Original Research

Niacin promotes the efflux of lysosomal cholesterol from macrophages via the CD38/NAADP signaling pathway

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Impact Statement

Niacin treatment was found to reduce lysosomal free cholesterol levels in oxidized low-density lipoprotein-containing macrophages in a concentration-dependent manner; Niacin decreased the formation of atherosclerotic lesion-causing cholesterol crystal clefts in LDLr$^{-/-}$ mice fed a Western diet; Niacin may promote the efflux of lysosomal cholesterol from macrophages via the CD38/NAADP signaling pathway.

Abstract

The accumulation of free cholesterol in macrophage lysosomes significantly enhances atherogenesis. Our recent study demonstrated that the cluster of differentiation 38 (CD38)/nicotinic acid adenine dinucleotide phosphate (NAADP)/Ca^{2+} signaling pathway plays a critical role in the efflux of lysosomal free cholesterol from macrophages in atherosclerosis. Niacin, known as nicotinic acid, is one of the oldest lipid-lowering medications showing unique anti-atherosclerotic activity. However, it is unknown whether this anti-atherosclerosis activity is associated with the efflux of lysosomal compartmentalized cholesterol in macrophages. In this study, we investigated the anti-atherosclerotic effects of niacin on the reduction of lysosomal free cholesterol via CD38/NAADP signaling in macrophages derived from low-density lipoprotein receptor (LDLr$^{-/-}$) mice. Fluorescent filipin and Nile red labeling coupled with confocal microscopy demonstrated that niacin reduced free cholesterol accumulation in lysosomes in a concentration-dependent manner. Transmission electron microscopy also showed that niacin markedly decreased cholesterol crystal formation in lysosomes in oxidized LDL-containing LDLr$^{-/-}$ bone marrow–derived macrophages. Enzyme-linked immunosorbent assays showed that niacin increased NAADP production in a concentration-dependent manner, which was inhibited by small interfering RNA interference of CD38. Therefore, niacin may promote the efflux of lysosomal cholesterol from macrophages via the CD38/NAADP signaling pathway.

Keywords: Niacin, cholesterol, lysosome, atherosclerosis, cluster of differentiation 38/nicotinic acid adenine dinucleotide phosphate, low-density lipoprotein receptor $^{-/-}$ mice

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Introduction

Atherosclerosis is characterized by the accumulation of cholesterol in macrophages. In atherosclerotic plaques, lesional macrophages endocytose oxidized low-density lipoprotein (oxLDL) and deliver it to endo/lysosomal systems for hydrolysis. Under normal conditions, the hydrolyzed (free) cholesterol is transported out of lysosomes and serves as cell-building blocks, is secreted from cells, and/or is re-esterified into cholesteryl ester and stored in cytosol lipid droplets.[1,2] Disruption in cholesterol metabolism and trafficking can induce free cholesterol accumulation in macrophages. Several studies have shown that the accumulation of free cholesterol in the lysosomes of macrophages can greatly enhance atherosclerosis progression by eliciting inflammation, promoting lesion core formation, and destabilizing plaque.[3–5] In this regard, our recent study demonstrated that free cholesterol accumulation in lysosomes of macrophages results in coronary atherosclerosis development in cluster of differentiation 38 (CD38$^{-/-}$) mice, in which the CD38/nicotinic acid adenine dinucleotide phosphate (NAADP) signaling pathway plays an important role in the regulation of macrophage lysosomal cholesterol homeostasis in atherosclerosis. NAADP is a secondary Ca^{2+} messenger that targets lysosomes and mediates Ca^{2+} release, which facilitates free cholesterol transportation out of lysosomes.[6,7] Moreover, NAADP is produced by the CD38 enzyme in a substitution

reaction using nicotinic acid as a substrate and substituent for the nicotinamide moiety in the NAADP molecule.[8–10]

Niacin, commonly known as nicotinic acid, is a widely used lipid-modulating agent in atherosclerosis and is second only to β-hydroxy β-methylglutaryl coenzyme A reductase inhibitors of statins.[11] Niacin treatment increases levels of plasma high-density lipoprotein (HDL), which is an anti-atherogenic apolipoprotein that is capable of transferring peripheral cholesterol to the liver for elimination during reverse cholesterol transport (RCT).[12] Egression of macrophage cholesterol represents a critical step in achieving regression of atherosclerotic lesions via RCT.[13] It has been reported that niacin promotes the release of cholesterol from macrophages.[14,15] However, it is unknown whether the anti-atherosclerosis activity of niacin is associated with the metabolism of cholesterol in macrophages via CD38/NAADP signaling.

Given the fundamental requirement of nicotinic acid in the production of NAADP and the functional significance of the CD38/NAADP signaling pathway in the efflux of lysosomal cholesterol, it is possible that nicotinic acid directly affects lesional cholesterol by enhancing the efflux of lysosomal cholesterol via CD38/NAADP. Our results show that the nicotinic acid–mediated reduction in atherosclerotic lesions may be associated with the reduction of macrophage lysosomal free cholesterol in LDL receptor ($LDLr^{-/-}$) mice, which is mediated by the CD38/NAADP signaling pathway.

Materials and methods

Materials

The reagents and biochemical kits used in this study included: Nile red, filipin, and BAPTA-AM (Sigma-Aldrich; St. Louis, MO, USA); GenMute™ siRNA Transfection Reagent (SignaGen Laboratories; Gaithersburg, MD, USA); oxLDL (thiobarbituric acid reactive substances: 29–44 nmoles malondialdehyde/mg), 1,1′-dioctadecyl-3,3,3′,3′-tetramethylin docarbocyanine (Dil)-labeled oxLDL (Dil-oxLDL), and HDL (Alfa Aesar; Ward Hill, MA, USA); NED-19, lysosomal-associated membrane protein 1 (LAMP-1) rat monoclonal antibody (Santa Cruz biotechnology; Dallas, TX, USA); Alexa Fluor-594 chicken anti-rat IgG and Amplex red cholesterol assay kit (Life Technologies; Carlsbad, CA, USA); *Cd38* siRNA (OriGene Technologies; Rockville, MD, USA); and oil red O staining kit (American Mastertech; Lodi, CA, USA). *LDLr*-knockout C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME, USA). Western-type diet (gm%: protein 20, carbohydrate 50, and fat 21) was obtained from Research Diets (New Brunswick, NJ, USA). Animal experiments were approved by the Animal Research Committee of Guilin Medical College (No. GLMC-202005035). All animal experiments were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Primary culture of bone marrow macrophages and cell treatment

The isolation and differentiation of bone marrow cells into macrophages were performed as previously described.[6,16] In brief, the $LDLr^{-/-}$ mice were euthanized via exsanguination under deep anesthesia (50 mg/kg pentobarbital, intraperitoneal), and the femur and tibia bones were dissected. Bone marrow cells were collected from the femur and tibia medullary cavities, and differentiation into macrophages was induced by culturing the cells in RPMI-1640 media supplemented with 15% L-929 conditional media, 10% fetal bovine serum, 100 IU/mL penicillin, and 100 μg/mL streptomycin at 37 °C and 5% CO_2. The macrophage phenotype was attained after 7 days of differentiation in differential culture and confirmed via CD68-positive staining; the differentiated cells were used for experiments from day 8 to day 10. For inducing the accumulation of free cholesterol in macrophage lysosomes, the cells were incubated for 48 h with 60 μg/mL oxLDL in RPMI-1640 media of the same composition as the differential media mentioned above except L-929 growth media was not included. The effects of niacin on macrophage lysosomal cholesterol homeostasis were examined at a concentration of 2.0 mmol/L, except when indicated otherwise. The cells were treated with CD38/NAADP/Ca^{2+} signaling inhibitors, including NED-19 (10 μM, NAADP antagonist) and glycyl-L-phenylalanine 2-naphthylamide (GPN; 0.5 mM, lysosome disruptor), 1 h prior to the addition of niacin. The transfection of *Cd38* siRNA was performed as described previously, and the efficacy of gene interference was confirmed via real-time polymerase chain reaction (PCR) analysis of the transcription levels of the related genes.[6] Western blot was performed to further clarify the expression. The cells subjected to gene interference were used for experiments 48 h after transfection.

Confocal microscopic analysis of lipid droplets and lysosomal free cholesterol in macrophages

The effects of niacin on cholesterol in macrophages were determined via simultaneous staining of free cholesterol and lipid droplets using filipin and Nile red fluorescent dyes, respectively, as well as by immunolabeling using the lysosomal marker protein LAMP1. After different treatments, macrophages were subcultured into eight-well chamber slides, fixed, permeabilized, and labeled with filipin and lysosomal LAMP1. The cells were stained with Nile red as described previously.[17] Fluorescence images were taken using a multi-photon laser scanning microscope (Zeiss LSM 510 NLO META; Carl Zeiss Microcopy, Thornwood, NY, USA) equipped with a 63×1.4 NA Plan Achromat oil immersion objective lens. Multi-channel images were collected sequentially to ensure the absence of cross-talk between channels, and the detector offset and gain settings were maintained for all the images collected. The dyes were imaged at the following wavelengths: Alexa Fluor 633, excitation (ex) 633 nm/emission (em) 650–710 nm; filipin, ex 740 nm/em 435–485 nm; and Nile red, ex 514 nm/em 535–590 nm. Image Pro 9.1 software (Media Cybernetics, Rockville, MD, USA) was used to quantify the intensities of Nile red- (lipid droplet) and filipin-stained sections (free cholesterol) and to analyze the co-localization coefficient (Pearson's) between filipin and lysosomal LAMP1 as described previously.[6]

Enzyme-linked immunosorbent assay analysis of NAADP production by macrophages in culture

Macrophages were sub-cultured in six-well plates at a cell density of 1.2×10^6 cells/well. After different treatments, the culture medium was collected for NAADP quantification using the NAADP enzyme-linked immunosorbent assay (ELISA) kit (MyBioSource, Inc. SD, USA) according to the manufacturer's instructions. In brief, NAADP-containing culture medium was added to a microplate strip (50 µL/well), incubated for 1 h at 37 °C, mixed with horseradish peroxidase-conjugated antibody (100 µL/well), and then incubated for another 1 h at 37 °C. Four washes were performed after the two incubations. Subsequently, 100 µl of the substrate solution was applied to generate chemiluminescence. Chemiluminescence absorbance was determined using a microplate reader (BMG Labtech, Ortenberg, Germany) at $\lambda = 450$ nm, corrected to read at $\lambda = 570$ nm. NAADP was quantified by relating the sample readings to the generated standard curve.

Transmission electron microscopy of cholesterol crystal clefts

Cholesterol crystal clefts in atherosclerotic lesions and macrophages were examined via transmission electron microscopy (TEM) according to a previous method.[6] Briefly, LDLr$^{-/-}$ mice fed a Western diet for 16 weeks were anesthetized using pentobarbital (50 mg/kg, intraperitoneal). After perfusion through the heart using a fixative solution containing 2% formaldehyde and 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4), the aortic arch was dissected and placed in fixative solution overnight, followed by postfixing using 1% osmium and 2% uranyl acid. After dehydration, the aortic arch was embedded in Durcupan (Merk KGaA, German), cut into ultra-thin sections (60 nm), and stained using uranyl acetate and lead. Images were captured using an FEI Spirit Tecnai transmission electron microscope (Nikon, Japan) at 80 kV with an Eagle 4K × 4K camera (Hillsboro, OR, USA). TEM was used to examine the formation of cholesterol crystal clefts in the cultured macrophages in which the cells were incubated with 60 µg/mL oxLDL for 144 h with and without 5 mM niacin. After treatment, the cells were fixed using 2% glutaraldehyde in 0.1 M cacodylate at 4 °C for 48 h, collected via gentle scraping, and pelleted into 2% agarose. The remaining TEM procedures and microscopic imaging for these cells were the same as those performed for the aortic arch.

Atherosclerosis model and aorta root sections from LDLr$^{-/-}$ mice

LDLr$^{-/-}$ mice (age, 8 weeks) were randomly assigned to groups that were fed a Western diet with and without nicotinic acid water (0.12%) for 16 weeks to establish an atherosclerosis model. The mice were fasted overnight before being euthanized for tissue collection. While the mice were anesthetized with pentobarbital (50 mg/kg, intraperitoneal), blood was collected for cholesterol biochemical assay followed by *in situ* heart phosphate-buffered saline perfusion, heart collection, and aortic dissection. The isolated aorta roots were used for Oil Red O staining, and slides were cut for examination.

Statistical analysis

Data are presented as the mean ± standard error. Significant differences between and within multiple groups were examined using analysis of variance (ANOVA). Student's *t*-tests were used to evaluate the significance of differences between the two groups of observation. $P < 0.05$ was considered significant.

Results

Niacin reduced free cholesterol accumulation in oxLDL-containing macrophages

To investigate the effects of niacin on cholesterol accumulation in lysosomes, we first examined the accumulation of lysosomal free cholesterol in macrophages treated with oxLDL. Filipin and Nile red were used to label compartmentalized cholesterol in lysosomes and cholesteryl ester in cytosolic lipid droplets, respectively, and Alexa Fluor 633-labeled LAMP1 was used to label lysosomes. A total of 60 µg/ml oxLDL was applied to construct an atherosclerosis cellular model of LDLr$^{-/-}$ macrophages. Confocal imaging showed that niacin treatment reduced lysosomal free cholesterol levels in oxLDL-containing macrophages in a concentration-dependent manner, as determined by the intensity of blue filipin staining and co-localization coefficient analysis between filipin and LAMP1 (Figure 1(A) to (C)). However, niacin treatment showed a minor effect on cholesteryl ester-containing lipid droplets compared with that observed for macrophages containing oxLDL alone (Figure 1(D)).

Niacin decreased lysosomal free cholesterol accumulation and cholesterol crystal formation *in vivo* and *in vitro*

To investigate the effects of niacin on atherosclerosis and free cholesterol accumulation *in vitro* and *in vivo*, LDLr$^{-/-}$ macrophages treated with oxLDL and LDLr$^{-/-}$ mice fed a Western diet were used to construct cellular and animal atherosclerosis models. *In vivo*, niacin successfully decreased filipin-labeled free cholesterol, which co-localized with Lamp1-marked lysosomes (Figure 2(A)); TEM showed that niacin reduced accumulation of lipid and cholesterol crystals (Figure 2(B)). Reduced cholesterol crystals were also observed in oxLDL-treated LDLr$^{-/-}$ macrophages (Figure 2(C)). The data implied that niacin increased the efflux of lysosomal free cholesterol *in vitro* and *in vivo*.

Niacin treatment increased CD38 expression and NAADP production in oxLDL-containing LDLr-knockout macrophages

To investigate whether niacin functions via the CD38/NAADP signaling pathway to accelerate efflux of lysosomal cholesterol, CD38 mRNA and protein were assayed by RT-PCR and Western blot, and NAADP was detected by ELISA assays. As shown in Figure 3, niacin increased CD38 expression and NAADP production in oxLDL-containing

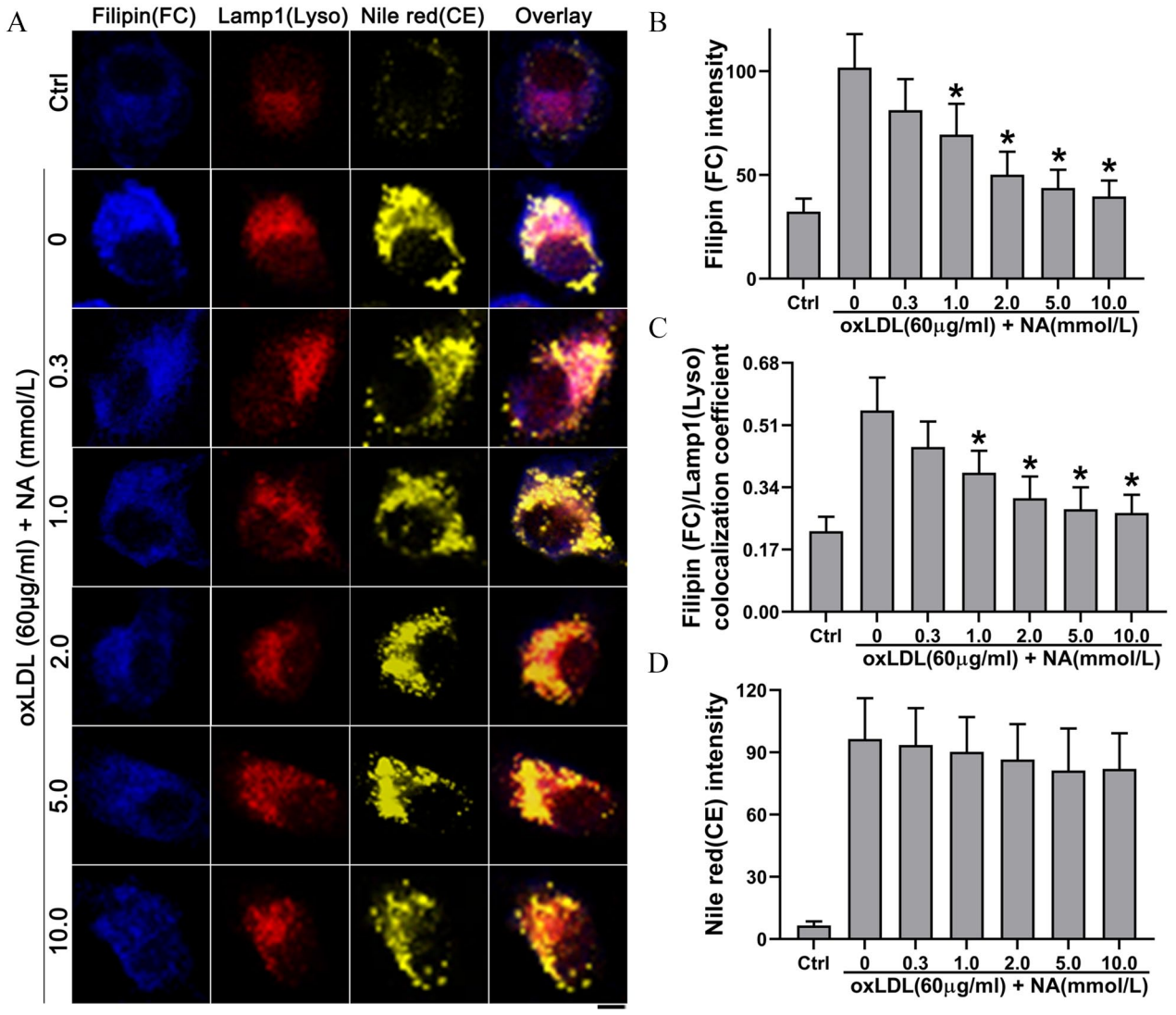

Figure 1. Confocal microscopy images showed that niacin treatment reduced lysosomal free cholesterol in oxLDL-treated LDLr$^{-/-}$ macrophages in a dose-dependent manner. Macrophages were incubated with oxLDL (60 μg/ml) in the presence of different NA concentrations (0.3 mmol/L to 10 mmol/L) for 48 h and labeled with filipin (free cholesterol, blue), Lamp1 antibody (Lysosomes, red), and Nile red (lipid droplet of cholesteryl ester, yellow). (A) Free cholesterol gradually decreased in oxLDL-containing macrophages as the NA concentration increased. In contrast, cholesteryl ester showed no change in oxLDL-treated macrophages (Bar, 5 μm). (B) Intensities from lysosomal free cholesterol stained by filipin. (C) Co-localization coefficient between filipin (free cholesterol) and Lamp1 (lysosomes). (D) Intensities of Nile red staining (cholesteryl ester). (A color version of this figure is available in the online journal.)
NA: niacin; oxLDL: oxidized low-density lipoprotein; LDLr$^{-/-}$: low-density lipoprotein receptor knock-out.
*$p < 0.05$ versus oxLDL group ($n = 6$).

LDLr$^{-/-}$ macrophages. Meanwhile, knockdown of CD38 successfully inhibited niacin-induced upregulation of NAADP. The data implied that niacin could activate the CD38/NAADP signaling pathway in LDLr$^{-/-}$ macrophages.

Inhibition of CD38/NAADP signaling successfully reversed niacin-induced efflux of lysosomal free cholesterol in oxLDL-treated LDLr$^{-/-}$ macrophages

To investigate whether CD38/NAADP mediates niacin-induced efflux of free cholesterol in lysosome of macrophages, specific inhibitors and siRNA were used to block CD38/NAADP signaling. As shown in Figure 4(A), oxLDL successfully induced an influx of free cholesterol and increase in cholesterol ester in LAMP1-labeled lysosomes of LDLr$^{-/-}$ mouse macrophages. Niacin promoted the efflux of free cholesterol and then decreased lysosomal cholesterol ester. In addition, NED-19 (an NAADP antagonist), GPN (a lysosome disruptor), and CD38 siRNA partially rescued niacin-induced efflux of free cholesterol and decrease in cholesterol ester. These data implied an essential role of CD38/NAADP in niacin-mediated efflux of lysosomal cholesterol.

Discussion

Niacin has been found to show multiple beneficial effects on lipid metabolism, particularly by elevating the level of HDL-cholesterol (HDL-C), which is one of the most important factors in atherosclerosis.[11,12] Therefore, niacin is used as an anti-atherogenic agent to treat cardiovascular disease.

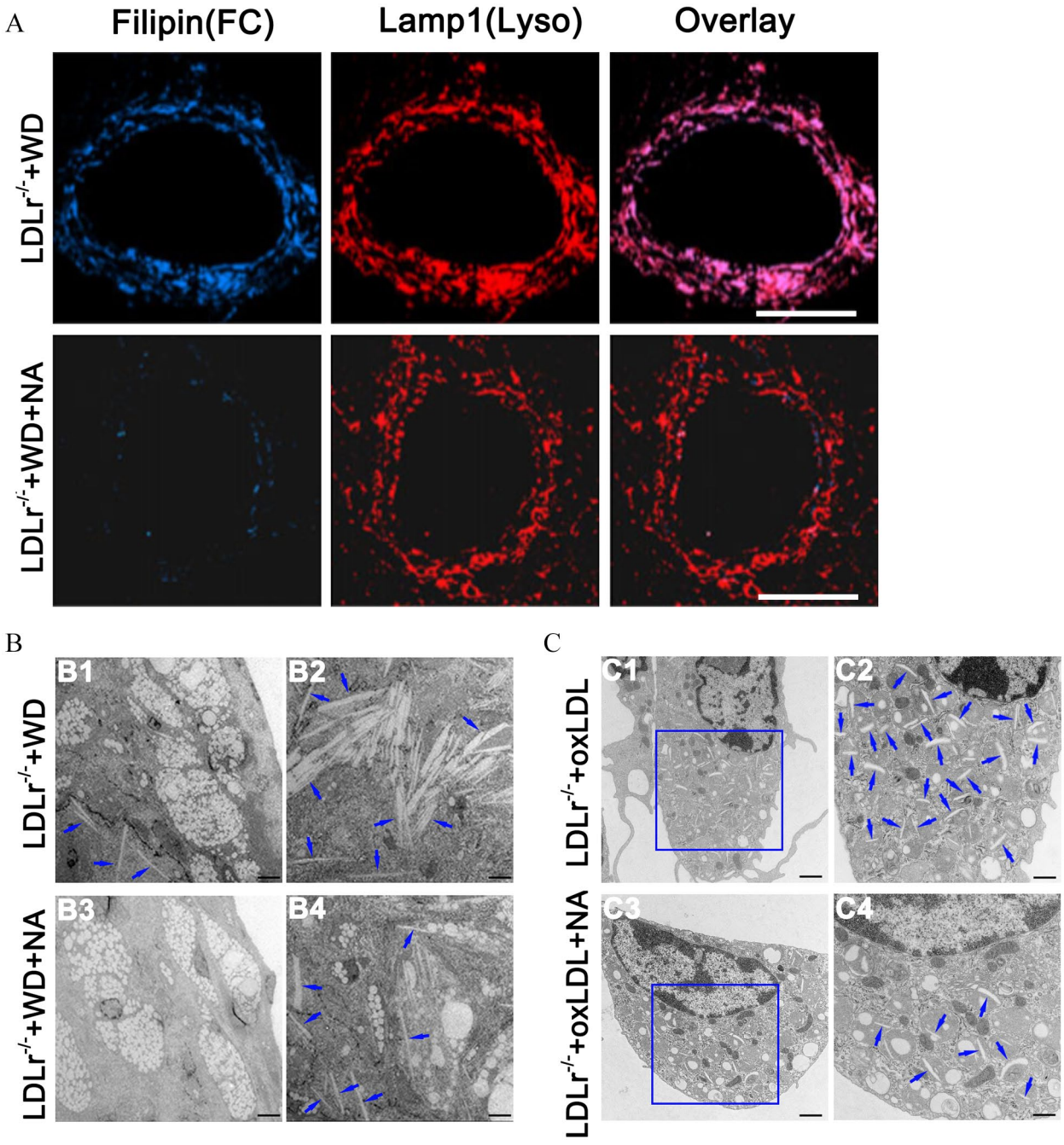

Figure 2. Niacin decreased lysosomal free cholesterol accumulation and cholesterol crystals formation *in vivo*. (A) Confocal microscopy imaging shows the decrease of lysosomal cholesterol crystals in aorta root of NA-treated LDLr$^{-/-}$ mice (Bar, 50 μm). (B) Transmission electron microscopic examination of lesional cholesterol crystal clefts in LDLr$^{-/-}$ mice fed with/without NA in water along with a Western diet. (B1 and B2) Cholesterol crystal clefts in macrophages of LDLr$^{-/-}$ mice fed with Western diet (LDLr$^{-/-}$ + WD, $n = 3$). (B3 and B4) Cholesterol crystal clefts in macrophages from LDLr$^{-/-}$ mice fed with Western diet and niacin (LDLr$^{-/-}$ + WD + NA, $n = 3$; Bar, 5 μm). (C) Transmitted electron microcopy examination of free cholesterol accumulation in LDLr$^{-/-}$ macrophages treated with oxLDL in culture without/with niacin (2.0 mmol/L). (C1) LDLr$^{-/-}$ macrophages treated with oxLDL (LDLr$^{-/-}$ + oxLDL). (C2) Amplified area of the squared portion in A1. (C3) LDLr$^{-/-}$ macrophages treated with oxLDL with niacin (LDLr$^{-/-}$ + oxLDL + NA). (C4) Amplified area of the squared portion in A3. Cholesterol crystals (arrow) in lysosomes (an abundant single membrane-bounded electron-dense structure, $n = 3$) (C1 and C3, Bar, 1 μm; C2 and C4, Bar, 500 nm). (A color version of this figure is available in the online journal.) NA: niacin; Ctrl: control; oxLDL: oxidized low-density lipoprotein; LDLr$^{-/-}$: low-density lipoprotein receptor knockout; WD: Western diet.

Two recent trials on niacin, namely AIM-HIGH and HPS2-THRIVE, obtained negative results in which niacin failed to reduce the occurrence of cardiovascular events (CVEs); however, a meta-analysis on both trials further showed that niacin still reduces CVEs without affecting HDL-C.[18] Similarly, data from an apoE$^{-/-}$ mouse model indicate that long-term administration of niacin affects advanced atherosclerotic plaques by reducing the accumulation of macrophages independently of lipid-mediated actions.[13] In our study, we found that niacin treatment significantly alleviated atherosclerotic lesions by reducing the formation of cholesterol crystal clefts and macrophage-derived foam cells.

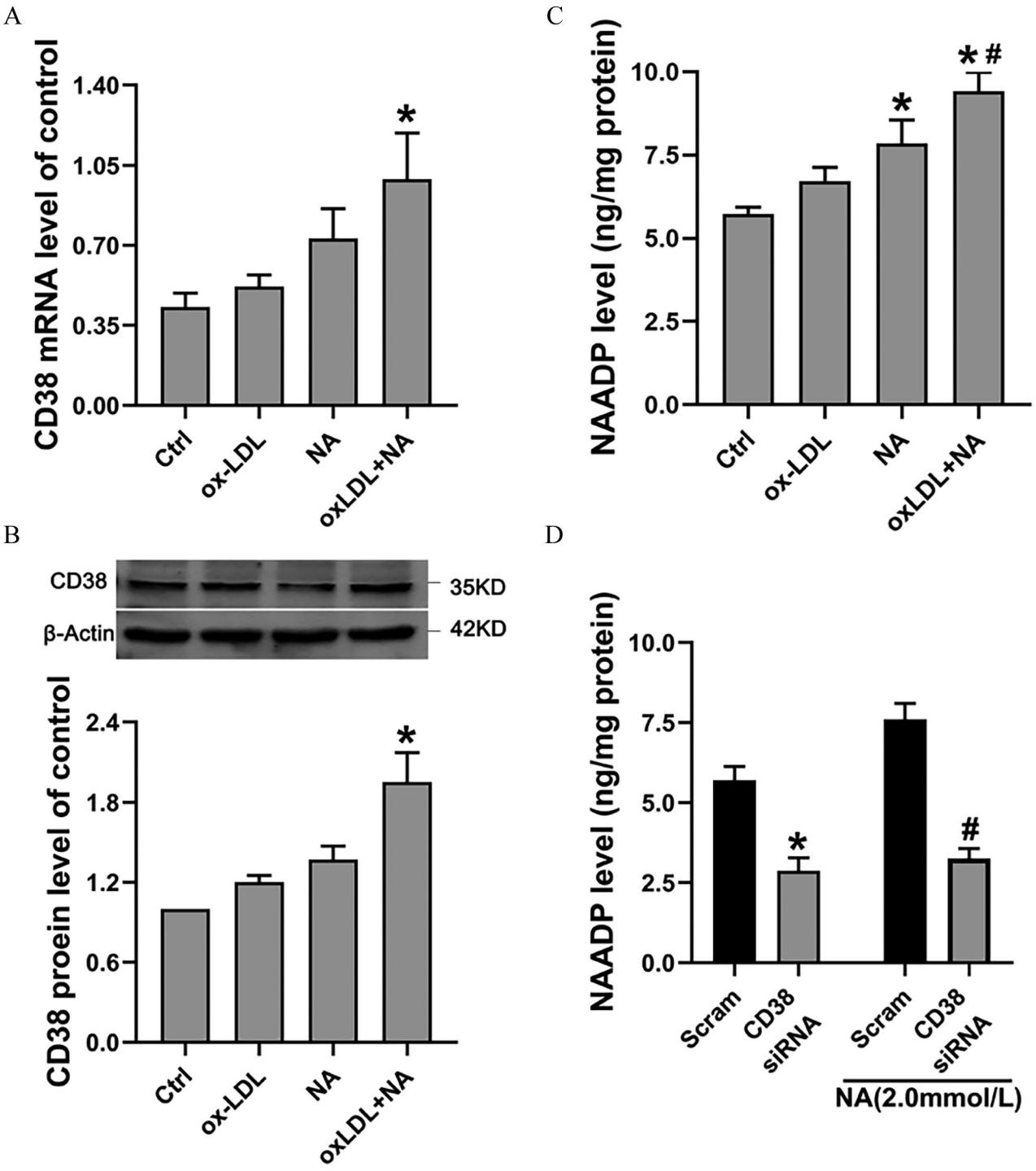

Figure 3. Niacin treatment increased CD38 expression and NAADP secretion in ox-LDL-containing LDLr-knockout macrophages. (A) CD38 mRNA levels determined via quantitative reverse transcription-polymerase chain reaction analysis. (B) Representative western blot images of CD38 bands and intensity of CD38 bands in western blots which were normalized to the control. *$p < 0.05$ versus Ctrl or oxLDL group; #$p < 0.05$ versus Ctrl group ($n = 5$). (C) NA treatment markedly increased NAADP production in oxLDL-containing LDLr$^{-/-}$ macrophages. *$p < 0.05$ versus Ctrl group; #$p < 0.05$ versus oxLDL group ($n = 5$). (D) Enzyme-linked immunosorbent assay analysis of NAADP production after treatment with Cd38 siRNA in LDLr$^{-/-}$ macrophages. *$p < 0.05$ versus Scram group. #$p < 0.05$ versus Scram in NA group ($n = 5$).
oxLDL: oxidized low-density lipoprotein; LDLr: low-density lipoprotein receptor; LDLr$^{-/-}$: low-density lipoprotein receptor knockout; NAADP: nicotinic acid adenine dinucleotide phosphate; Ctrl: control; NA: niacin.

Regarding the effects of niacin on macrophage cholesterol homeostasis, a few studies have reported that treatment with niacin can promote cholesterol efflux from macrophages in different animal models.[7–9] In this study, we found that niacin significantly promoted the efflux of lysosomal free cholesterol in oxLDL-containing macrophages. Moreover, niacin showed a minor effect on cytosolic cholesteryl ester-containing lipid droplets. Therefore, the reduction in levels of lysosomal compartmentalized cholesterol may be related to enhanced egression of cellular cholesterol. In this regard, several studies have reported that transportation of lysosomal free cholesterol to the plasma membrane represents a

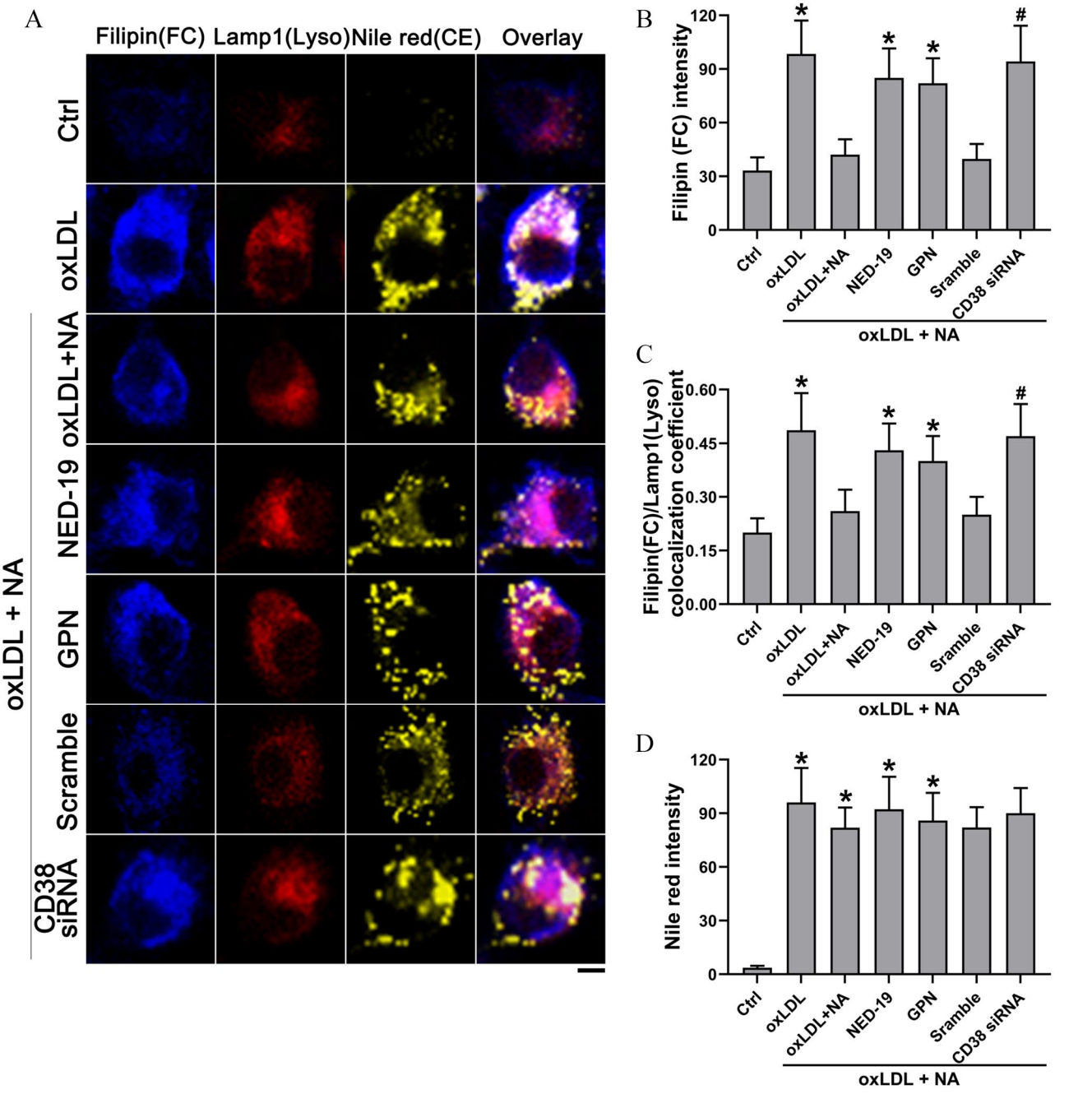

Figure 4. Inhibition of the CD38/NAADP signaling pathway affected lysosomal free cholesterol in LDLr$^{-/-}$ macrophages. (A) Lysosomal free cholesterol levels increased in oxLDL-treated LDLr$^{-/-}$ macrophages treated with CD38/NAADP signaling pathway inhibitor NED-19, GPN, and CD38 siRNA. In contrast, cholesteryl ester showed no change in oxLDL-treated macrophages (Bar, 5 μm). (B) Intensities from lysosomal free cholesterol stained with filipin. (C) Co-localization coefficient between filipin (free cholesterol) and Lamp1 (lysosomes). (D) Intensities of Nile red staining (cholesteryl ester). (A color version of this figure is available in the online journal.)
NA: niacin; oxLDL: oxidized low-density lipoprotein; LDLr$^{-/-}$: low-density lipo-protein receptor knockout; CD38: cluster of differentiation 38; NAADP: nicotinic acid adenine dinucleotide phosphate.
*$p < 0.05$ versus Ctrl or oxLDL + NA group ($n = 6$).

major pathway that mediates the removal of excess cholesterol generated from endocytosed modified LDL.[11] Our TEM images showed that niacin significantly reduced the formation of cholesterol crystal clefts in atherosclerotic lesions, indicating that the effect of niacin on increasing HDL-C levels can be attributed to the reduction in lesional free cholesterol via RCT. This result may be explained by the fact that reduction in lysosomal compartmentalized cholesterol leads to the mitigation of atherosclerotic lesions.

Regarding the mechanism via which niacin promotes the efflux of lysosomal free cholesterol, previous studies have shown that the CD38/NAADP signaling pathway plays a critical role in removal of free cholesterol from lysosomes in macrophages.[10] We found that niacin acts as a substrate in the reaction of NAADP production via the CD38 enzyme, which facilitates the increased production of NAADP due to upregulation of CD38 in oxLDL-containing macrophages. Our confocal microscopy images illustrate that niacin

reduces free cholesterol accumulation in lysosomes in a concentration-dependent manner. However, the mechanism by which niacin modulates the expression of CD38 remains to be elucidated.

Conclusions

Recent studies have suggested the importance of accumulation of free cholesterol, which plays a critical role in initiating and maintaining the foamy morphology of macrophages during atherosclerosis. Our study suggests that niacin may promote the efflux of lysosomal free cholesterol from macrophages via the CD38/NAADP signaling pathway in $LDLr^{-/-}$ mice. These results indicate that niacin has a beneficial effect in reducing plaque lesions in atherosclerosis. These findings will advance our knowledge of the use of niacin in the prevention and treatment of cardiovascular diseases.

AUTHORS' CONTRIBUTIONS

SY conducted the experiments and wrote the original draft. FZ designed and interpreted the study. QL analyzed the data. QL supervised the study and reviewed the manuscript. All authors reviewed and approved the manuscript.

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DECLARATION OF CONFLICTING INTERESTS

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