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CD36-Dependent 7-Ketocholesterol Accumulation in Macrophages Mediates Progression of Atherosclerosis in Response to Chronic Air Pollution Exposure

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Abstract

Rationale—Air pollution exposure has been shown to potentiate plaque progression in humans and animals. Our previous studies have suggested a role for oxidized lipids in mediating adverse vascular effect of air pollution. However, the types of oxidized lipids formed in response to air pollutants and how this occurs and their relevance to atherosclerosis is not fully understood.

Objective—To investigate the mechanisms by which particulate matter< 2.5μm ($PM_{2.5}$) induces progression of atherosclerosis.

Methods and Results—Atherosclerosis-prone $ApoE^{-/-}$ or $LDLR^{-/-}$ mice were exposed to filtered air or concentrated ambient $PM_{2.5}$ using a versatile aerosol concentrator enrichment system for 6 months. $PM_{2.5}$ increased 7-ketocholesterol (7-KCh), an oxidatively modified form of cholesterol, in plasma IDL/LDL fraction and in aortic plaque concomitant with progression of atherosclerosis and increased CD36 expression in plaque-macrophages from $PM_{2.5}$-exposed mice. Macrophages isolated from $PM_{2.5}$-exposed mice displayed increased uptake of oxidized lipids without alterations in their efflux capacity. Consistent with these finding, CD36-positive macrophages displayed a heightened capacity for oxidized lipid uptake. Deficiency of CD36 on

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hematopoietic cells diminished the effect of air pollution on 7-KCh accumulation, foam cell formation, and atherosclerosis.

Conclusions—Our results suggest a potential role for CD36-mediated abnormal accumulations of oxidized lipids such as 7-KCh in air pollution induced atherosclerosis progression.

Keywords

Air pollution; 7-ketocholesterol; particulate matter; atherosclerosis; CD36; lipid metabolites; foam cells; macrophage

INTRODUCTION

Both epidemiologic and empirical evidence suggest an important effect of inhaled particular matter (<2.5μm, $PM_{2.5}$) in mediating cardiovascular events.[1, 2] The impact of air pollution on global cardiovascular disease and daily mortality is significant and substantial,[1, 2] given its pervasive presence and ubiquitous exposure, with recent data suggesting that >17% of global adult mortality is directly attributable to anthropogenic components of $PM_{2.5}$, primarily through ischemic heart disease events.[3] In 2005, 89% of the world's population lived in areas where the WHO Air Quality Guideline of 10 μg/m^3 $PM_{2.5}$ (annual average) was exceeded.[4] We and others have previously demonstrated that controlled exposure to air pollution results in potentiation of atherosclerosis in genetically susceptible models of atherosclerosis, which have been corroborated in human studies using surrogate measures such as coronary artery calcium and carotid intima media thickness.[2, 5, 6] We have also previously shown that chronic exposure to $PM_{2.5}$ increases generation of derivatives of oxidized phospholipids with resultant mobilization of mononuclear cells from bone marrow to the circulation, lungs, and the vascular wall via a toll-like receptor 4 (TLR4) mechanism, providing a direct pathway by which air pollution may potentiate atherosclerosis.[7] However, important questions remain, including the role of other oxidation such as oxidatively modified forms of cholesterol which are fundamental to atherosclerosis lesion progression.[8, 9] In the present study, we found an increase of 7-ketocholesterol (7-KCh), a major component of oxysterols, in the intermediate density lipoproteins (IDL) and low density lipoprotein (LDL) fraction of plasma lipoproteins and within aortic plaque. In addition, air pollution enhanced CD36-mediated uptake of 7-KCh by macrophages. Disruption of CD36 in hematopoietic cells abolished air pollution-induced 7-KCh accumulation in aortic wall and foam cell formation. Our study thus provides new insights into adverse cardiovascular effects of air pollution exposure by linking air pollution mediated lipoprotein oxidation with CD36 mediated abnormal deposition of oxidized lipids in atherosclerotic plaque and vascular dysfunction.

METHODS

For detailed methods please refer to supplementary section.

Animal studies and exposure protocol

Eight-week-old Apolipoprotein $E^{-/-}$ ($ApoE^{-/-}$) or $LDLR^{-/-}$ male mice were purchased from Jackson Laboratory (Bar Harbor, ME) and housed at constant temperature (22 ± 2°C) on a

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12-h light/dark cycle. The mice were maintained on regular chow (catalog #862, Harlan, Haslett, MI). Assignments to $PM_{2.5}$ group vs. filtered air (FA) group were randomized. The animal exposure and the monitoring of the exposure environment and ambient aerosol were performed. Exposure to concentrated ambient $PM_{2.5}$ was performed at the Ohio Air Pollution Exposure System for Interrogation of Systemic Effects (OASIS-1) using a versatile aerosol concentrator and exposure system (VACES) as previously described.[6, 10, 11] In this system, ultrafine (PM < 0.1 μm) particles are not excluded. We have previously demonstrated that atherosclerosis progression enhanced in $ApoE^{-/-}$ mice fed high-fat chow and concomitantly exposed to $PM_{2.5}$ in Tuxedo, NY.[6] However, since the biological effects of $PM_{2.5}$ may differ based on concomitant diet and composition and size of particulate matter (which in turn is dependent on emission sources), we designed our experiments with a different exposure environment (Columbus, OH regional) and in the context of normal diet conditions[12]. Throughout this manuscript, concentrated ambient $PM_{2.5}$ exposure is referred to as $PM_{2.5}$ unless specified otherwise.

$PM_{2.5}$ concentration and composition analysis in the exposure chamber

The overall mean ambient $PM_{2.5}$ level during the exposure periods was 9.1 ± 7.3 $\mu g/m^3$. Mean exposure enrichment over ambient levels in the chamber was ≈10-fold higher. Particles from the exposures were collected on Teflon filters (Gelman Teflo, 37 mm, 0.2-mm pore, Gelman Sciences, Ann Arbor, MI). Filters were weighed before and after collecting samples. Analysis of $PM_{2.5}$ composition was performed as previously described.[11]

7-KCh and cholesterol levels

Liquid chromatography/mass spectrometry was used to determine the level of 7-KCh and cholesterol in the serum and tissues as described previously.[13, 14] Lipoproteins were isolated by fast protein liquid chromatography. 7-KCh and cholesterol level in lipoprotein fraction were determined by Liquid chromatography/mass spectrometry.

Bone marrow transplantation

Bone marrow transplantation was performed as previously described.[15] Briefly, $LDLR^{-/-}$ mice were irradiated at the dose of 950 Rad. Mice were i.v. injected with 15×10^6 bone marrow cells from C57BL/6 or $CD36^{-/-}$ mice 24 h after irradiation.

Data analyses

All the data in the manuscript are expressed as mean ± SEM unless otherwise specified (Supplemental Table I). Difference between two groups was tested by student's t test. Differences among groups were tested by two-way ANOVA and Bonferroni's post hoc test using Graphpad Prizm 5.01 software. P values of < 0.05 were considered statistically significant.

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RESULTS

$PM_{2.5}$ concentrations during the study period

ApoE$^{-/-}$ mice were exposed to filtered air or concentrated $PM_{2.5}$ for a total duration of 6 months. The daily ambient air $PM_{2.5}$ concentration (mean ± SD) during exposure hours was 9.1 ± 7.3 μg/m^3. The concentration of $PM_{2.5}$ in the exposure chamber during exposure hours was approximately 11 fold higher than the corresponding ambient level (101.6 ± 30.7 μg/m^3). The mean elemental composition of exposure, as measured by energy-dispersive X-ray fluorescence (ED-XRF) analysis, is presented in Supplemental Table I.

Atherosclerosis burden with $PM_{2.5}$.

After exposure with filtered air (FA) or $PM_{2.5}$ for 6 months, ApoE$^{-/-}$ mice were euthanized and atherosclerotic plaque burden was quantified at the level of the aortic sinus. Starting at the level of the aortic valve leaflet, serial 5μm sections, covering a total length of 600 μm were stained with hematoxylin and eosin (H&E) to evaluate the severity of atherosclerosis. $PM_{2.5}$ exposure significantly increased lesion area, lipid and collagen content of atherosclerotic plaque (Fig. 1A-D). The plaque distribution and lipid content in the aorta is depicted from representative FA and $PM_{2.5}$ exposed animals stained with Oil-Red-O (Fig. 1E).

7-KCh levels in mice exposed to $PM_{2.5}$.

A preponderance of lipids present in plaques is composed of oxidized cholesterol derivatives (oxysterols).[16] 7-Ketocholesterol (7-KCh), a major component of oxysterols in advanced plaque, has been postulated to play an important role in endothelial dysfunction and atherogenesis.[17-20] We therefore evaluated levels of 7-KCh in the tissues of exposed mice. No significant difference of 7-KCh was detected in liver and plasma between $PM_{2.5}$-exposed and FA-exposed group (Supplemental Fig. I). The level of 7-KCh was ≈7-fold higher in the aorta, while levels in the lung did not differ in response to $PM_{2.5}$ exposure (8.58 ± 2.08 vs. 1.25 ± 0.45 μg/g tissue, $p<0.05$, n=5 in aorta; 4.55 ± 0.28 vs. 4.98 ± 0.35 μg/g lung tissue in $PM_{2.5}$ vs. FA, $p>0.05$, n=5 in lung, Fig. 2A & B). We also assessed the amount of cholesterol (CHL) in the same tissues. We failed to detect differences in CHL content between $PM_{2.5}$ and FA exposure group in either the aorta or lung (Fig. 2C & D). When adjusted for levels of cholesterol, the ratio of 7-KCh/CHL increased ≈10- fold in the aorta but not in the lung of $PM_{2.5}$ group (Fig. 2E & F). As 7-KCh has been demonstrated to accumulate in the plasma lipoproteins, with this representing a facile pathway of delivery to the arterial vessel wall, we quantified 7-KCh level in the lipoprotein fraction. As depicted in Fig. 2G, plasma very low density lipoprotein (VLDL) and IDL/LDL fractions contained the bulk of 7-KCh, with these levels being confined to the IDL/LDL fraction once the cholesterol content of the lipoprotein fractions were adjusted for. The 7-KCh/ CHL in plasma LDL was ≈2-fold higher in $PM_{2.5}$-exposed mice (Fig. 2G & H).

Effect of $PM_{2.5}$ on 7-KCh and cholesterol uptake and efflux

The transport and uptake of oxysterols into macrophages and endothelial cells results in progressive accumulation of lipids in the arterial wall in atherosclerosis.[21] To test the impact

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of $PM_{2.5}$ exposure on macrophage mediated sterol accumulation, we used peritoneal macrophages derived from $PM_{2.5}$/FA exposed mice. Prior studies have demonstrated that these cells are a reasonable surrogate for plaque macrophages and can be isolated in large enough quantities for our assays.[22, 23] As shown in Fig. 3A, the uptake of 7-KCh by peritoneal exudate macrophages (PEM) derived from $PM_{2.5}$-exposed mice was higher than that of FA-exposed mice (9.4 ± 1.19% vs. 8.18 ± 0.76%, $p<0.05$; n=6, Fig. 3A). To additionally validate these results, we also used bone marrow derived macrophages (BMDM) to investigate the effect of collected $PM_{2.5}$ particles on cholesterol/oxysterol transport. After overnight treatment with $PM_{2.5}$, the ability of 7-KCh uptake by BMDM increased (Supplemental Fig. II). In contrast, no significant difference in 7-KCh uptake by cultured endothelial cells (HUVECs) was detected between $PM_{2.5}$-treated and vehicle-treated group (Supplemental Fig. III-A). These results suggest that $PM_{2.5}$ exposure promotes uptake of 7-KCh selectively in macrophages but not in endothelial cells. Unmodified cholesterol uptake by PEM was not affected by $PM_{2.5}$ treatment (Fig. 4B). Pretreatment with 7-KCh did not increase the cholesterol uptake by PEM (Fig. 3B)

In addition to uptake, efflux is another process that affects the accumulation of oxysterols in the cell. $PM_{2.5}$ did not impact the efflux of 7-KCh in PEM derived from mice exposed to $PM_{2.5}$ (Fig. 3C). Efflux of cholesterol in PEM was also not influenced by the treatment of $PM_{2.5}$ (Fig. 3D). However, pretreatment of 7-KCh reduced the cholesterol efflux in PEM (Fig. 3D).

$PM_{2.5}$ promotes CD36-mediated 7-KCh uptake by macrophages

Scavenger receptors such as CD36 have been reported to mediate oxLDL internalization in macrophages, and have been implicated in the pathogenesis of atherosclerosis.[24, 25] To verify the involvement of CD36 in air pollution-induced 7-KCh accumulation, we evaluated the total expression of CD36 on circulating monocytes after $PM_{2.5}$ exposure for 12 weeks. $PM_{2.5}$ exposure doubled CD36 expression on CD11b+ monocytes (Figure 4A). To assess the expression of CD36 on plaque-infiltrating macrophage, aortic sinus sections of FA- and PM -exposed $ApoE^{-/-}$ 2.5 mice were stained with F4/80 and CD36. Most $F4/80^{+}$ macrophages infiltrating the plaque expressed CD36, with an increased number of CD36-expressing macrophages observed in the plaque of $PM_{2.5}$-exposed mice (Figure 4B & C).

Since CD36 expression was increased in the monocytes and macrophages of $PM_{2.5}$-exposed mice, we investigated whether $PM_{2.5}$ facilitates 7-KCh uptake through a CD36 dependent mechanism. First, we tested the association between CD36 expression and 7-KCh uptake. Bone marrow derived macrophages (BMDMs) of WT mice were incubated with 7-KCh-loaded LDL (labeled with fluorescent dye, BODIPY) for 8 h and subjected to flow cytometric analysis of CD36 expression and 7-KCh-LDL uptake after extensive washes. CD36-possitive BMDMs were more efficient in taking up 7-KCh-LDL compared to CD36-negative BMDMs (Fig. 4D). Next, we investigated whether $PM_{2.5}$ could promote intracellular translocation of CD36. We detected cell surface and total CD36 expression on BMDMs after *in-vitro* treatment with $PM_{2.5}$. Surface expression of CD36 on macrophages decreased after in-vitro treatment with $PM_{2.5}$ (50 μg/ml) for 24 hours (Supplemental Fig. IV-A). Further, we detected total expression of CD36 by staining for CD36 after

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permeabilization. There was a small but statistically significant increase in total CD36 (Supplemental Fig. IV-B), suggesting that the decrease of surface CD36 is caused by internalization rather than suppression of synthesis of CD36. To exclude the non-specific effect of lipopolysaccharide (LPS) that is usually found in PM particles, cells were incubated with LPS inhibitor polymyxin (25 μg/ml) and $PM_{2.5}$ particles (50 μg/ml) together. Blockade of LPS with polymyxin did not reverse the effect of PM particles on CD36 internalization (Supplemental Fig. V). In contrast, no difference in the expression of SR-A (scavenger receptor class A, also known as CD204) was observed between $PM_{2.5}$- and vehicle-treated cells (Supplemental Fig. IV-C & -D). In line with this, non-specific phagocytosis induced by microsphere (1 μm latex beads) did not affect 7-KCh uptake (Supplemental Fig. VI).

Loss of CD36 attenuates air pollution effects on atherosclerosis

To demonstrate the role of CD36 in mediating the effects of $PM_{2.5}$ *in vivo*, $LDLR^{-/-}$ mice were transplanted with bone marrow cells isolated from WT or $CD36^{-/-}$ mice followed by 3-month exposure to $PM_{2.5}$. $PM_{2.5}$ exposure promoted atherosclerotic plaque development in $LDLR^{-/-}$ mice transplanted with WT bone marrows while deficiency of CD36 on hematopoietic cells reduced plaque volume in response to $PM_{2.5}$ (Fig. 5A & B). Lipid deposition in response to $PM_{2.5}$ exposure was reduced in mice transplanted with $CD36^{-/-}$ bone marrows in comparison with mice transplanted with WT marrow (Fig. 5C & D).

Confocal analysis of aortic sinus sections suggested $PM_{2.5}$ exposure elevated 7-KCh level in the plaque in $LDLR^{-/-}$ mice transplanted with WT bone marrows compared with FA, while 7-KCh level in the plaque of $LDLR^{-/-}$ mice transplanted with $CD36^{-/-}$ bone marrows was comparable to mice transplanted with WT marrow and exposed to FA (Fig. 6A & B). Most macrophages were co-localized with 7-KCh and there were also a lot of 7-KCh single positive areas surrounding macrophages (Fig. 6A). To test whether the increased 7-KCh level was caused by increased plaque size, 7-KCh positive area was normalized by plaque area. As shown in Figure 6C, percentage of 7-KCh positive area increased in the plaque of $LDLR^{-/-}$ mice transplanted with WT bone marrows and exposed to $PM_{2.5}$ when compared to FA, but not in $LDLR^{-/-}$ mice with $CD36^{-/-}$ bone marrows, suggesting that $PM_{2.5}$ increased 7-KCh level independent of plaque lesion size and CD36 deficiency can blunt the effect of $PM_{2.5}$. Similar results were obtained when normalized with macrophage area ($F4/80^{+}$), indicating increase of 7-KCh in aortic plaque is not due to the increase of macrophage (Fig. 6D). To further confirm the role of CD36 in oxysterol uptake, PEMs isolated from FA- or $PM_{2.5}$-exposed $LDLR^{-/-}$ mice with WT or $CD36^{-/-}$ bone marrows were incubated with DiI-labeled LDL, oxLDL, or 7-KCh-loaded LDL for 8 h. Fluorescence intensity of the cells was measured by flow cytometry after extensive washes. As depicted in Fig. 6E, similar levels of LDL uptake were observed in all 4 groups. However, uptake of oxLDL or 7-KCh-LDL was enhanced by $PM_{2.5}$ exposure in WT macrophages and loss of CD36 reduced the uptake of oxLDL or 7-KCh-LDL by macrophages. The effect of air pollution on oxLDL or 7-KCh-LDL uptake was markedly diminished by CD36 deficiency (Fig. 6E).

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$PM_{2.5}$ increases foam cell formation, which is blunted by CD36 deficiency

Foam cell formation from macrophages with subsequent fatty streaks plays an important role in the pathogenesis of atherosclerosis. Modified LDL including oxidized LDL (oxLDL) or acetylated LDL promotes foam cell formation while LDL in its native form is not atherogenic.[26, 27] Likewise, there was a marked increase in foam cell formation when macrophages were incubated with 7-KCh-LDL compared with LDL (Fig. 7A & 7D). Dil-labeled 7-KCh-LDL was prepared by loading 7-KCh (2mM) to Dil-labeled LDL. Fluorescence intensity of macrophages incubated with Dil-labeled 7-KCh-LDL was significantly higher compared with that incubated with Dil-labeled LDL, suggesting 7-KCh-LDL rather than native LDL accumulates in macrophages and promotes foam cell formation (Fig. 7A & 7D). To determine the effect of $PM_{2.5}$ and CD36 in the foam cell formation, PEMs isolated from FA- or $PM_{2.5}$-exposed $LDLR^{-/-}$ mice with WT or $CD36^{-/-}$ bone marrows were incubated with 7-KCh-LDL for 36 h. $PM_{2.5}$ exposure promoted foam cell formation as evidenced by Oil Red O staining and confocal visualization of Dil-labeled 7-KCh-LDL. There was no difference in foam cell formation between FA and $PM_{2.5}$ in CD36 deficient macrophages suggesting that this effect of $PM_{2.5}$ in increasing 7-KCh uptake occurs via CD36 pathways (Fig. 7B, C, E & F).

DISCUSSION

In this study we provide evidence for a novel mechanism by which inhaled particulate matter may promote atherosclerosis. 7-KCh, an oxysterol extensively implicated in atherosclerosis, increased with chronic exposure to $PM_{2.5}$ and preferentially accumulated in the aortic plaque in two different hypercholesterolemic rodent models. 7-KCh was almost exclusively present in the IDL/LDL fraction of plasma lipoproteins and selectively accumulated in macrophages through a CD36 dependent process. Consistent with a role for CD36 in air pollution mediated effects, hematopoietic deficiency of CD36 exerted a protective effect on atherosclerosis, inflammation and lipid accumulation in response to air pollution. $CD36^{+}$ macrophages derived from $PM_{2.5}$ exposed animals preferentially accumulated 7-KCh without demonstrating abnormalities in cholesterol efflux pathways. Our results provide new insights into mechanisms underlying air pollution-induced atherosclerosis progression by linking air pollution with vascular lipid dysregulation.

Multiple studies have confirmed an important pro-atherogenic effect of diesel exhaust particles, $PM_{2.5}$, and ultrafine components of air pollution.[5, 6, 10] Limited human cross-sectional studies have demonstrated higher carotid intima-media thickness (CIMT) and coronary artery calcium scores in patients exposed to higher levels of air pollution.[28, 29] Abnormalities in endothelial function have also been described in both acute and chronic exposure studies (primarily in animal models).[30, 31] We and others have demonstrated increased lipid accumulation within plaques of animals exposed to air pollution together with macrophage accumulation.[6, 32-34] Increased expression of oxidative stress markers such as iNOS, nitrotyrosine and CD36 have been previously demonstrated in plaques after both $PM_{2.5}$ and diesel exhaust exposure.[34, 35] While the pro-atherogenic effects of $PM_{2.5}$ in animal models are consistent with the epidemiologic evidence linking $PM_{2.5}$ with atherosclerotic events such as acute myocardial infarction and stroke, the precise pathways

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that sustain progression of plaque with exposure resulting in unstable coronary syndromes are poorly understood.[2] In atherosclerosis, a critical step is recognition and internalization of oxidized LDL (oxLDL) by specific macrophage scavenger receptors (with CD36 accounting for 60-70% of oxLDL uptake).[36] CD36 has been shown to mediate oxLDL internalization in macrophages, with its expression correlating with exposure measures such as alveolar macrophages positive for inhaled particles [34]. We therefore investigated this pathway and the mechanisms that may lead to CD36 mediated foam cell formation in response to $PM_{2.5}$ exposure. CD36 expression was increased in plaque macrophages from $PM_{2.5}$ exposed animals. In our studies, we found a marked increase in 7-KCh in the aorta of exposed hyperlipidemic animals with co-localization primarily in plaque macrophages. Interestingly, nonspecific phagocytosis mediated by in-vitro exposure to microspheres did not enhance 7-KCh uptake. $PM_{2.5}$ exposure did enhance the uptake of 7-KCh. These results suggest that $PM_{2.5}$ promotes the accumulation of 7-KCh by enhancing CD36-mediated internalization rather than non-specific phagocytosis. Consistent with our findings, other air pollutants have also been shown to increase CD36 expression. Bai et al. reported a 1.5 to 2-fold increase of CD36 in plaque, accompanying with 1.5 to 3-fold increase of lipid content after exposure to diesel exhaust.[34] CD36 expression was correlated positively with increased entry of monocytes into atherosclerotic plaques.[34] Robertson et al have suggested that CD36 mediates the vasoconstrictor effect of circulating vasoactive factors released in response to ozone exposure.[37] However, our study does not exclude other scavenger receptors such as LOX-1, CD68, and SR-BI that may also be involved in enhanced atherogenesis. Exposure to engine emissions increased lectin-like oxLDL receptor (LOX-1), plasma oxLDL, and vascular infiltration of monocyte/macrophage in $ApoE^{-/-}$ mice, which were attenuated by the blockade of LOX-1 using neutralizing anti-LOX-1 Ab.[38] These findings raise the possibility that air pollutants such as $PM_{2.5}$ as well as gaseous components of vehicle emission may also influence atherogenesis through similar mechanisms (scavenger receptors-mediated dysregulation of oxidized lipids).

We have previously demonstrated that chronic exposure to $PM_{2.5}$ increased the generation of oxidized phospholipid derivatives of 1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine (oxPAPC) such as 1-palmitoyl-2-(5-oxovaleroyl)-sn-glycero-3-phosphocholine (POVPC) and 1-palmitoyl-2-glutaroyl-sn-glycero-3-phosphocholine (PGPC) in the lungs.[7] The generation of oxPAPC resulted in activation of NF B and pro-inflammatory genes including chemokines such as chemokine (CC motif) ligand 2 (CCL2) through a TLR4/NADPH oxidase dependent pathway.[7] Mice deficient in NADPH oxidase 2 (NOX2) and TLR4 demonstrated reduced vascular inflammation and improved vascular dysfunction seen with $PM_{2.5}$ exposure.[7] TLR4 deficiency also abolished circulating increase of $Ly6C^{hi}$ monocytes in response to long term $PM_{2.5}$ exposure, providing a cellular mechanism through which exposure may facilitate inflammation in atherosclerosis.[7] We have postulated previously that since PAPC is abundantly present in tissues such as the lung (dominant phospholipid in the surfactant fluid of lung), the generation of oxidized forms of PAPC could easily occur with air pollution exposure resulting in TLR4 activation and transcription of pro-inflammatory genes and chemokines. The role of oxidation products other than oxPAPC that may arise with air pollution exposure such as oxidatively modified forms of cholesterol has not been investigated thus far. Since air pollution exposure serves

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as a potent redox trigger, facilitating the generation of a range of oxidatively modified products, it is entirely possible that other oxidation products of cholesterol are also generated. In atherosclerosis, lesion formation and progression is fundamentally dependent on the generation of oxidized forms of cholesterol, which are rapidly taken up by macrophages in lesions via scavenger receptor dependent and independent pathways, resulting in lipid accumulation within the vessel wall. 7-KCh is a major oxidation product present in oxLDL and within atherosclerotic plaque.[16, 17] In current study, we detected elevated levels of 7-KCh in plasma lipoproteins (IDL/LDL) after chronic exposure to airpollution. 7-KCh but not CHL increased in the LDL/IDL fraction of plasma lipoproteins of $PM_{2.5}$-exposed mice, raising the possibility that elevated 7-KCh in the aorta may result from increased accumulation, through delivery of ApoB lipoproteins enriched in 7-KCh. Consistent with these findings, deficiency of CD36 markedly decreased the internalization of oxidized LDL and 7-KCh-loaded LDL but had no effect on the uptake of native LDL. Macrophages with low expression of CD36 had a significantly lower ability to uptake oxidized and 7-KCh-loaded LDL compared with macrophages with high expression of CD36. Furthermore, *ex vivo* macrophages uptake of 7-KCh-loaded LDL or oxLDL as well as foam cell formation were enhanced by $PM_{2.5}$ exposure, results that were partially abolished by CD36 deficiency. We further demonstrated in carefully performed in-vivo experiments that bone marrow deficiency of CD36 attenuates $PM_{2.5}$ mediated effects on atherosclerotic plaque and lipid accumulation. F4/80+ macrophages in plaque co-localized with 7-KCh within the plaque with abundant 7-KCh surrounding macrophage, suggesting release of 7-KCh presumably from apoptotic macrophages containing the oxysterol. CD36 deficiency in bone marrow derived cells also reduced atherosclerotic lesion in FA-exposed mice. This result is in accordance with previous reports.[36, 39, 40] Although Moore et al reported that CD36 deficiency in $ApoE^{-/-}$ background did not affect atherosclerotic progression in their work[41], subsequent studies including those from Sheedy et al suggest CD36 deletion did reduce atherosclerotic plaque in $ApoE^{-/-}$ mice.[40, 42, 43] The differences noted in these studies may be attributable to the use of two different mouse strains by the groups as pointed out previously.[44] Our result that $LDLR^{-/-}$ mice with CD36-null bone marrow were protected from atherosclerosis is indeed consistent with another previous report that transplantation of CD36-null bone marrow reduces atherosclerotic lesion formation.[39]

The finding in the current investigation that CD36 mediates air pollution induced 7-KCh accumulation and atherosclerosis progression provides complementary mechanisms to our prior studies linking TLR4 to abnormal vascular effects and cellular inflammation associated with air pollution exposure.[7] Oxidized phospholipids such as ox-PAPC may result in pro-inflammatory effects through NF B mechanisms that may synergistically interact with accumulation of lipids such as 7-KCh in macrophages to accelerate atherogenesis. Based on our findings of increased 7-KCh in lipoproteins such as VLDL and LDL/IDL and lack of increase in lung derived 7-KCh, it is likely that 7-KCh is continually formed in the vascular compartment during air pollution exposure particularly on the surface of cholesterol loaded lipoproteins such as LDL and IDL. We posit that the continual exposure of LDL to air pollution particularly at the alveolar-capillary membrane, likely plays a role in this process. LDL/IDL containing oxidized lipids such as 7-KCh and oxPAPC in response to air pollution

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may be delivered to the atherosclerotic aorta expressing scavenger receptors and CD36. A recent study suggested CD36 may also activate TLR4 and induces sterile inflammation by forming a TLR4/TLR6 heterodimer, thus providing additional pathways by which TLR4 may interact with pathways involving CD36.[45] In the current study we did not measure oxPAPC in LDL fraction although prior studies have demonstrated increased oxPAPC in circulating lipoproteins including LDL and HDL.[46, 47] Although oxPAPC may not be the major component of oxLDL or oxidized lipids within plaque, it certainly may represent an additional oxidation product that is delivered via scavenger receptors and may accumulate within atherosclerosis. Additionally the presence of oxPAPC may result in pro-inflammatory effects within the vascular wall.[48] The relative contribution of 7-KCh versus other oxidized lipids in air pollution mediated atherosclerosis progression will require additional studies.

Mass transfer of 7-KCh can also occur in the opposite direction from within the cell to a wide range of phospholipid-containing acceptors, including high density lipoprotein (HDL).[49] Recently, ATP-binding cassette sub-family G member 1 (ABCG1) has been suggested to promote the export of 7-KCh and 7β-hydroxycholesterol (7β-HC) to HDL, and to exert cytoprotective effects.[17, 50] In our study, efflux assays performed with both 7-KCh and CHL demonstrated that pre-treatment with $PM_{2.5}$ does not influence 7-KCh and CHL efflux in either macrophages or endothelial cells. Interestingly, we observed increased CHL uptake in endothelial cells and a decrease of CHL efflux in macrophages when cells were pre-treated with 7-KCh. These findings suggest a feed-forward effect on lipid accumulation following 7-KCh exposure/accumulation. These results are consistent with those of Gelissen and co-workers who demonstrated that the presence of 7-KCh suppresses the export of CHL from macrophage to Apolipoprotein A-I acceptor.[51] Additionally, studies have suggested that 7-KCh suppresses incorporation of CHL to plasma membrane and may thus reduce CHL efflux to cholesterol acceptors.[52-54]

Our study has a number of important limitations that must be acknowledged. Peritoneal macrophages from mice exposed to $PM_{2.5}$ were used to assess 7-KCh uptake in plaque macrophage in light of the large amount of cells to perform the assays. Prior studies however have demonstrated that these cells are however reasonable surrogates for plaque macrophages.[22, 23] We believe that despite this limitation, these studies help provide a framework of understanding of how exposure to $PM_{2.5}$ may modulate uptake of 7-KCh. Accumulation of 7-KCh in plaque depends importantly on circulating levels of 7-KCh in lipoproteins, 7-KCh uptake and retention of 7-KCh in the sites of lesion formation. In the present study, we have not provided insights on to how 7-KCh accumulates selectively in lipoproteins, nor have we provided information on the precise locus of 7-KCh formation in LDL. We postulate that plasma lipoproteins may undergo oxidation at the alveolar-capillary membrane interface in response to repetitive $PM_{2.5}$ exposure. This may potentially explain lack of parenchymal lung accumulation of 7-KCh as was noted in the study.

In summary our findings provide a novel mechanism by which air pollution may enhance the development of atherosclerosis via linking CD36-mediated abnormal accumulation of oxidized lipid in vasculature with air pollution induced lipid oxidation. Further studies are required to determine which components of $PM_{2.5}$ are required for lipid oxidation, other

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receptors involved in the deposition of oxidized lipid in vascular wall, and the precise compartment where 7-KCh is generated.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

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Nonstandard Abbreviations and Acronyms

7-KCh	7-ketocholesterol
7β-HC	7β-hydroxycholesterol
ABCG1	ATP-binding cassette sub-family G member 1
BMDM	bone marrow derived macrophage
CHL	cholesterol
CIMT	carotid intima-media thickness
ED-XRF	energy-dispersive X-ray fluorescence
FA	filtered air
H&E	hematoxylin and eosin
HDL	high density lipoprotein
IDL	intermediate density lipoprotein
LDL	low density lipoprotein
LPS	lipopolysaccharide
OASIS	Ohio Aerosol Exposure System for Interrogation of Systemic Effects
oxLDL	oxdized LDL
PAPC	1-palmitoyl-2-arachidonyl-sn-glycero-3-phosphorylcholine
PEM	peritoneal exudate macrophage
PM	particulate matter
SR-A	scavenger receptor class A
TLR4	Toll-like receptor 4
VACES	Versatile Aerosol Concentrator Enrichment System

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VLDL very low density lipoproteins

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Novelty and Significance

What Is Known?

- Exposure to air pollution contributes to cardiovascular morbidity and mortality.
- Oxidized lipids promote atherosclerosis.
- Formation of oxidized lipid in the lung is increased in animals exposed to air pollution.

What New Information Does This Article Contribute?

- In mice, exposure to air pollution increases accumulation of oxidized lipids such as 7-ketocholesterol in macrophages and the aortic wall.
- CD36 links air pollution and abnormal accumulation of oxidized lipid in macrophages and atherosclerotic lesion.
- Deficiency of CD36 reduces ability of macrophage to take up oxidized lipids and it diminishes the effect of air pollution on atherogenesis.

Both epidemiologic and empirical evidence suggest an adverse effect of air pollution on cardiovascular disease. However, the underlying mechanisms remain poorly understood. Previous studies have provided evidence of acceleration of atherosclerosis by air pollution in experimental models. Limited human studies using surrogate markers of subclinical cardiovascular disease such as coronary artery calcium and carotid intima media thickness support these findings. We asked whether air pollution contributes to the formation oxidized lipids in plasma lipoproteins and whether these are deposited in to the arterial wall. We found that the levels of 7-ketocholesterol were increased in plasma lipoproteins in response to air pollution exposure in atherosclerosis-prone mice. These results suggest that air pollution increases CD36-mediated accumulation of oxidized lipids in macrophages and the arterial wall and thereby, promotes atherogenesis. This study provides new insights into mechanisms underlying air pollution-induced atherosclerosis progression by linking air pollution with the formation of oxidized lipids.

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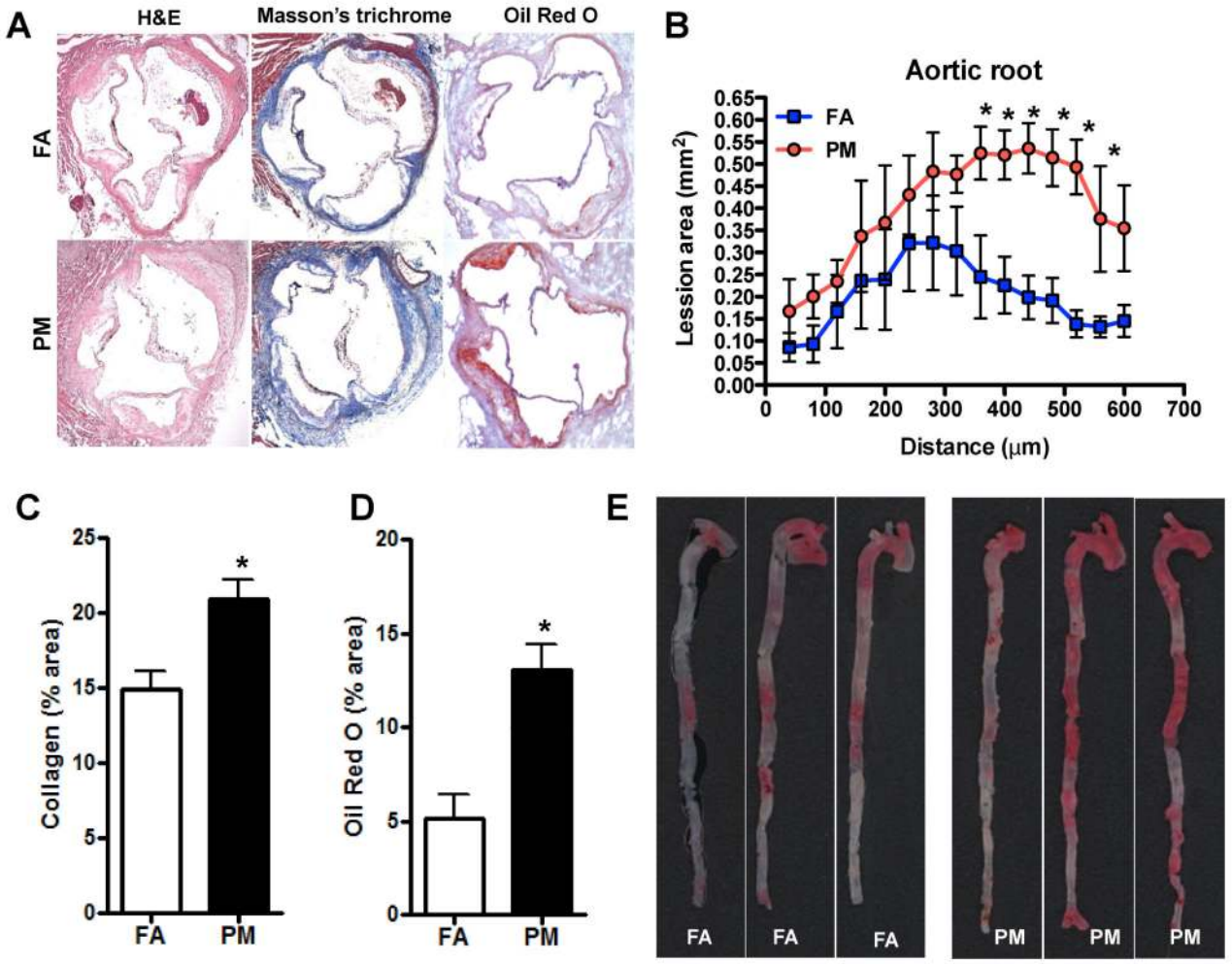

Figure 1. Plaque burden increased upon PM exposure

ApoE$^{-/-}$ **2.5** mice were exposed to FA or $PM_{2.5}$ for 6 months and plaque burden was evaluated. A, Representative images of H&E, Masson's trichrome and Oil-Red-O staining indicate plaque area, collagen and lipid content in the aortic sinus of FA- or $PM_{2.5}$-exposed ApoE$^{-/-}$ mice. B, Statistical analysis indicates the plaque area in the aortic root (600 μm sections starting from aortic valve leaflets) of FA- or $PM_{2.5}$-exposed mice. C, Bar graph shows collagen content in the aortic sinus of $PM_{2.5}$- or FA-exposed mice. D, Statistical analysis of Oil-Red-O staining indicates level of lipid content in the sinus of FA- or $PM_{2.5}$-exposed mice. E, Representative images show Oil-Red-O staining of plaque at the surface of the entire aorta. n=6. Results are shown as mean ± SEM. *, $P<0.05$.

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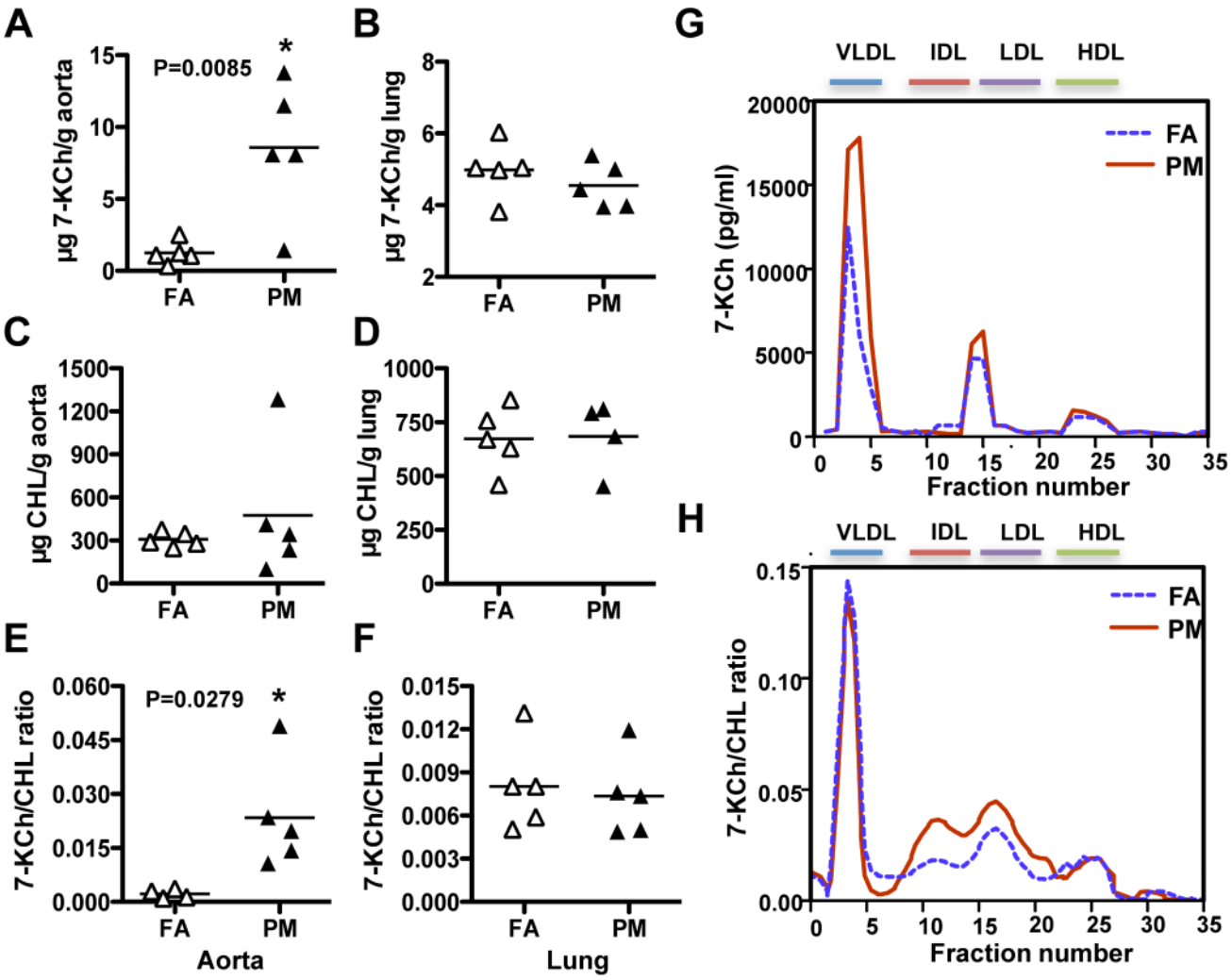

Figure 2. $PM_{2.5}$ exposure increased the accumulation of 7-ketocholesterol (7-KCh) but not cholesterol level in aortic wall

Aorta, lung and plasma were collected from mice exposed to $PM_{2.5}$ or FA. VLDL/LDL/IDL/HDL fractions were isolated from plasma by fast protein liquid chromatography (FPLC). 7-KCh and cholesterol were detected by liquid chromatography/ mass spectrometry (LC/MS). A, 7-KCh level in the aorta of $PM_{2.5}$-exposed mice. B, 7-KCh level in the lung of $PM_{2.5}$-exposed mice. C, Cholesterol (CHL) content in aorta in FA- and PM-exposed mice. D, Lung CHL level in FA and $PM_{2.5}$-exposed mice. E, Ratio of 7-KCh/CHL in the aorta of FA/ $PM_{2.5}$-exposed mice. F, Ratio of 7-KCh/CHL in the lung of FA/ $PM_{2.5}$-exposed mice. G, 7-KCh level increased in the VLDL/IDL/LDL fraction of $PM_{2.5}$-exposed mice. H, The ratio of 7-KCh/CHL in the LDL fraction of FA and $PM_{2.5}$-exposed mice. n=5/group. *, $P<0.05$.

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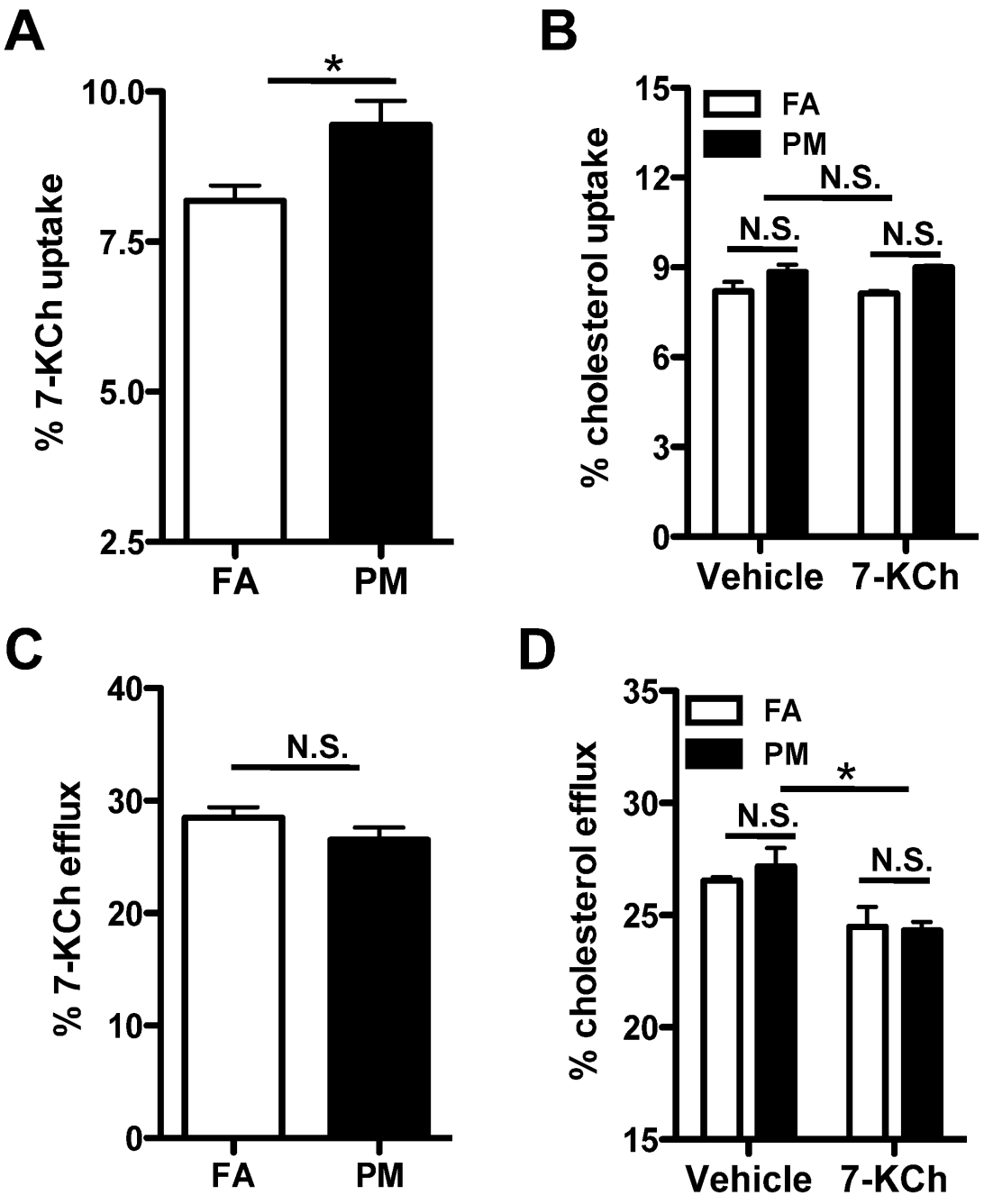

Figure 3. Effects of PM exposure on 7-KCh/CHL uptake and efflux

A, 7-KCh uptake in peritoneal exudate macrophages (PEMs): PEMs isolated from mice exposed to FA or $PM_{2.5}$ for 3 months were incubated with ^{3}H-labeled 7-ketocholesterol (1 μCi/ml) for 3 hours. Cells and medium were then harvested for the detection of radioactivity. B, Cholesterol uptake in PEMs: PEMs isolated from mice exposed to FA or PM for 3 months were incubated with H^{3}-labeled cholesterol (1 μCi/ml) for 3 hours with or without the presence of 7-KCh (20 ug/ml). Cells and medium were then harvested for the detection of radioactivity. C, 7-KCh efflux in PEMs: PEMs isolated from mice exposed to

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FA or $PM_{2.5}$ were incubated with ^{3}H-labeled 7-ketocholesterol (1 μCi/ml) for 6 hours. After washing out the remaining 7-ketocholesterol, cells were incubated with HDL (50 ug/ml) overnight. Cells and medium were then collected to detect 7-ketocholesterol. D, Cholesterol efflux in PEMs: PEMs isolated from mice exposed to FA or $PM_{2.5}$ were incubated with H^{3}-labeled cholesterol (1 μCi/ml) for 6 hours, followed by 1 hour incubation with 7-KCh or vehicle. After washing out the remaining H^{3}-labeled cholesterol and 7-KCh, cells were incubated with 50 ug/ml HDL overnight. Cells and medium were then collected to detect radioactivity. n=6. *, $P<0.05$.

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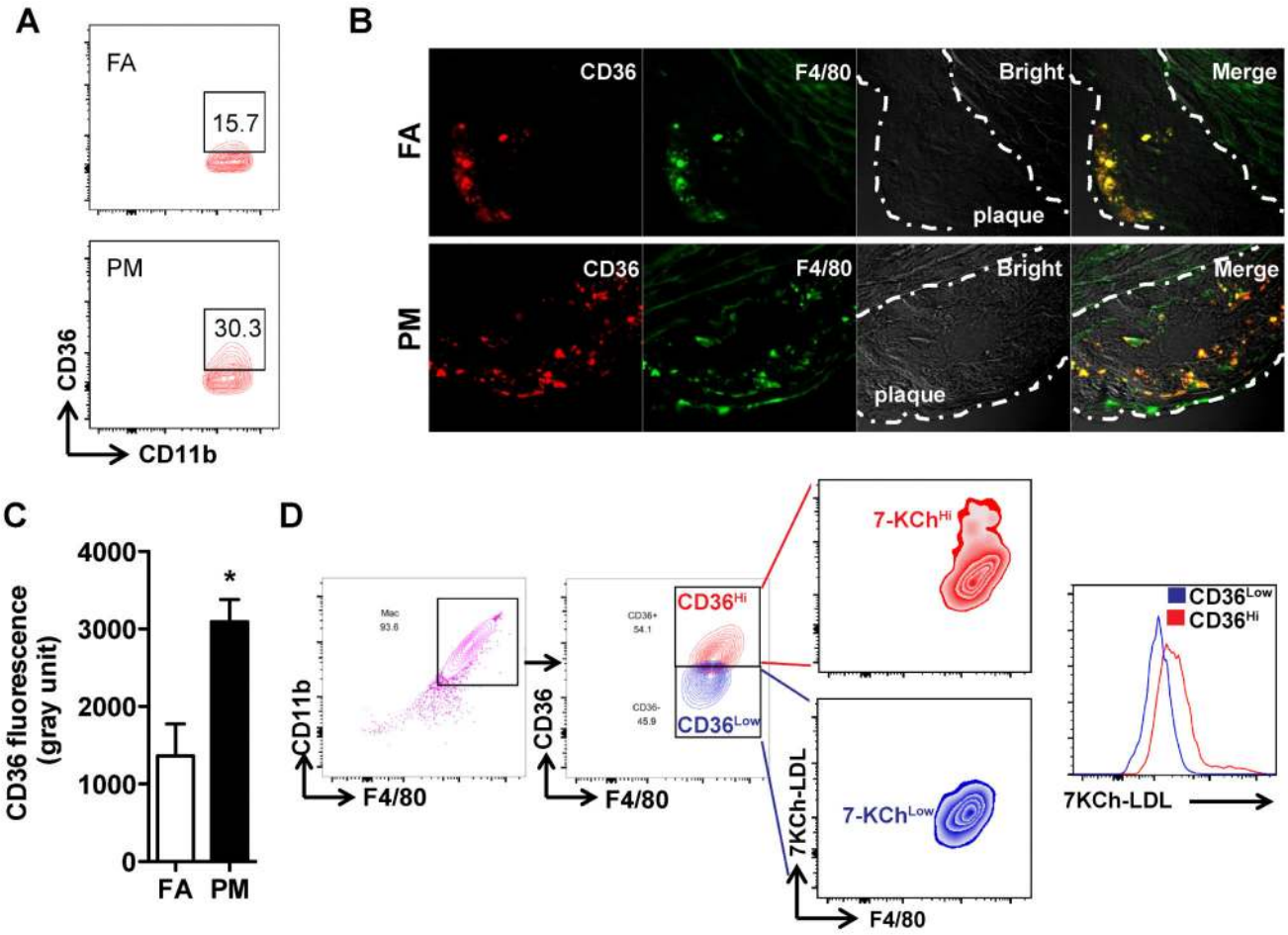

Figure 4. PM exposure enhances CD36-mediated uptake in macrophages

A, CD36 expression was analyzed on circulating monocytes which were gated based on expression of CD11b and F4/80. B & C, CD36-expressing macrophages in the plaque of $PM_{2.5}$-exposed mice: sinus sections of FA- or $PM_{2.5}$-exposed mice were used for the confocal detection of CD36 (Red) and F4/80 (Green). Representative images (B) and statistical analysis (C) were shown. D, BMDMs were incubated with bodipy-labeled 7-ketocholesterol LDL (7-KCh-LDL, 10 μg/ml) for 8 hours. Cells were then harvested and stained with PE-labeled anti-CD36, PE-Cy5-labeled CD11b, and APC-labeled F4/80, followed by analyzing on LSRII flow cytometer. CD36-positive and -negative macrophages were gated for the analysis of bodipy intensity.

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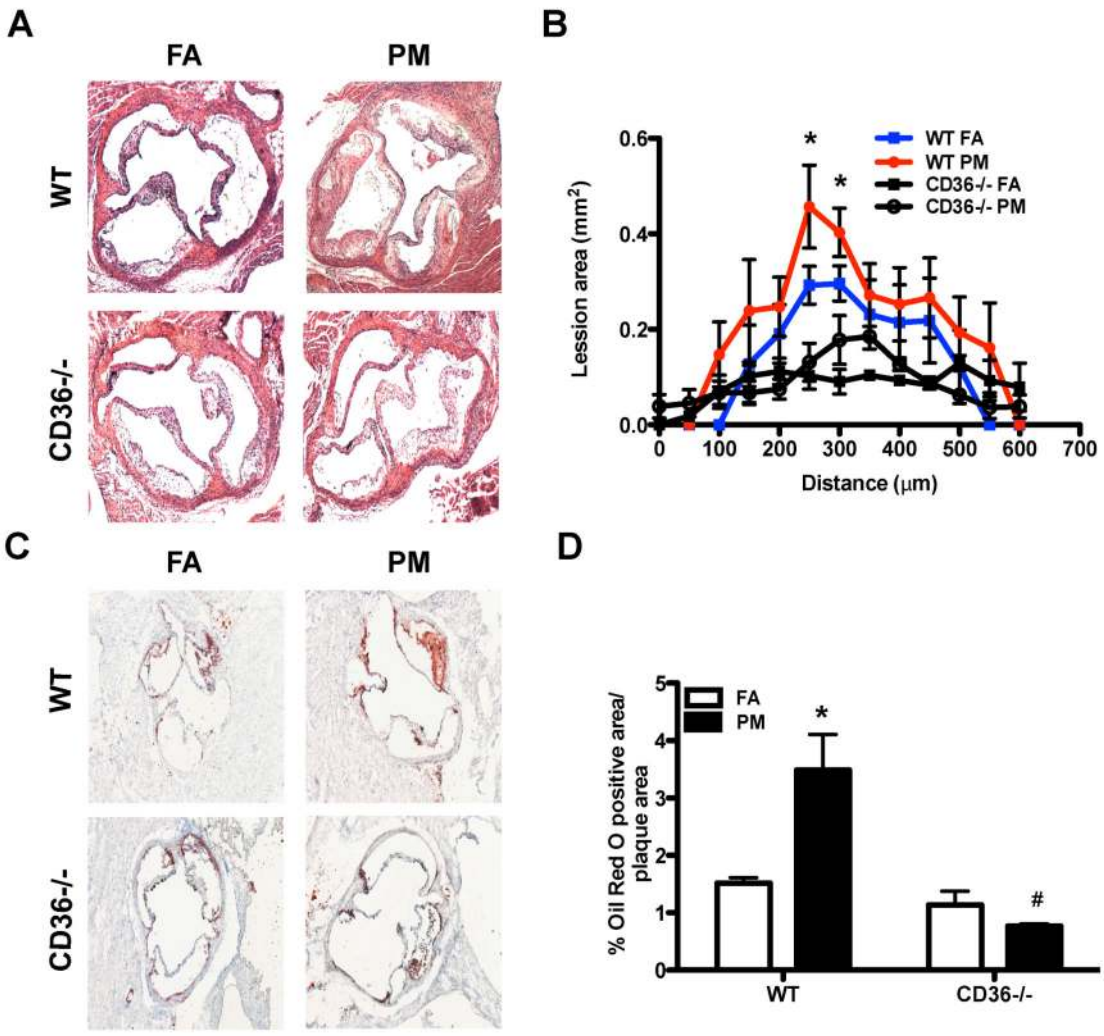

Figure 5. Loss of CD36 diminished promotive effect of air pollution on atherosclerosis

A, LDLR$^{-/-}$ mice transplanted with WT or CD36$^{-/-}$ bone marrows were exposed to FA or $PM_{2.5}$ for 3 months. Aortic sinus sections were used for the detection of plaque burden by H&E staining and lipid content detection by Oil-Red-O staining. A, representative image of H&E staining. B, statistical analysis of plaque volume. C, representative images of Oil-Red-O staining. D, statistical analysis of Oil-Red-O positive area. *, $P<0.05$ when compared with FA; #, $P<0.05$ when compared with WT.

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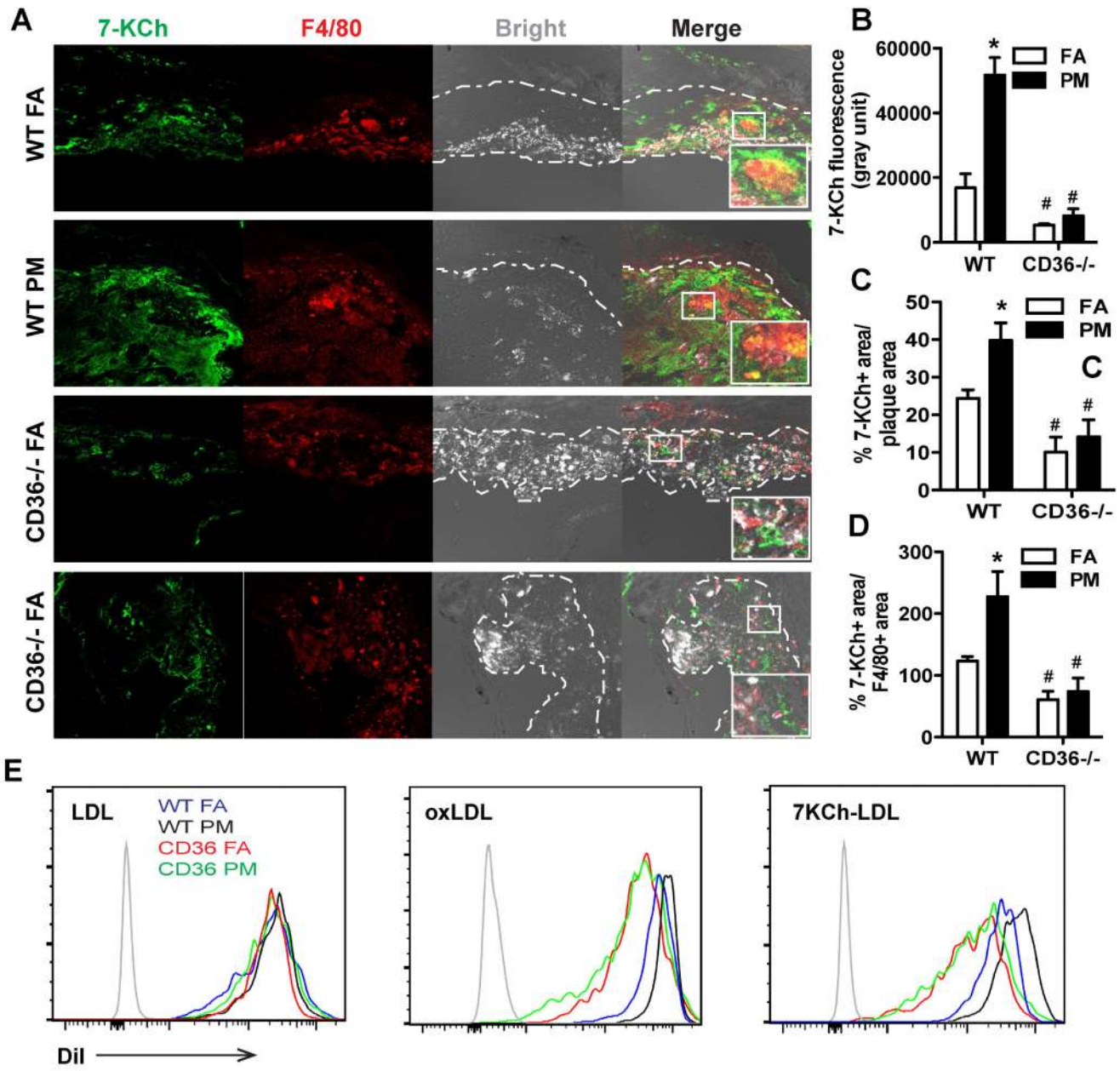

Figure 6. Loss of CD36 reduces $PM_{2.5}$-mediated uptake of oxysterol

A - D, Aortic sinus sections from FA- or $PM_{2.5}$-exposed $LDLR^{-/-}$ mice with WT or $CD36^{-/-}$ bone marrows were used for the detection of 7-KCh (Green) and macrophage (F4/80, Red). Representative images (A) and statistical analysis of 7-KCh area (B), 7-KCh/ Plaque ratio (C), and 7-KCh/F4/80 ration (D) were shown. E, PEMs isolated from FA- or $PM_{2.5}$-exposed $LDLR^{-/-}$ mice with WT or $CD36^{-/-}$ bone marrows were incubated with DiI-labeled LDL (10 μg/ml), oxLDL (10 μg/ml), or 7-KCh-LDL (10 μg/ml) for 8 hours. Cells were then harvested for the analysis of DiI intensity on LSRII flow cytometer. Flow histograms are shown. n=6. *, $P<0.05$ when compared with FA; #, $P<0.05$ when compared with WT.

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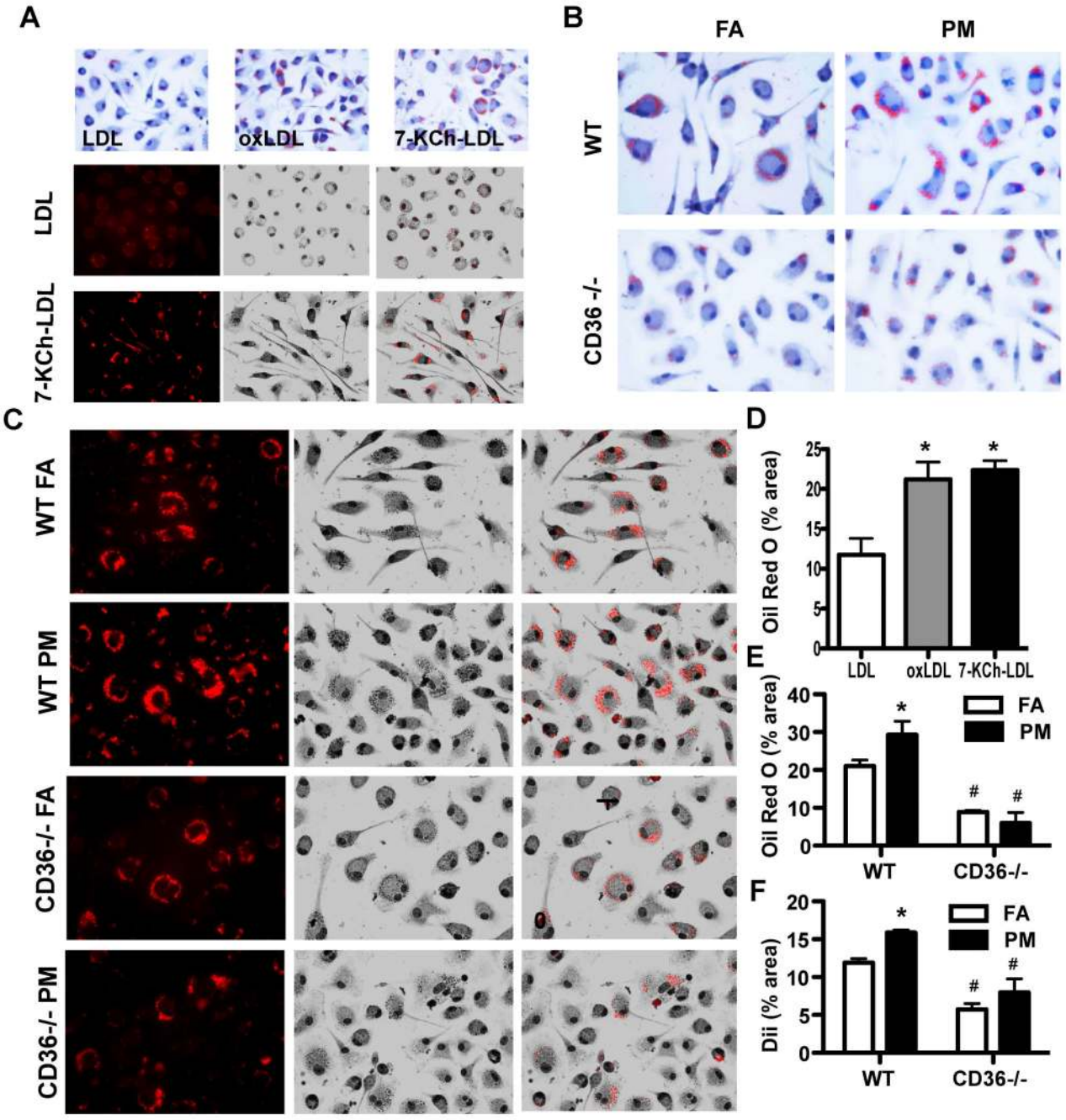

Figure 7. $PM_{2.5}$ increases foam cell formation in response to 7-ketocholesterol LDL (7-KCh-LDL) and CD36 siRNA transfection can blunt this effect

A, PEMs isolated from WT mice were incubated with Dil-labeled LDL (5 μg/ml), Dil-labeled oxLDL (5 μg/ml), or Dil-labeled 7-KCh-LDL (5 μg/ml) for 36 hours. Cells were then fixed with 4% paraformaldehyde and stained with Oil-red-O. Representative images of Oil-red-O staining and Dil fluorescence imaging were shown. B, PEMs isolated from FA- or $PM_{2.5}$-exposed $LDLR^{-/-}$ mice with WT or $CD36^{-/-}$ bone marrows were incubated with 7-KCh-LDL (5 μg/ml) for 36 hours. Cells were then fixed with 4% paraformaldehyde and stained with Oil-red-O. Representative images were shown. C, PEMs isolated from FA- or

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$PM_{2.5}$-exposed $LDLR^{-/-}$ mice with WT or $CD36^{-/-}$ bone marrows were incubated with DiI-labeled 7-KCh-LDL (5 μg/ml) for 36 hours. Cells were then visualized under confocal microscope and representative images of DiI fluorescence were shown. D. Statistical bar graph of % Oil-red-O positive area/cell area for figure A. E, Statistical bar graph of % Oilred-O positive area/cell area for figure B. F, Statistical bar graph of % fluorescence positive area/cell area for figure C.