell basal medium (EBM, Promocell) supplemented with 1% FCS without growth factors prior to and throughout experiments.

For the experiments, HUVECs were stimulated with or without ox-LDL (0.1 mg/mL). In additional experiments, IRS869 or ODN1826 was added to HUVECs 6 h prior to adding ox-LDL. The treatment time was 24 hours.

Real-time polymerase chain reaction (PCR)

Total RNA was separated from HUVECs adopting the Qiazol reagent (Qiagen, Germany). Intracellular RNA was reverse-transcribed with SuperScript reverse transcriptase kit (Applied

Biosystems, Germany). Real-time PCR was carried out utilizing Power SYBR Green PCR master mixture on a StepOne Plus real-time PCR system (Applied Biosystems, Germany). Target genes' expression were normalized to GAPDH. Target genes' relative expression were determined utilizing the $2^{-\Delta\Delta Ct}$ approach. Primer sequences are as follows: TLR9 forward (5'-TGGACGGGAACTGCTACT-3') and reverse (5'-GCCACATTCTATACAGGGATT-3'); LOX-1 forward (5'-GAGCTGCAAACTTTTCAGG-3') and reverse (5'-GTCTTTCATGCAGCAACAG-3'); GAPDH forward (5'-TGGAC GGGAACTGCTACT-3') and reverse (5'-GCCACATTCTATACAGGGATT-3').

Western blotting analysis

Total protein was separated from HUVECs utilizing RIPA buffer that was supplied with the protease inhibitor cocktail (Roche, Germany). Total protein concentration was gauged utilizing the BCA kit (Thermo Scientific, USA) in accordance with guidance of manufacturer. An equivalent quantity of total protein (20 μg) was subjected to 10% denaturing SDS gel and transferred to the polyvinylidene difluoride (PVDF) membrane (Amersham Biosciences, UK). After blocking with 5% non-fat dry milk, membranes were sequentially probed with primary antibody against proteins of interest that was followed by horseradish peroxidase (HRP)-linked secondary antibody. Blots were formulated utilizing chemiluminescence substrate (BioRad Laboratories, USA). Chemiluminescence signal intensity was determined utilizing Quantity One software (BioRad).

CCK8 analysis

Transduced HUVECs were plated at a density of 3,000 cells/well in 96-well plates. After placing for 16 hours, cells were serum starved or treated with ox-LDL (0.1 mg/mL) for an extra 24 hours. Throughout the last 4 hours of growth, WST-1 (Roche) was added to the media to gauge cell activity following the manufacturer's protocol. No less than four points were averaged for each condition, and the experiment was repeated three times with a representative experiment chosen.

Mitochondrial membrane potential (MTP) changing analysis

HUVECs were plated at a density of 3,000 cells/well in 96-well plates, and at the same
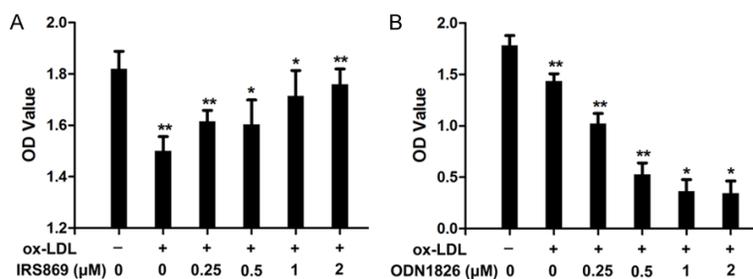
Figure 1. The proliferative impact of ODN1826 and IRS869 on ox-LDL-mediated Human umbilical vein endothelial cells (HUVECs). A. IRS869's proliferative effects on ox-LDL-mediated HUVECs after 24 hour treatment with diverse concentrations (0-2 μM) of dynamic substances. Cell viability was determined via the CCK8 assay. B. ODN1826's anti-proliferative effects on ox-LDL-mediated HUVECs after 24 hour treatment with various concentrations (0-2 μM) of active substances. The cell viability was assessed by the CCK8 assay. (*, P<0.05; **, P<0.01)

time, cells in each group were treated with the mitochondrial membrane potential detection reagent JC-1 (100 μl medium were added 10 μl JC-1 reagent), incubated at 37°C for 15 minutes in the CO_2 incubator. In accordance with recommendations from the manufacturer's protocol, the wavelength of the FITC channel was utilized for dynamic observation. JC-1 dye was collected within the mitochondria followed by dependent potential. In normal mitochondria, JC-1 in the form of polymer cumulated in the mitochondrial matrix, demonstrated a strong red fluorescence (Ex = 585 nm, Em = 590 nm). As reported by color changes directly that reflect the change of mitochondrial membrane potential, mitochondrial depolarization was measurable via red fluorescence intensity.

Nitrite assay

As previously reported [15], Nitrite accumulation was measured in the culture medium of confluent HUVECs. Nitrite was colorimetrically quantified after adding 100 μL of Griess reagent (1% sulfanilamide and 0.1% naphthylenediamine in 5% phosphoric acid) to 100 μL culture medium. Absorbance at 550 nm was determined with a microplate reader (Molecular Devices). Nitrite concentrations were calculated by comparison with the absorbance of standard solutions of sodium nitrite prepared in cell culture medium.

Statistical analysis

The data are expressed as mean with standard error (X ± SD). Data were compared utilizing Student's t-test or one-way ANOVA followed by Tukey multiple comparison tests while more than two groups were compared (GraphPad Prism, version 5.01, GraphPad Software, Inc., USA). *P* value of below 0.05 was considered to be statistically significant.

Results

TLR9 inactivation increased the viability of ox-LDL-mediated HUVECs

To investigate whether TLR9 activation had a direct effect on the viability of ox-LDL-mediated HUVECs. cell proliferation assays (Cell Counting Kit 8, CCK8) were performed in ox-LDL-mediated HUVECs, which were treated with TLR9 ligand C-phosphate-G (CpG) oligodeoxynucleotide ODN1826 (TLR9 agonist) and/or IRS869 (a special antagonist of TLR9). CCK-8 proliferation assay illustrated that cell proliferation was greatly reduced in HUVECs that were treated with ox-LDL in comparison with negative control (non ox-LDL treated HUVECs) (**Figure 1A** and **1B**). Furthermore, compared with ox-LDL-mediated HUVEC, cell proliferation abilities were significantly promoted following the treatment of IRS869 in ox-LDL-mediated HUVECs (**Figure 1A**). In contrast, treatment of ODN1826 in ox-LDL-mediated HUVECs inhibited the cell growth rate (**Figure 1B**). Overall, these results illustrate that ox-LDL exerts anti-proliferative effects on HUVECs and TLR9 inactivation may ameliorate the viability of HUVECs injured by ox-LDL.

TLR9 inactivation inhibited the decrease of mitochondrial transmembrane potential induced by ox-LDL

Mitochondrial membrane potential plays an important role in maintain normal physiological functions of cells. Decreased cell viability may be caused by corresponding reduction in mitochondrial membrane potential. In order to confirm our speculation, the change of mitochondrial membrane potential (MTP) of HUVECs were observed by dynamic imaging system. Higher positions of mitochondrial membrane potential were marked by red fluorescence. The

A

B
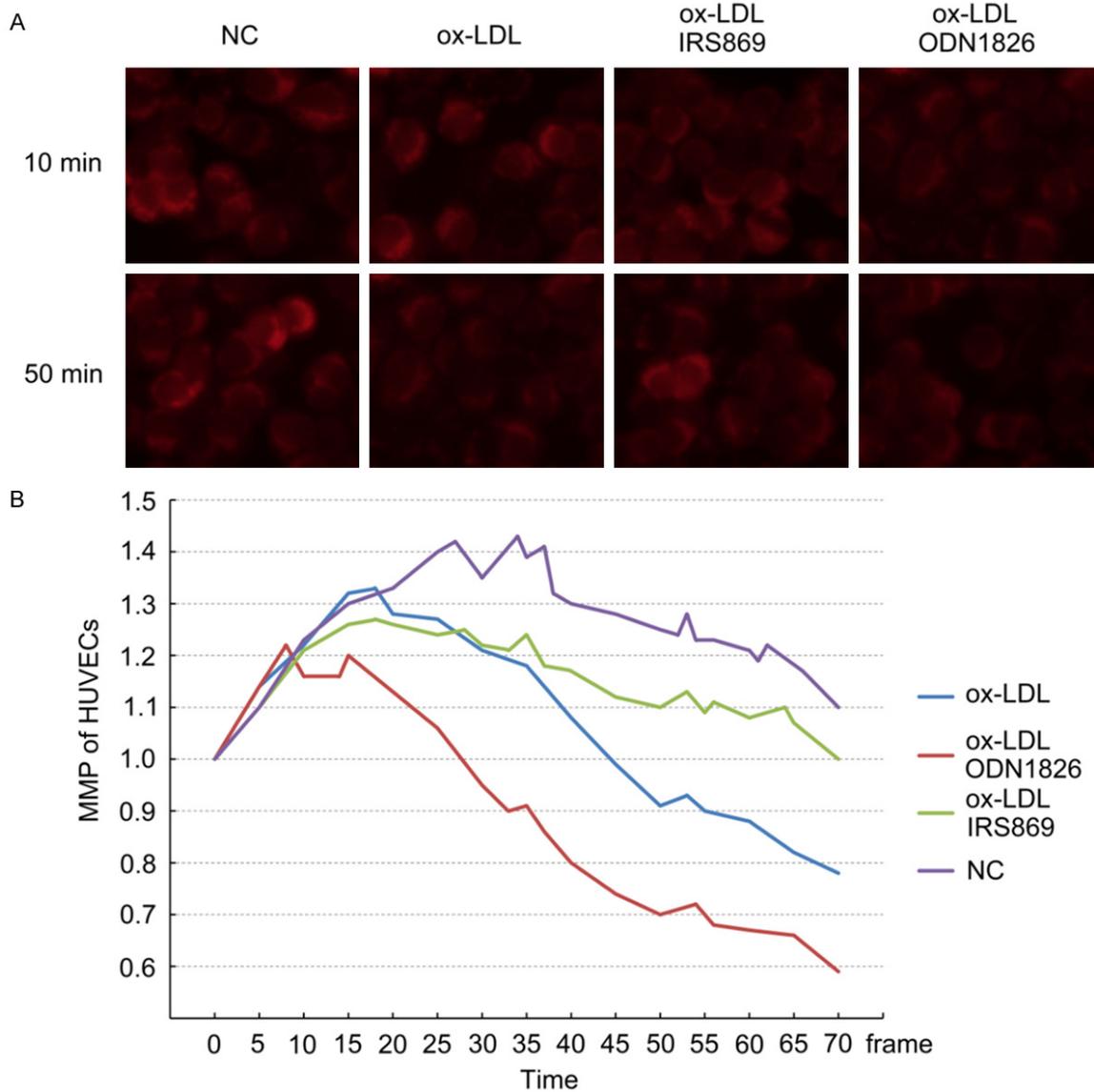
Figure 2. Mitochondrial transmembrane potential changes of IRS869 and ODN1826 on ox-LDL-mediated HUVECs. A. Detection of mitochondrial transmembrane potential changes of IRS869 (2 μM) and ODN1826 (2 μM) on ox-LDL (0.1 mg/mL)-mediated HUVECs at 10 and 50 minutes by fluorescent staining. B. Living cell imaging system showed mitochondrial transmembrane potential levels of IRS869 (2 μM) and ODN1826 (2 μM) on ox-LDL (0.1 mg/mL)-mediated HUVECs from 0 to 70 minutes.

red fluorescence was not changed under the treatment of IRS869 or ODN1826 in ox-LDL-mediated HUVECs at first 10 minutes (**Figure 2A** and **2B**). While red fluorescence signal persisted in HUVECs and later weakened rapidly in ox-LDL-mediated HUVECs. Furthermore, a lesser red fluorescence signal occurred following the treatment of ODN1826 in ox-LDL-mediated HUVECs and more red fluorescence signal was detected under the treatment of IRS869 in ox-LDL-mediated HUVECs (**Figure 2A** and **2B**). These results show that IRS869 co-

uld reduce and delay the decrease of MTP induced by ox-LDL but have the opposite effect in ODN1826.

TLR9 inactivation attenuates iNOS expression and decreased NO production in ox-LDL-mediated HUVECs

To assess whether TLR9 induces iNOS expression in ox-LDL-mediated HUVECs, the cells were incubated IRS869 (a special antagonist of TLR9) with and/or ODN1826 (TLR9 agonist) for
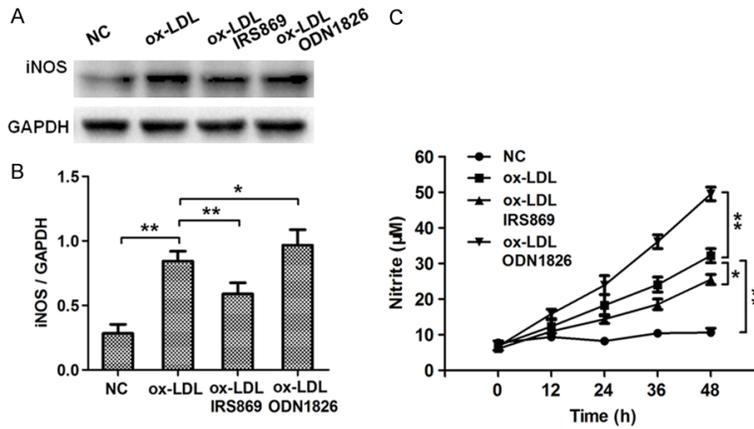
Figure 3. iNOS expression and NO production under the treatment of ODN1826 and IRS869 in ox-LDL-mediated HUVECs. A. HUVECs were stimulated with ox-LDL (0.1 mg/mL, 24 hours) with or without pretreatment with IRS869 (2 µM, 12 hours) or ODN1826 (2 µM, 12 hours) and then, protein expression of iNOS was determined by Western blot. B. iNOS protein levels were analyzed by Western blotting gray value statistics. C. Following pretreatment of HUVECs with IRS869 (2 µM) or ODN1826 (2 µM) for 0, 12, 24, 36 or 48 hours, nitrite concentration in the medium was measured using Griess reaction. *, $P<0.05$; **, $P<0.01$.

24 hours and then iNOS expression was measured. mRNA and protein expression of iNOS was hardly detectable in unstimulated HUVECs, but expression of iNOS was markedly upregulated in cells stimulated with ox-LDL (**Figure 3A** and **3B**). While iNOS expression was attenuated in ox-LDL and IRS869-mediated HUVECs, in contrast, it was increased in ox-LDL and ODN1826-mediated HUVECs (**Figure 3A** and **3B**). Consistent with iNOS, NO generation was time-dependently increased after ox-LDL and ODN1826 stimulating HUVECs and decreased in ox-LDL and IRS869-mediated HUVECs (**Figure 3C**).

TLR9 inactivation inhibited expression of LOX-1 and inactivated NF-κB signaling pathway

The molecules of LOX-1, IκBα, and NF-κBp65 are critical downstream signaling components in TLR9 pathway. To determine whether those molecules are involved in TLR9 inactivation, the mRNA and protein levels of TLR9 and the downstream molecules (LOX-1, IκBα, pIκBα, NF-κBp65 and pNF-κBp65) were determined by qRT-PCR and Western blotting. qRT-PCR results showed that the expression of TLR9, LOX-1, IκBα, and NF-κBp65 were significantly upregulated in HUVECs that were treated with ox-LDL compared to negative control (**Figure 4A-D**). Furthermore, under treatment of IRS869

or ODN1826 in ox-LDL-mediated HUVECs, expression of TLR9, LOX-1, IκBα, and NF-κBp65 was significantly downregulated or upregulated compared to those in ox-LDL-mediated HUVECs, respectively (**Figure 4A-D**). Consistent with the qRT-PCR results, the protein levels of TLR9, LOX-1, IκBα, NF-κBp65, pNF-κBp65 were upregulated in ox-LDL-mediated HUVECs except for the downregulation of pIκBα in ox-LDL-mediated HUVECs (**Figure 4E** and **4F**). Meanwhile, compared with negative control, the markedly lower protein levels of TLR9, LOX-1, IκBα, NF-κBp65, pNF-κBp65 and higher protein level of pIκBα were detected following IRS869 treatment in ox-LDL-mediated HUVECs than those in non-treatment of IRS869 (**Figure 4E** and **4F**). Altogether, these results demonstrate that TLR9 inactivation could inhibit the expression of LOX-1 and inactivate NF-κB signaling pathway induced by ox-LDL in HUVECs.

Discussion

Vascular endothelial cells are the main barrier of the blood vessel wall, while its dysfunction and injury are the first steps of atherosclerosis. It is already well known that ox-LDL plays a key role in the development of atherosclerosis, including endothelial dysfunction, macrophage invasion, and foam cell formation [16, 17]. In our study, the model of ox-LDL induces HUVECs injury had been successfully performed in the experiment. Our data demonstrate that ox-LDL exerts anti-proliferative effects on HUVECs and TLR9 inactivation may ameliorate the viability of HUVECs injured by ox-LDL.

Mitochondria as cellular "power plants", is the basis of eukaryotic cell survival. Mitochondrial membrane potential changes could lead to electron transport and oxidative phosphorylation obstacles, resulting in decreasing ATP production [18]. Furthermore, the decrease of the ATP can decrease ATP dependent ion channels open, causing gain of ion balance disor-
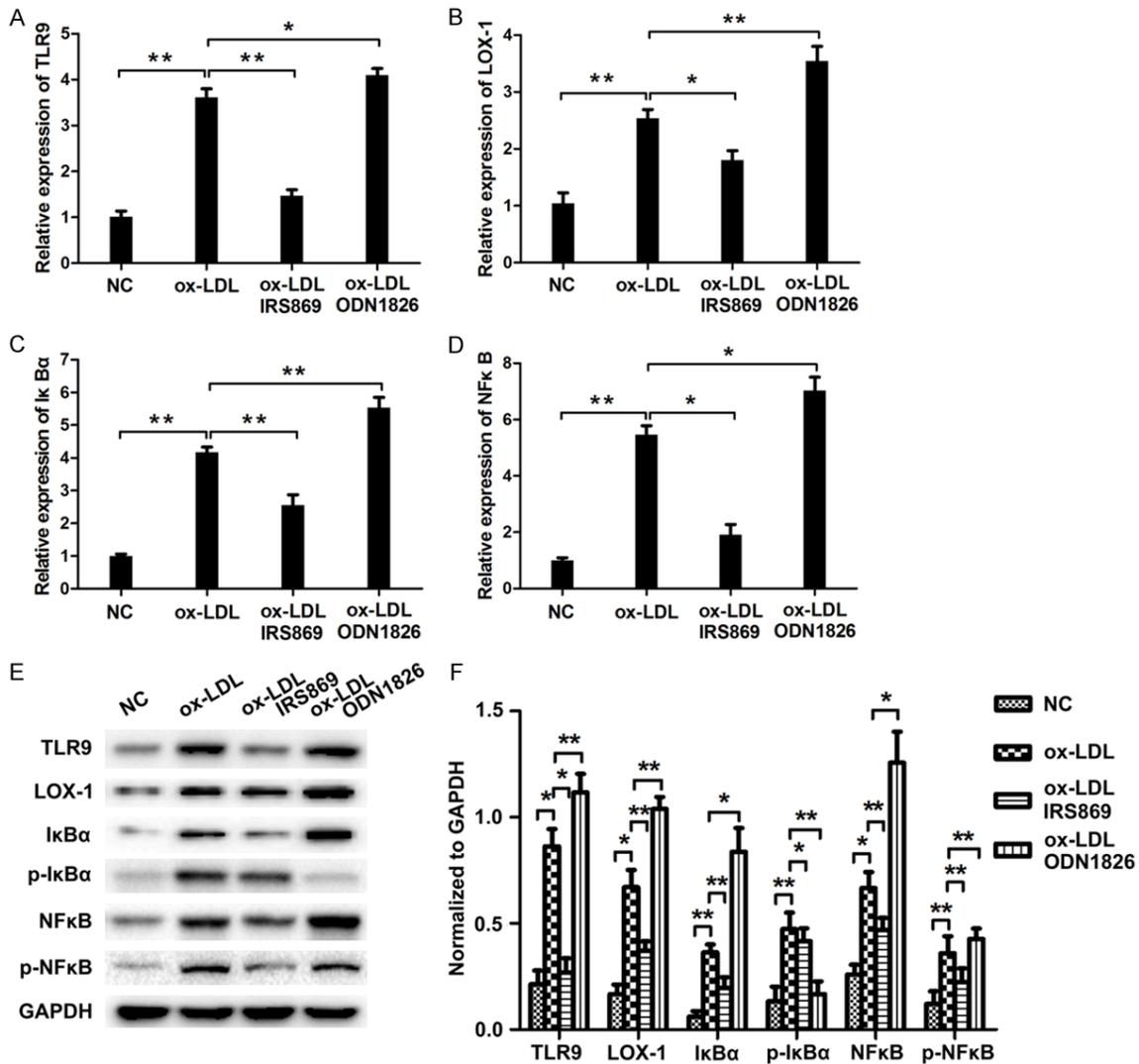
Figure 4. Expression levels of TLR9, LOX-1, IκBα, pIκBα, NF-κBp65, and pNF-κBp65 protein of ODN1826 and IRS869 on ox-LDL-mediated HUVECs. HUVECs were dealt with ox-LDL (0.1 mg/mL, 24 hours) with or without pretreatment with IRS869 (2 μM, 12 hours) or ODN1826 (2 μM, 12 hours), and the expression levels of TLR9 (A), LOX-1 (B), IκBα (C), NF-κB (D) mRNA were determined by quantitative real-time PCR analysis. (E) The protein levels of TLR9, LOX-1, IκBα, pIκBα, NF-κBp65, pNF-κBp65 were analyzed by Western blotting. (F) TLR9, LOX-1, IκBα, pIκBα, NF-κBp65, pNF-κBp65 protein levels were analyzed by Western blotting gray value statistics. *, P<0.05; **, P<0.01.

ders, as well as *in vivo* and *in vitro* calcium overload in the mitochondria [19], increased mitochondrial damage, and even cause apoptosis or necrosis [20]. Our experiment results show that mitochondrial membrane potential was reduced in HUVECs treated with ox-LDL. While TLR9 inactivation could reduce and delay the decrease of mitochondrial membrane potential induced by ox-LDL.

The damage of endothelial cells induced was affected by binding of ox-LDL and its receptor LOX-1 on endothelial cells [21]. The expression

of LOX-1 mRNA and protein were upregulated through ox-LDL in a dose-dependent manner. After the combination of both, the production of a large number of reactive oxygen species and reduction of NO was generated by cells through the mitochondrial electron transport chain. Previous studies showed that iNOS expression is upregulated with subsequent synthesis of large quantity of NO following injury to the vasculature [22]. NO is an important pro-inflammatory and pro-atherogenic mediator. Enhancement of NO level likely plays a significant role in the development of atherosclerosis

[23]. It is well known that TLR9 inactivation inhibits iNOS expression in M1 macrophage infiltration [24]. The results in the study show that TLR9 inactivation remarkably attenuates iNOS in the mRNA and protein level, and decreases NO production in ox-LDL-mediated HUVECs.

Although pro-inflammatory effect of TLR9 has firmly been established, its pro-inflammatory signaling pathway, especially in ox-LDL-mediated HUVECs, remains to be completely defined. It is reported that CpG ODN activates the TLR9-MyD88-ERK1/2 pathway in formation of foam cells [25]. Additionally, ODN1826, the agonist ligand of TLR9, can significantly enhance perilipin 3 expression in RAW264.7 cells [26]. Alternatively, compelling evidence has suggested that activation of TLR9 facilitates formation of foam cells in an NF-κB- and IRF7-dependent manner [27, 28]. Consistent with those findings, our results indicate that inactivation of TLR9 downregulated LOX-1, IκBα, NF-κBp65 and alleviated atherosclerosis progression.

In conclusion, our data provide novel observations that TLR9 inactivation increases mitochondrial transmembrane potential, decreases NO production, and promotes cell viability. Our study may be valuable for deciphering cross talk between the autoimmune response and atherosclerosis and provide a promising therapeutic strategy for atherosclerosis, given that atherosclerosis is a multifactorial disease in which diverse mechanisms are involved.

Acknowledgements

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References

[1] Libby P. Inflammation in atherosclerosis. Nature 2002; 420: 868-874.

[2] Skålén K, Gustafsson M, Rydberg EK, Hultén LM, Wiklund O, Innerarity TL, Borén J. Subendothelial retention of atherogenic lipoproteins in early atherosclerosis. Nature 2002; 417: 750-754.

[3] Pirillo A, Norata GD, Catapano AL. LOX-1, ox-LDL, and Atherosclerosis. Mediators Inflamm 2013; 2013: 152786.

[4] Renie G, Maingrette F, Li L. Diabetic vasculopathy and the lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1). Curr Diabetes Rev 2007; 3: 103-110.

[5] Bhaskar S, Shalini V, Helen A. Quercetin regulates oxidized LDL induced inflammatory changes in human PBMCs by modulating the TLR-NF-kappa B signaling pathway. Immunobiology 2011; 216: 367-373.

[6] Kannan Y, Sundaram K, Aluganti Narasimhulu C, Parthasarathy S, Wewers MD. Oxidatively modified low density lipoprotein (LDL) inhibits TLR2 and TLR4 cytokine responses in human monocytes but not in macrophages. J Biol Chem 2012; 287: 23479-23488.

[7] Takeuchi O, Akira S. Pattern recognition receptors and inflammation. Cell 2010; 6: 805-820.

[8] Hemmi H, Takeuchi O, Kawai T, Kaisho T, Sato S, Sanjo H, Matsumoto M, Hoshino K, Wagner H, Takeda K, Akira S. A Toll-like receptor recognizes bacterial DNA. Nature 2000; 408: 740-745.

[9] Barrat FJ, Meeker T, Gregorio J, Chan JH, Uematsu S, Akira S, Chang B, Duramad O, Coffman RL. Nucleic acids of mammalian origin can act as endogenous ligands for Toll-like receptors and may promote systemic lupus erythematosus. J Exp Med 2005; 202: 1131-1139.

[10] Akira S, Uematsu S, Takeuchi O. Pathogen recognition and innate immunity. Cell 2006; 124: 783-801.

[11] El Kebir D, József L, Pan W, Wang L, Filep JG. Bacterial DNA activates endothelial cells and promotes neutrophil adherence through TLR9 signaling. J Immunol 2009; 182: 4386-4394.

[12] Fitzner N, Clauberg S, Essmann F, Liebmann J, Kolb-Bachofen V. Human skin endothelial cells can express all 10 TLR genes and respond to respective ligands. Clin Vaccine Immunol 2008; 15: 138-146.

[13] Pegu A, Qin S, Fallert Junecko BA, Nisato RE, Pepper MS, Reinhart TA. Human lymphatic endothelial cells express multiple functional TLRs. J Immunol 2008; 180: 3399-3405.

[14] Edfeldt K, Swedenborg J, Hansson GK, Yan ZQ. Expression of toll-like receptors in human atherosclerotic lesions: a possible pathway for plaque activation. Circulation 2002; 105: 1158-1161.

[15] Granger DL, Taintor RR, Boockvar KS, Hibbs JB Jr. Measurement of nitrate and nitrite in biological samples using nitrate reductase and

Griess reaction. Methods Enzymol 1996; 268: 142-151.

[16] Xu RX, Sun XC, Ma CY, Yao YH, Li XL, Guo YL, Zhang Y, Li S, Li JJ. Impacts of berberine on oxidized LDL-induced proliferation of human umbilical vein endothelial cells. Am J Transl Res. 2017, 9: 4375-4389.

[17] Goyal T, Mitra S, Khaidakov M, Wang X, Singla S, Ding Z, Liu S, Mehta JL. Current concepts of the role of oxidized LDL receptors in atherosclerosis. Curr Atheroscler Rep 2012; 14: 150159.

[18] Iannetti EF, Willems PH, Pellegrini M, Beyrath J, Smeitink JA, Blanchet L, Koopman WJ. Toward high-content screening of mitochondrial morphology and membrane potential in living cells. Int J Biochem Cell Biol 2015; 63: 66-70.

[19] Tkatch T, Greotti E, Baranauskas G, Pendin D, Roy S, Nita LI, Wettmarshausen J, Prigge M, Yizhar O, Shirihai OS, Fishman D, Hershfinkel M, Fleidervish IA, Perocchi F, Pozzan T, Sekler I. Optogenetic control of mitochondrial metabolism and Ca2+ signaling by mitochondria-targeted opsins. Proc Natl Acad Sci U S A 2017; 114: E5167-E5176.

[20] Dulak J, Jozkowicz A, Dembinska-Kiec A, Guevara I, Zdzienicka A, Zmudzinska-Grochot D, Florek I, Wójtowicz A, Szuba A, Cooke JP. Nitric oxide induces the synthesis of vascular endothelial growth factor by rat vascular smooth muscle cells. Arterioscler Thromb Vasc Biol 2000; 20: 659-666.

[21] Chen M, Masaki T, Sawamura T. LOX-I, the receptor for oxidized low-density lipoprotein indentified from endothelial cells: implications in endothelial dysfunction and aterosclerosis. Pharmacol Ther 2009; 95: 89100.

[22] Marsch E, Theelen TL, Janssen BJ, Briede JJ, Haenen GR, Senden JM, van Loon LJ, Poeze M, Bierau J, Gijbels MJ, Daemen MJ, Sluimer JC. The effect of prolonged dietary nitrate supplementation on atherosclerosis development. Atherosclerosis 2016; 245: 212-21.

[23] Ma C, Ouyang Q, Huang Z, Chen X, Lin Y, Hu W, Lin L. Toll-like receptor 9 inactivation alleviated atherosclerotic progression and inhibited macrophage polarized to M1 phenotype in apoE-/- mice. Dis Markers 2015; 2015: 909572.

[24] Zhang YX, Yu PF, Gao ZM, Yuan J, Zhang Z. Caffeic acid n-butyl ester-triggered necrosis-like cell death in lung cancer cell line A549 is prompted by ROS mediated alterations in mitochondrial membrane potential. Eur Rev Med Pharmacol Sci 2017; 21: 1665-1671.

[25] Khallou-Laschet J, Varthaman A, Fornasa G, Compain C, Gaston AT, Clement M, Dussiot M, Levillain O, Graff-Dubois S, Nicoletti A, Caligiuri G. Macrophage plasticity in experimental atherosclerosis. PLoS One 2010; 5: e8852.

[26] Gu JQ, Wang DF, Yan XG, Zhong WL, Zhang J, Fan B, Ikuyama S. A Toll-like receptor 9-mediated pathway stimulates perilipin 3 (TIP47) expression and induces lipid accumulation in macrophages. Am J Physiol Endocrinol Metab. 2010; 299: E593-600.

[27] Sorrentino R, Morello S, Chen S, Bonavita E, Pinto A. The activation of liver X receptors inhibits toll-like receptor-9-induced foam cell formation. J Cell Physiol. 2010; 223: 158-167.

[28] Bjorkbacka H, Kunjathoor VV, Moore KJ, Koehn S, Ordija CM, Lee MA, Means T, Halmen K, Luster AD, Golenbock DT, Freeman MW. Reduced atherosclerosis in MyD88-null mice links elevated serum cholesterol levels to activation of innate immunity signaling pathways. Nat Med 2004; 10: 416-421.