Modification of HDL by reactive aldehydes alters select cardioprotective functions of HDL in macrophages

Rebecca L. Schill[1]*, Darcy A. Knaack[1], Hayley R. Powers[1], Yiliang Chen[2], Moua Yang[2,3], Daniel J. Schill[3†], Roy L. Silverstein[2,3,4,5] and Daisy Sahoo[1,4,5] iD

1 Department of Biochemistry, Medical College of Wisconsin, Milwaukee, WI, USA
2 Blood Research Institute, BloodCenter of Wisconsin, Milwaukee, WI, USA
3 Department of Cell Biology, Neurobiology & Anatomy, Medical College of Wisconsin, Milwaukee, WI, USA
4 Department of Medicine, Medical College of Wisconsin, Milwaukee, WI, USA
5 Cardiovascular Center, Medical College of Wisconsin, Milwaukee, WI, USA

While increased levels of high-density lipoprotein (HDL)-cholesterol correlate with protection against cardiovascular disease, recent findings demonstrate that HDL function, rather than HDL-cholesterol levels, may be a better indicator of cardiovascular risk. One mechanism by which HDL function can be compromised is through modification by reactive aldehydes such as acrolein (Acro), 4-hydroxynonenal, and malondialdehyde (MDA). In this study, we tested the hypothesis that modification of HDL with reactive aldehydes would impair HDL's athero-protective functions in macrophages. Compared to native HDL, Acro- and MDA-modified HDL have impaired abilities to promote migration of primary peritoneal macrophages isolated from C57BL6/J mice. Incubation of macrophages with MDA-HDL also led to an increased ability to generate reactive oxygen species. Our studies revealed that the changes in HDL function following aldehyde modification are likely not through activation of canonical nuclear factor-kappa B signaling pathways. Consistent with this finding, treatment of either noncholesterol-loaded macrophages or foam cells with modified forms of HDL does not lead to significant changes in expression levels of inflammatory markers. Importantly, our data also demonstrate that changes in HDL function are dependent on the type of modification present on the HDL particle. Our findings suggest that modification of HDL with reactive aldehydes can impair some, but not all, of HDL's athero-protective functions in macrophages.

Introduction

Decades of epidemiological studies show that higher circulating levels of high-density lipoprotein (HDL)-cholesterol associate with lower risk for cardiovascular disease (CVD). However, therapeutic interventions [1] and genetic predisposition [2] that raise HDL-cholesterol levels have failed to protect against CVD. These observations have led to the hypothesis that HDL function, rather than HDL-cholesterol levels, may be a better predictor of CVD risk [3].

Macrophages play a major role in the development and progression of atherosclerosis. Macrophages express several scavenger receptors (SR) including SR-BI and CD36, the primary receptors for HDL [4] and oxidized low-density lipoprotein (oxLDL) [5], respectively, as well as members of the ATP-binding cassette (ABC) superfamily of transporters, including ABCA1 and ABCG1. These receptors and transporters play vital roles in controlling cellular cholesterol levels, inflammatory responses, and a variety of other macrophage processes. Through interactions with CD36, oxLDL promotes the accumulation of excess esterified cholesterol in macrophages that results in the formation of lipid-laden 'foam cells' that are the precursor cells for plaque formation in the arterial wall (reviewed in [6]). OxLDL can also promote pro-inflammatory cytokine release, generate reactive oxygen species (ROS), as well as inhibit macrophage migration that results in macrophage trapping and eventually promotion of apoptosis (reviewed in [7]).

On the other hand, HDL helps to protect against CVD. The most commonly studied antiatherogenic function of HDL is its role in the early stages of the reverse cholesterol transport pathway, where HDL mediates the removal of cholesterol from peripheral macrophages for delivery to the liver for net excretion [8]. In macrophages, HDL also protects against the generation of ROS [9] and promotes the production of anti-inflammatory cytokines [10]. Furthermore, by interacting with SR-BI, HDL can promote macrophage migration [11] and prevent foam cell formation (reviewed in [6]).

An emerging body of literature suggests that many of HDL's cardioprotective functions can become compromised in several chronic inflammatory diseases. For example, HDL isolated from patients with coronary artery disease has a decreased ability to promote endothelial nitric oxide synthase activation, nitric oxide production, and endothelial cell protection when compared to HDL isolated from control patients [12]. Similarly, HDL from patients with chronic kidney disease is unable to protect endothelial cells against monocyte adhesion and triggers a heightened cytokine response [13]. HDL isolated from lupus patients induces pro-inflammatory effects in macrophages [14]. In fact, in many of these diseased states, 'dysfunctional HDL' has been proposed as a potential biomarker for risk of atherosclerosis [15]. However, the mechanisms responsible for rendering HDL dysfunctional are not well understood.

One potential mechanism by which HDL can become dysfunctional is by modification with reactive carbonyl aldehydes, many of which have been implicated in various disease states, including atherosclerosis [16]. Acrolein, 4-hydroxynonenal (HNE), and malondialdehyde (MDA) are reactive aldehydes that are found in circulation and play a role in altering lipoprotein function [17–19]. Furthermore, the main protein component of HDL, apolipoprotein A-I (apoA-I), can be modified by these oxidants in plasma [20] and in isolated human atheroma [21,22].

Recently, our group has shown that modification of HDL with acrolein (Acro) impairs SR-BI–mediated cholesterol transport [23]. These findings led us to hypothesize that Acro-modified HDL, and perhaps other reactive aldehyde-modified forms of HDL, have pro-atherogenic effects on macrophages. In this study, we describe how oxidative modification with three different reactive aldehydes (Acro, HNE, and MDA) can impair some of HDL's cardioprotective functions in macrophages. Further, we demonstrate that changes in HDL function are dependent on the type of aldehyde modification present on the HDL particle.

Results

Reactive aldehydes modify HDL *in vitro*

To test our hypothesis that a family of reactive aldehydes impairs HDL's cardioprotective functions, we first confirmed *in vitro* oxidative modification of HDL, similar to what we had shown in our previous studies with Acro-modified HDL [23]. As shown in Fig. 1, immunoblot analyses demonstrated that aldehyde adduct formation promoted cross-linking of apoA-I. Furthermore, using antibodies that recognize specific adducts formed by these aldehydes, immunoblot analyses confirmed the presence of Acro, HNE, and MDA on the HDL particles.

Modified HDL inhibits macrophage migration

During the early stages of plaque formation, macrophages play a critical role in a variety of pro-atherogenic pathways. For example, oxLDL inhibits macrophage migration by promoting macrophage trapping [24], a process that helps mediate foam cell formation and pro-inflammatory signaling. On the other hand, HDL promotes the migration of macrophages through an SR-BI–dependent process [11]. Therefore, we performed a Boyden Chamber assay to determine the impact of aldehyde modification on HDL's ability to promote macrophage migration. Compared to native HDL, both Acro- and MDA-modified HDL failed to promote macrophage
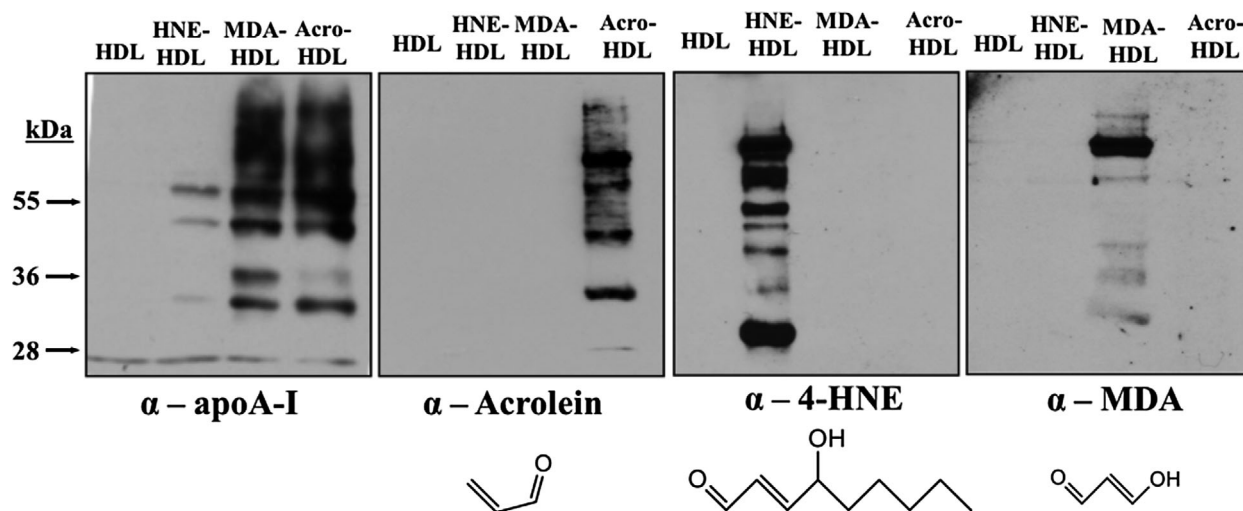
Fig. 1. Immunoblot analysis verifies apoA-I protein cross-linking and modification of HDL with reactive aldehydes. HDL was incubated with either 250 μM Acro, 500 μM HNE, or 15 μM MDA for 18 h. The reaction was stopped with a 20-fold excess of aminoguanidine-HCl. HDL proteins (20 μg total protein) were separated by 4–15% SDS/PAGE and following transfer to a nitrocellulose membrane, proteins were visualized by immunoblot analysis using antibodies directed against apoA-I or adducts formed by modification with Acro, HNE, or MDA. Reactive aldehyde structures are shown below each immunoblot. Data are representative of four independent experiments.
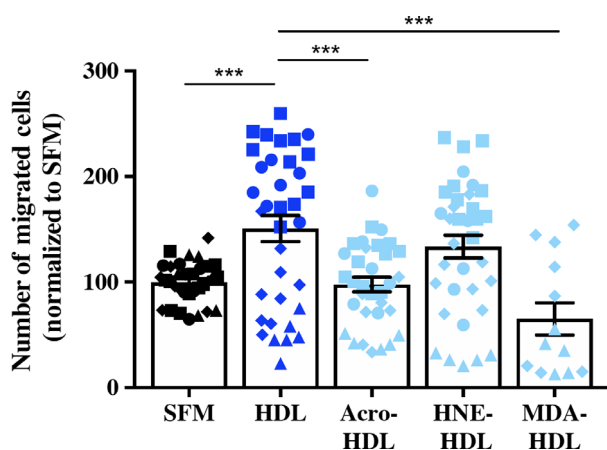

Fig. 2. Aldehyde-modified HDL has an impaired ability to mediate macrophage migration. Murine peritoneal macrophages were placed on the top of a Boyden Chamber. SFM or SFM containing native or modified HDL (50 μg·mL^{-1}) was placed on the bottom of the well. Following 24 h incubation, the bottom of the membrane was DAPI-stained and fluorescence quantified. Values represent four independent experiments (two in the case of MDA-HDL), each performed in duplicate, with 3–5 fields quantified from each sample. Values are shown as mean ± SEM and normalized to SFM treatment for comparison. Replicates within individual experiments are designated by different symbols. Statistical analyses were determined by one-way ANOVA. ***$P < 0.001$ vs. HDL.

migration (Fig. 2). Macrophage migration in the presence of HNE-HDL was similar to that of native HDL.

MDA-modified HDL generates reactive oxygen species in macrophages

Interaction of oxLDL with macrophages generates intracellular ROS, a step which appears to be critical to downstream signaling events. To measure ROS generation, we used a coumarin-7-boronic (CBA) probe, which is specific for hydrogen peroxide (H_2O_2) and peroxynitrite ($ONOO^-$) generation. The product formed is 7-hydroxycoumarin (COH) and was detected using HPLC methods [25]. Our data demonstrate that, like oxLDL, incubation with MDA-HDL generated a 1.5-fold increase in COH levels over treatment with serum-free media (SFM) alone (Fig. 3). Incubation of acro- or HNE-modified HDL did not lead to statistically significant increases in ROS generation. To identify the specific ROS oxidizing the probe following incubation with MDA-HDL, we measured COH production in the presence of polyethylene glycol (PEG)-catalase, which selectively converts H_2O_2 to H_2O and O_2. Our data showed that in the presence of catalase, MDA-HDL–induced COH production was suppressed, demonstrating that H_2O_2, rather than $ONOO^-$, is the major ROS formed by macrophages following treatment with MDA-HDL.

Aldehyde-modified HDL does not activate NF-κB signaling

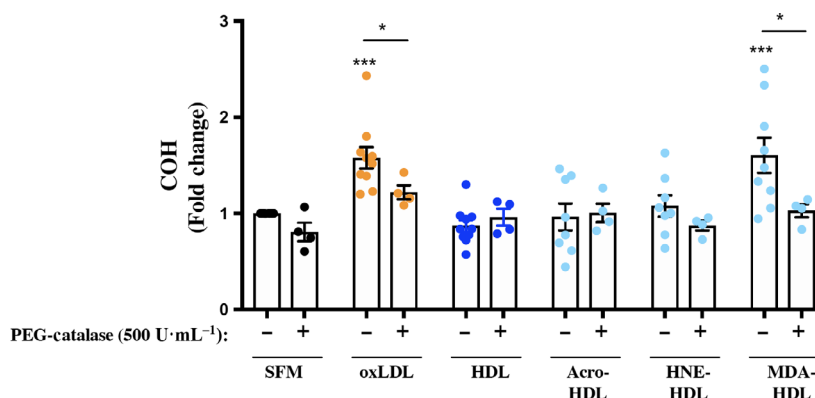When oxLDL interacts with macrophages, several downstream signaling cascades are activated, including

Fig. 3. MDA-HDL increases ROS generation. Cholesterol-loaded primary peritoneal macrophages were treated with SFM or SFM containing 50 μg·mL^{-1} oxLDL, HDL, acro-HDL, HNE-HDL, and MDA-HDL for 1 h at 37 °C ± 500 U·mL^{-1} of polyethylene glycol-catalase. ONOO$^-$ and/or H$_2$O$_2$ generation were quantified by measuring the conversion of CBA to COH. Statistical analyses were determined by one-way ANOVA for comparison of minus catalase samples. ***$P < 0.01$ vs. HDL in the absence of catalase. Statistical analyses were determined using an unpaired *T*-test for +/− catalase experiments. *$P < 0.05$. Data are the average of at least eight experiments (four in the case of ± polyethylene glycol-catalase addition), each performed in duplicate (each dot represents the average of the duplicates). Values are shown as mean ± SEM.

pathways related to nuclear factor-kappa B (NF-κB) signaling [26]. Additionally, others have reported that HDL modified by myeloperoxidase can activate NF-κB signaling [27]. To determine if aldehyde modification alters HDL function by activating NF-κB signaling pathways, we measured the phosphorylation status of p65 in peritoneal macrophages treated with 50 μg·mL^{-1} of oxLDL, native HDL or aldehyde-modified HDL for 0 (SFM), 5, 10, or 30 min incubations (Fig. 4A,B). Treatment with lipopolysaccharide (LPS) or TNFα served as positive controls for phosphorylation of the p65 subunit. As expected, our results demonstrated that oxLDL was able to stimulate phosphorylation of p65. On the other hand, incubation of macrophages with either native HDL or aldehyde-modified HDL did not lead to increased phosphorylation of p65. Additionally, we utilized a THP-1/secreted alkaline phosphatase reporter cell line to measure NF-κB activation. These data confirmed that, unlike oxLDL, none of the aldehyde-modified forms of HDL activated the reporter line (Fig. 4C), suggesting that any changes in HDL function in macrophages caused by aldehyde-modified HDL are through alternative pathways.

Modified forms of HDL do not promote inflammation in macrophages or foam cells

Macrophages found in human atherosclerotic plaque produce a large number of cytokines [28–30]. To determine if aldehyde-modified HDL affects inflammatory cytokine production, peritoneal macrophages were treated with SFM or SFM containing 50 μg·mL^{-1} of oxLDL, HDL, acro-HDL, HNE-HDL, or MDA-HDL for 6 h. Transcript levels of interleukin (IL)-1β, inducible nitric oxide synthase (iNOS), IL-10, IL-6, and TNFα were measured using qRT-PCR. Our results showed that treatment of noncholesterol-loaded macrophages with oxLDL induced expression of these cytokines as expected [31–33]. On the other hand, incubation of macrophages with native HDL, acro-, HNE-, or MDA-HDL did not result in significant changes in mRNA levels of any of the measured inflammatory markers (Fig. 5A–E). Shorter (2 h) or longer (24 h) incubation times with modified forms of HDL also revealed no changes in inflammatory marker expression compared to native HDL (data not shown). Treatment of cells with LPS or oxLDL served as positive controls for cytokine production.

High-density lipoprotein can suppress pro-inflammatory cytokine levels in the presence of additional inflammatory stimuli, such as LPS [34] or oxLDL [35]. To determine if modified HDL can decrease inflammatory cytokine production in foam cells, macrophages were cholesterol-loaded with 50 μg·mL^{-1} oxLDL for 24 h, followed by incubation with SFM or SFM containing 50 μg·mL^{-1} oxLDL, HDL, acro-HDL, HNE-HDL, or MDA-HDL for 6 h and transcript levels of IL-1β, iNOS, IL-10, IL-6, and TNFα were measured as previously described (Fig. 5F–J). Similar to the results seen in noncholesterol-loaded macrophages, modification of HDL with reactive aldehydes does not impact inflammatory cytokine expression. Additionally, secreted cytokine levels of IL-1β, TNFα, and IL-6
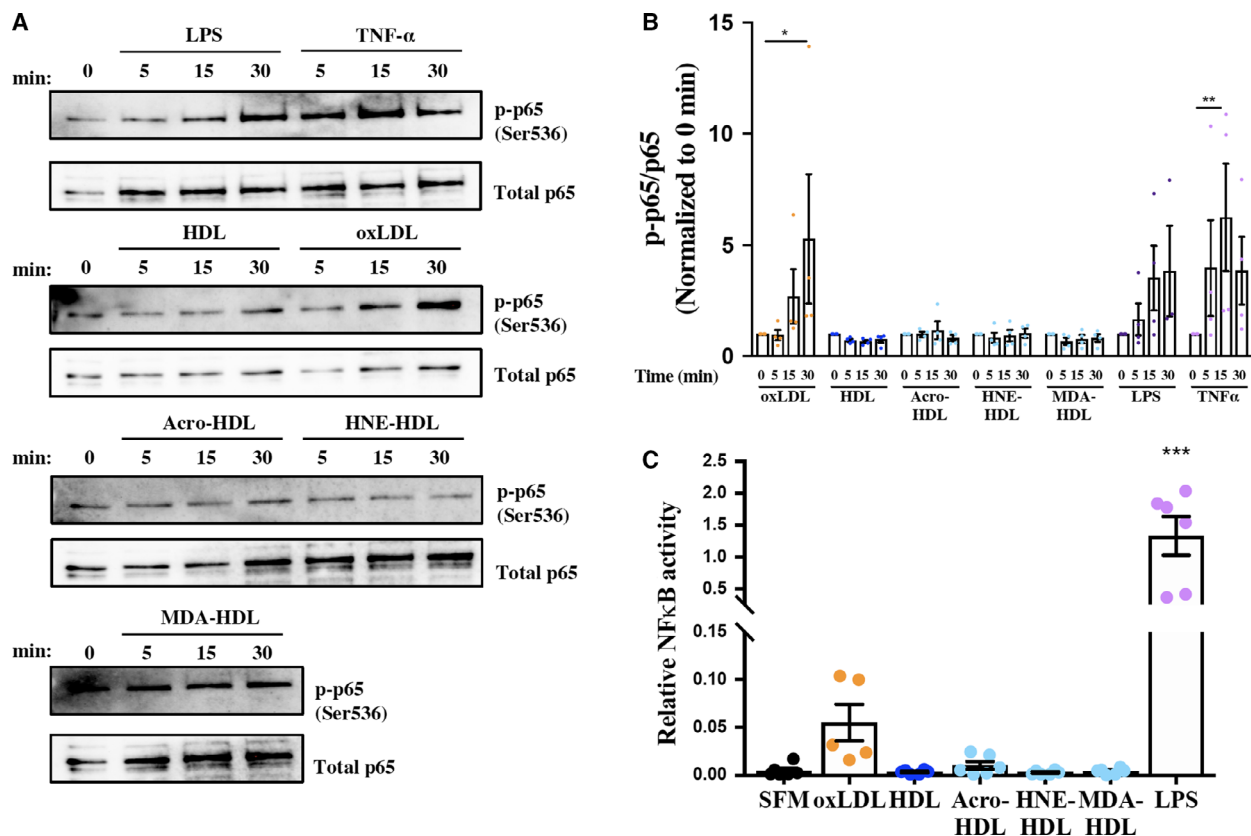
Fig. 4. Aldehyde-modified HDL does not activate NF-κB signaling. (A) Primary peritoneal macrophages were treated with SFM (0 time points) or SFM containing 50 µg·mL⁻¹ HDL, acro-HDL, HNE-HDL, or MDA-HDL for 5, 15, or 30 min at 37 °C. Cells were lysed and the level of phosphorylated p65 was determined by immunoblot analysis. Total p65 levels were measured as a loading control for phosphorylation. Treatment with oxLDL (50 µg·mL⁻¹), LPS (100 nM), or TNFα (10 nM) served as positive controls for phosphorylation. Data shown are representative of four immunoblots. (B) The ratio of phosphorylated p65 to total p65 was measured by densitometry and normalized to SFM (0 time point). Data are the mean ± SEM of four independent experiments. Statistical analyses were determined by two-way ANOVA. $*P < 0.05$, $**P < 0.001$. (C) NF-κB reporter THP-1 cells were treated for 1 h with SFM or SFM containing 50 µg·mL⁻¹ of HDL, oxLDL, acro-HDL, HNE-HDL, or MDA-HDL. Media was collected and the secreted reporter protein was measured by reading absorbance at 655 nm. Treatment with LPS (100 nM) for 1 h served as a positive control. Values are an average of six independent experiments and data are shown as mean ± SEM. Statistical analyses were determined by one-way ANOVA. $**P < 0.001$ vs. HDL.

were measured by ELISA following 24 h incubation of foam cells with SFM or SFM containing 50 µg·mL⁻¹ oxLDL, HDL, acro-HDL, HNE-HDL, or MDA-HDL (Fig. 6). There were no significant changes in secretion of IL-1β, TNFα, or IL-6 levels with any of the modified forms of HDL. To further determine if modified HDL impacts inflammatory gene expression in the presence of pro-inflammatory stimuli, we coincubated primary peritoneal macrophages with 50 µg·mL⁻¹ oxLDL and 50 µg·mL⁻¹ native or modified HDL for 2, 6, or 24 h. Our results demonstrated that coincubation with native HDL or the modified forms of HDL with oxLDL did not lead to major changes in inflammatory gene expression at any of the time points tested (Fig. 7).

Discussion

Recent studies suggest that enhancing HDL's cardioprotective functions, rather than raising circulating HDL-cholesterol levels, may be a better approach to reducing CVD risk. However, the circumstances that govern HDL function are not well understood. In the current study, we determined that oxidative modification of HDL with reactive aldehydes (Acro, HNE or MDA) alters HDL's athero-protective functions in macrophages. Specifically, we demonstrate that HDL modified by reactive aldehydes has an impaired ability to promote macrophage migration and promotes ROS generation, with no impact on inflammatory cytokine production. Our findings also suggest that changes in
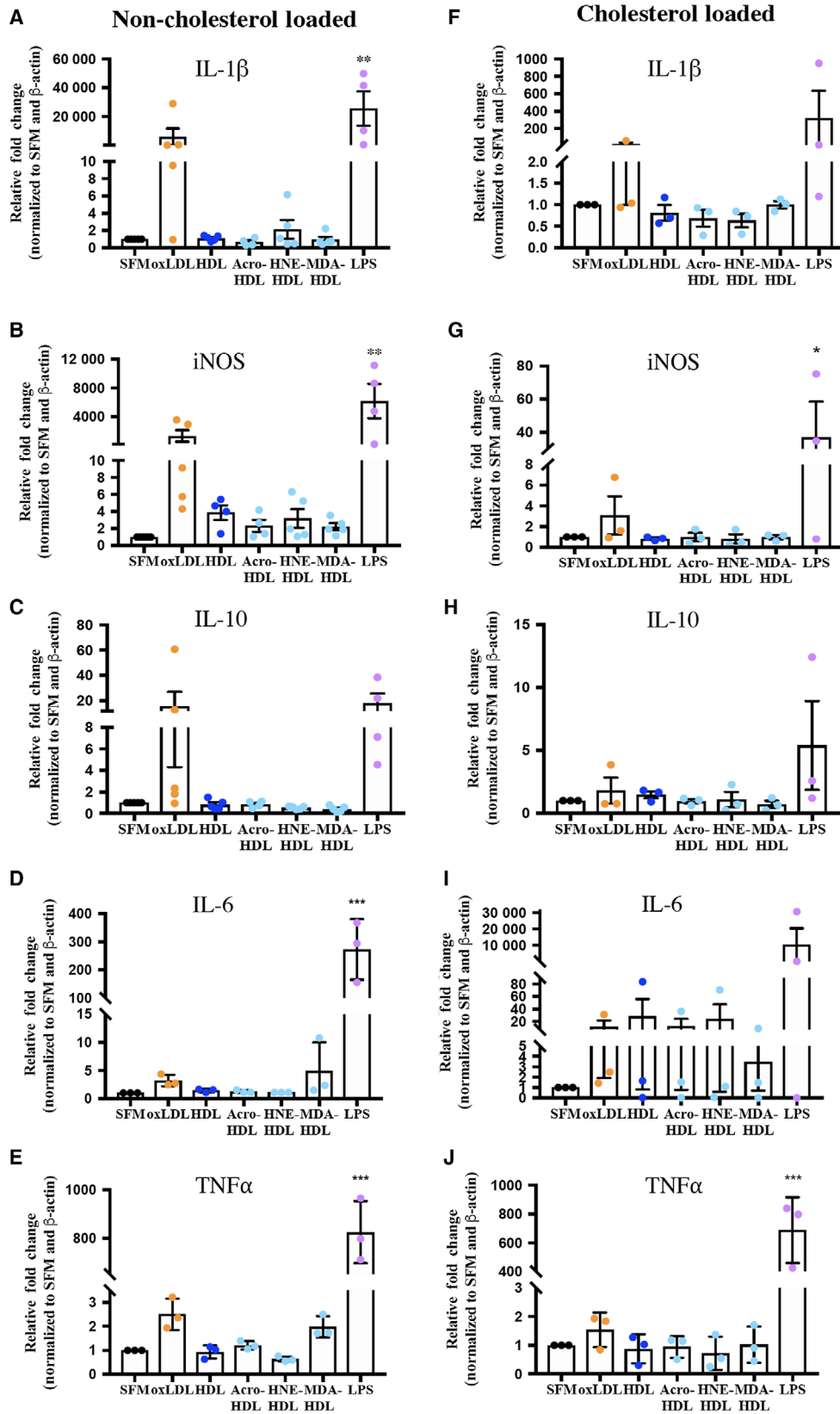
A **Non-cholesterol loaded**

IL-1β

F **Cholesterol loaded**

IL-1β

B

iNOS

G

iNOS

C

IL-10

H

IL-10

D

IL-6

I

IL-6

E

TNFα

J

TNFα

Fig. 5. Modification of HDL with reactive aldehydes does not alter HDL's inflammatory properties on macrophages or foam cells. (A–E) Primary peritoneal macrophages were treated with SFM or SFM containing 50 µg·mL^{-1} HDL, acro-HDL, HNE-HDL, MDA-HDL, or oxLDL for 6 h at 37 °C. Messenger RNA was isolated, converted to cDNA, and genes of interest were quantified using qRT-PCR. Murine β-actin was used as an internal control. Data are presented as a relative fold change to SFM treatment and β-actin levels. Data are the average of at least four independent experiments. (F-J) Primary peritoneal macrophages were pretreated with 50 µg·mL^{-1} oxLDL for 24 h to induce foam cell expression. Next, foam cells were incubated with SFM alone or SFM containing 50 µg·mL^{-1} oxLDL, HDL, acro-HDL, HNE-HDL, or MDA-HDL for an additional 6 h at 37 °C. Foam cells treated with LPS (100 nM) were used as positive controls. Gene expression was determined as described in A–C. Data are the average for three independent experiments and values are shown as mean ± SEM. All statistical analyses were determined by one-way ANOVA. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. HDL.
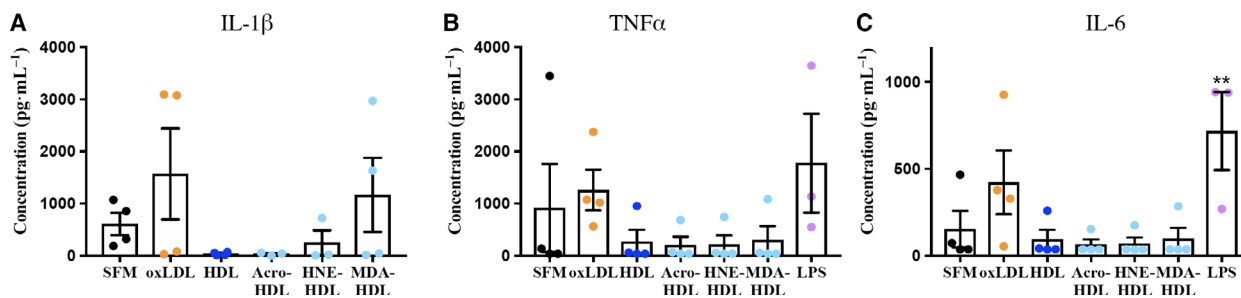

Fig. 6. Modification of HDL with reactive aldehydes does not alter inflammatory cytokine secretion in foam cells. Primary peritoneal macrophages, pretreated with 50 µg·mL^{-1} oxLDL for 24 h to induce foam cell expression. Next, foam cells were incubated with SFM alone or SFM containing 50 µg·mL^{-1} HDL, acro-HDL, HNE-HDL, or MDA-HDL for 24 h at 37 °C. Foam cells treated with a second incubation of 50 µg·mL^{-1} of oxLDL or LPS (100 nM) served as positive controls. Secreted levels of (A) IL-1β, (B) TNFα, and (C) IL-6 were measured by ELISA. Data are the average of at least three independent experiments and values are shown as mean ± SEM. All statistical analyses were determined by one-way ANOVA. **$P < 0.01$ vs. HDL.
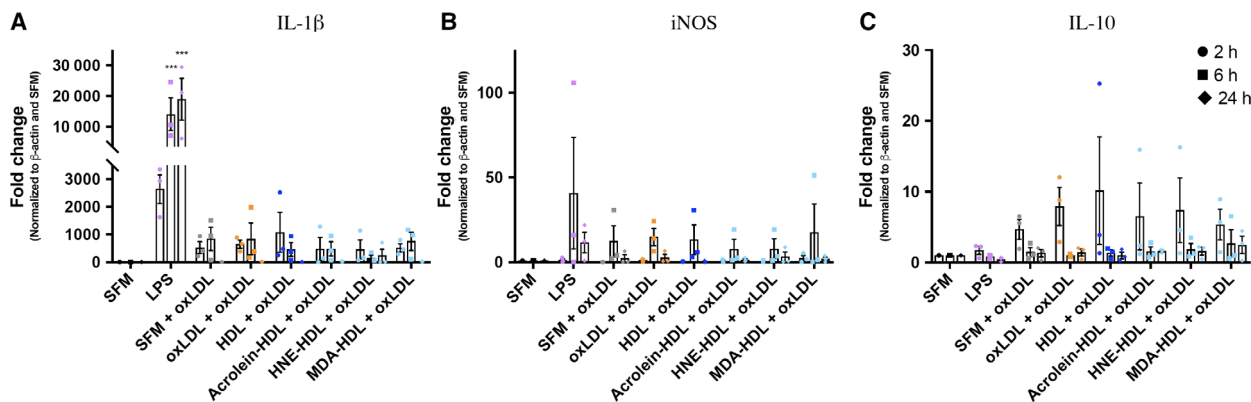

Fig. 7. Coincubation of oxLDL with native HDL or modified forms of HDL did not lead to major changes in inflammatory gene expression. Primary peritoneal macrophages were treated with 50 µg·mL^{-1} oxLDL in combination with 50 µg·mL^{-1} native or modified HDL for 2, 6, or 24 h at 37 °C. Messenger RNA was isolated, converted to cDNA, and (A) IL-1β, (B) iNOS, and (C) IL-10 transcript levels were quantified using qRT-PCR. Murine β-actin was used as an internal control. Data are presented as a relative fold change to SFM treatment and β-actin levels. Data are the average of at least three independent experiments and values are shown as mean ± SEM. All statistical analyses were determined by two-way ANOVA. ***$P < 0.001$ vs. SFM. Treatment with LPS (100 nM) served as positive control.

HDL function are dependent on the type of modification present on the HDL particle.

The reactive aldehydes tested in this study are physiologically relevant to CVD, and all are products of lipid peroxidation. Since Acro is a major component of cigarette smoke and other environmental atmospheric emissions, exposure to Acro is considered a CVD risk factor [36]. In mice, Acro consumption can induce dyslipidemia and modify lipoproteins [17]. In humans, Acro colocalizes with apoA-I in atherosclerotic plaque [37]. Related aldehydes such as HNE and MDA have also been implicated in

lipoprotein modification. HDL from patients with acute coronary syndrome or with stable angina carries both HNE and MDA adducts [20]. When plasma is exposed to HNE, the activity of lecithin : cholesterol acyltransferase, an enzyme required for HDL maturation is decreased [38].

The ability of reactive aldehydes to form covalent crosslinks in proteins has been well studied [39]. In our study, we demonstrated that modification with reactive aldehydes leads to apoA-I protein cross-linking on whole-particle HDL. Shao *et al.* [40] demonstrated that the specific lysine residues on apoA-I modified by Acro are similar between lipid-free apoA-I and whole-particle HDL [23]. Modification of apoA-I with MDA also predominantly leads to the modification of lysine residues 56 and 118 [40]. While HNE can modify lysines on other proteins, Shao *et al.* [40] demonstrated that HNE preferentially targets all five histidine residues present on apoA-I. To our knowledge, specific apoA-I residues modified by MDA or HNE on whole-particle HDL have not been identified. However, all three aldehydes are present in circulation and have been shown to modify lipoproteins [17,19,22,37,40–43]. In addition, apoA-II, the second most abundant HDL apolipoprotein, also presents crosslinked species when modified by Acro [23], HNE or MDA (data not shown).

Macrophage trapping is a critical step in the progression of atherosclerosis. As such, increasing mobilization of macrophages has been proposed as a therapeutic strategy to reverse atherosclerosis. OxLDL plays an important role in recruitment and retention of lipid-laden macrophages within the plaque [24]. On the other hand, HDL promotes macrophage migration [11], a process that could ultimately lead to plaque regression [6]. We demonstrated that, compared to native HDL, aldehyde-modified HDL could not promote migration of peritoneal macrophages. It is possible that aldehyde-modified HDL's inability to promote macrophage migration is due to defects in SR-BI–mediated signaling cascades that include PI3K and Akt [11].

Following incubation with oxLDL, macrophages can produce a variety of different ROS species including H_2O_2 [26]. We measured ROS generation using a CBA-to-COH probe that is specific for generation of $ONOO^-$ and H_2O_2 [25]. Like oxLDL, incubation with MDA-HDL led to increased COH levels. The presence of polyethylene glycol-catalase prevented COH production, demonstrating that the major form of ROS generated following incubation with MDA-HDL is H_2O_2. Future experiments will determine if ROS generation following incubation of macrophages with

MDA-HDL is primarily through NADPH oxidase. Our results also demonstrated that only MDA-HDL, but not acro- or HNE-HDL, lead to increased ROS generation in peritoneal macrophages. It is possible that Acro, HNE, and MDA preferentially modify different components of the HDL particle. While our studies demonstrated that all three aldehydes modify the protein component of HDL (as in Fig. 1), we cannot exclude the possibility that these aldehydes also modify specific HDL lipids. Previous work has shown that modification of LDL with MDA generates a modification similar to that of copper-modified LDL [44], a well-known inducer of lipid peroxidation [45]. Therefore, perhaps MDA has similar effects on HDL, contributing to the differential effects observed between different modified HDL species, and this hypothesis warrants further investigation.

When oxLDL interacts with macrophages, NF-κB signaling is activated [26]. It has also been suggested that HDL, too, can activate NF-κB signaling in macrophages [27], although others have reported that HDL inhibits inflammation by suppressing NF-κB signaling [46]. Interestingly, HDL modified by myeloperoxidase can activate NF-κB signaling in endothelial cells [27]. One group has reported that while HDL induced expression of NF-κB reporter genes, HDL did not lead to nuclear localization of p65 [47], suggesting that HDL-dependent regulation of NF-κB responses potentially implicates another subunit. Our data suggest that aldehyde-modified HDL does not activate NF-κB signaling due to lack of phosphorylation of the p65 subunit. As such, any changes to HDL function following modification are likely through alternative pathways.

Macrophages trapped within an atherosclerotic lesion have been shown to produce both pro- and anti-inflammatory cytokines [48]. Previous reports have shown that treatment of human lung lymphoblasts with unconjugated HNE (i.e., aldehyde alone) can activate NF-κB signaling and increase the expression of IL-8, IL-1β, and TNFα following a 6 h treatment [49]. In contrast, our results showed that modified HDL did not lead to increased expression of these cytokines in macrophages at 2, 6, or 24 h. In lipid-loaded macrophages, modified HDL also had no impact on inflammatory gene expression compared to native HDL. Additionally, when coincubated with a pro-inflammatory stimuli (oxLDL), modified forms of HDL showed no significant changes in inflammatory gene expression. These data suggest that modification of HDL with reactive aldehydes does not generate a particle with pro-inflammatory properties.

One interesting observation from our studies is that changes to HDL function were dependent on the type

of modification present on the particle, suggesting that not all types of modified HDL behave similarly. HDL is comprised of a large variety of lipids and proteins, and HDL species vary by size and shape. Thus, one potential contributor to differences in function with aldehyde-modified HDL could be the aldehyde's preferred target of modification. While our analysis of HDL modification was limited to detection of protein cross-linking, it is possible that some reactive aldehydes preferentially modify nonprotein components of HDL. Additionally, the majority of our studies was performed using primary peritoneal macrophages that were isolated from mice 4 days postinjection with 4% thioglycolate, a model system that has been routinely used for studies of lipoprotein metabolism [9,50–54]. We chose thioglycolate-elicited macrophages for our studies for several reasons. Firstly, we can isolate these cells in large quantities. Secondly, because primary peritoneal macrophages replicate at minimal levels [55], they serve as an ideal cell system for our migration studies. However, macrophages elicited by thioglycolate can have altered responses when compared to resident macrophages [56,57]. Therefore, confirmation of our data using alternative sources of macrophages, including murine resident peritoneal macrophages, murine bone marrow-derived macrophages, or human monocyte-derived macrophages is warranted.

Recent failures in HDL-raising therapies to provide cardiovascular benefit have led to the conclusion that we have much to learn about HDL's cardioprotective functions in the body. Understanding the impact of modification with reactive aldehydes on HDL function will help in developing new therapeutic approaches aimed at preventing HDL dysfunction and enhancing HDL's cardioprotective functions in the fight against CVD.

Materials and methods

1,1,3,3-Tetramethoxypropane for MDA generation, LPS, and catalase-polyethylene glycol were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Boyden chambers (6.5 mm insert, 5.0 μm polycarbonate membrane) were from Costar (Burlington, MA, USA) (3421). HNE was purchased from Cayman Chemicals (Ann Arbor, MI, USA). Acrolein was purchased from Ultra Scientific (North Kingstown, RI, USA). Aminoguanidine hydrochloride was purchased from Acros Organics (Morris Plains, NJ, USA). The CBA probe was generously provided by Balaraman Kalyanaraman, PhD (Free Radical Research Center, Medical College of Wisconsin). ELISA kits for IL-1β, IL-6, and TNFα were from Invitrogen (Carlsbad, CA, USA). Human LDL (1 mg·mL^{-1}) was purchased from Lee BioSolutions (Maryland Heights, MO, USA). Human HDL (10 mg·mL^{-1}), purified via sequential high-speed ultracentrifugation (density = 1.063–1.21 g·mL^{-1}), was purchased from Alfa Aesar (Ward Hill, MA, USA). It consists of 60–70% apoA-I, 20% apoA-II and 7–8% (or less) apoE. HDL preps contain ~ 55% protein, 3–15% triglycerides, 26–46% phospholipids, 15–30% cholesteryl esters and 2–10% free cholesterol. The following antibodies were used: Anti-Apo-AI (Santa Cruz Biotechnology, Dallas, TX, USA), anti-HNE Michael adducts (Thermo Fisher Scientific, Waltham, MA, USA) anti-MDA (Abcam, Cambridge, UK), anti-Acro (Abcam), anti-rabbit IgG (Amersham-GE Healthcare, Pittsburgh, PA, USA), anti-mouse IgG (Amersham-GE Healthcare), phospho-p65 at residue Ser536 (Cell Signaling, Danvers, MA, USA) and total p65 (Cell Signaling). All other reagents were of analytical grade.

Lipoproteins and their chemical modification

Native HDL (1 mg·mL^{-1}) was incubated with 500 μM HNE, 250 μM Acro, or 15 μM MDA at 37 °C for 18 h. MDA was made prior to each experiment as previously described [40]. Each reaction was stopped by adding 20-fold molar excess of aminoguanidine hydrochloride to scavenge excess aldehyde, and experimental assays were performed immediately. LDL was oxidized by dialysis against 5 μM CuSO$_4$ in PBS for 6 h at 37 °C. Oxidation was terminated by dialysis against PBS containing 0.54 mM EDTA overnight at 4 °C, and for another 6 h at 4 °C against PBS to eliminate remaining EDTA.

Animals and primary cell culture

All experimental procedures in mice conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication, 8th Edition, 2011) and all procedures were carried out according to protocols approved by the Institutional Animal Care and Use Committee of the Medical College of Wisconsin.

Wild-type C57BL6/J mice (~ 50 in total) were housed under a normal light–dark cycle and bred in a pathogen-free barrier facility in accordance with federal and institutional guidelines. At the time of macrophage isolation, mice were euthanized by CO$_2$ inhalation followed by cervical dislocation. Peritoneal macrophages were isolated as previously described [37]. Briefly, on day 4 following a 4% thioglycolate injection, male or female mice (8–12 weeks of age) were euthanized via CO$_2$ inhalation followed by cervical dislocation, and macrophages were isolated in PBS, centrifuged at 400 *g* for 10 min at 4 °C, and resuspended in complete RPMI media (RPMI 1640 containing 10% FBS, 2 mM L-glutamine, 50 units·mL^{-1} penicillin, 50 μg·mL^{-1} streptomycin, and 1 mM sodium pyruvate), with the exception of macrophages used for migration studies, which were

Table 1. Primer sequences of inflammatory-associated genes for qRT-PCR.

Genes	Forward primer (5′–3′)	Reverse primer (5′–3′)
β-Actin	TCCTAGCACCATGAAGATCAAG	GACTCATCGTACTCCTGCTTG
iNOS	CGAGACTTCTGTGACACACAGC	CATCTCCTGGTGGAACACAGGG
IL-1β	CCTGTGGCCTTGGGCCTCAA	GGTGCTGATGTACCAGTTGGG
IL-10	GTGGAGCAGGTGAAGAGTG	CATGTATGCTTCTATGCAGTTGATG
IL-6	CCGGAGAGGAGACCTCACAG	GGAAATTGGGGTAGGAAGGA
TNFα	TACTGAACTTCGGGGTGATTGGTCC	CAGCCTTGTCCCTTGAAGAGAACC

resuspended in RPMI lacking serum and antibiotics and directly plated in to chamber wells. All experiments were performed within 7 days postisolation of macrophages.

Immunoblot analysis

Modification of HDL

High-density lipoprotein or HDL modified *in vitro* with HNE, MDA, or Acro was electrophoresed by 4–15% SDS/PAGE. Separated proteins were transferred to a nitrocellulose membrane at 100 V for 1 h at 4 °C and proteins were detected by immunoblot analysis using antibodies directed against apoA-I (Santa Cruz) or adducts formed by HNE (Thermo Fisher Scientific), MDA (Abcam), or Acro (Abcam) modification.

p65 Expression

Primary peritoneal macrophages were treated with serum-free RPMI medium (SFM) or 50 µg·mL^{-1} native HDL or modified HDL for the time points indicated. Cells were lysed in RIPA lysis buffer (150 mM sodium chloride, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris, pH 7.5) containing protease inhibitors (1 µg·mL^{-1} pepstatin, 0.2 mM phenylmethylsulfonyl fluoride, 1 µg·mL^{-1} leupeptin, and 10 µg·mL^{-1} aprotinin) and phosphatase inhibitor cocktail (Sigma). Proteins were separated by 10% SDS/PAGE and transferred to a nitrocellulose membrane at 100 V for 1 h at 4 °C. Proteins were detected by immunoblot analysis using antibodies directed against phospho-p65 at residue Ser536 (Cell Signaling) or total p65 (Cell Signaling) as a loading control.

Macrophage migration

Migration of peritoneal macrophages was measured using a Boyden Chamber assay. Briefly, peritoneal macrophages (0.5×10^6 cells) in serum-free RPMI were placed in the upper chamber of a 6 µm transwell insert. The inserts were placed in wells containing SFM (RPMI 1640) or SFM with 50 µg·mL^{-1} of oxLDL, HDL, acro-HDL, HNE-HDL, or MDA-HDL in the bottom of the wells. Cells were allowed to migrate through the porous membrane for 24 h.

Membranes were washed twice in PBS for 5 min per wash. Membranes were stained with DAPI and the number of migrated cells was blindly quantified by fluorescence microscopy (7–8 images per condition).

ROS generation

Primary peritoneal macrophages were cholesterol-loaded with 50 µg·mL^{-1} oxLDL for 1 h at 37 °C in SFM. Cells were then stimulated with SFM or SFM containing 50 µg·mL^{-1} oxLDL, HDL, acro-HDL, HNE-HDL, or MDA-HDL for 1 h at 37 °C in the presence or absence of 500 U·mL^{-1} polyethylene glycol-catalase. At 30 min of stimulation, 0.1 mM CBA was added in the dark for the remaining 30 min. Cells were collected and pelleted by centrifugation. The pellets were frozen at −80 °C until required for HPLC analysis as previously described [25].

NF-κB reporter assay

Nuclear factor-kappa B reporter THP-1 cells were treated with SFM or SFM containing 50 µg·mL^{-1} of HDL, oxLDL, acro-, HNE-, and MDA-HDL for 1 h at 37 °C. Media was collected and secreted alkaline phosphatase activity was measured using a colorimetric assay as described in the manufacturer's protocols (InvivoGen, San Diego, CA, USA).

Inflammatory cytokine analysis

RNA isolation and quantitative RT-PCR

Primary peritoneal macrophages (some of which were pretreated with 50 µg·mL^{-1} oxLDL for 24 h to induce foam cell formation) were incubated for 2, 6, or 24 h with SFM or SFM containing native or modified forms of HDL (50 µg·mL^{-1}) at 37 °C. Total RNA was isolated from cells using Trizol (Thermo Fisher Scientific) per manufacturer's instructions. cDNA was synthesized from up to 2 µg total RNA using the reverse transcriptase MMLV and random primers (Invitrogen). Quantitative RT-PCR was performed using an iCycler (Biorad, Hercules, CA, USA). Relative gene expression was determined using the $2^{-\Delta\Delta Ct}$ method with the murine β-actin gene serving as the internal control. Primer sequences are detailed in Table 1.

Secreted cytokine levels

Primary peritoneal macrophages were cholesterol-loaded with 50 $\mu g \cdot mL^{-1}$ oxLDL for 24 h and then incubated again with either RPMI 1640 SFM, SFM containing 50 $\mu g \cdot mL^{-1}$ oxLDL, or SFM containing 50 $\mu g \cdot mL^{-1}$ of native or modified HDL for an additional 24 h at 37 °C. Media was collected and cytokine levels were measured by ELISA using undiluted samples per manufacturer's protocols.

Statistics

Each experiment was repeated at least three times. Statistical analyses were determined by one-way or two-way ANOVA with Bonferroni *post hoc* analyses using GRAPHPAD PRISM7 Software (Graphpad, San Diego, CA, USA). Significance was set at $P < 0.05$.

Acknowledgements

Conflict of interest

All authors have read the journal's authorship agreement and policy on disclosure of potential conflict of interest and have nothing to disclose. This article has been reviewed and approved by all authors.

Author contributions

RLSc, RLSi, and DS conceived and designed research; RLSc, DAK, HRP, YC, MY, and DJS performed research and analyzed data. RLSc and DS wrote the manuscript. All authors contributed to manuscript editing.

References

1 Kosmas CE, DeJesus E, Rosario D & Vittorio TJ (2016) CETP inhibition: past failures and future hopes. *Clin Med Insights Cardiol* **10**, 37–42.

2 Voight BF, Peloso GM, Orho-Melander M, Frikke-Schmidt R, Barbalic M, Jensen MK, Hindy G, Holm H, Ding EL, Johnson T *et al.* (2012) Plasma HDL cholesterol and risk of myocardial infarction: a mendelian randomisation study. *Lancet* **380**, 572–580.

3 Ansell BJ, Watson KE, Fogelman AM, Navab M & Fonarow GC (2005) High-density lipoprotein function recent advances. *J Am Coll Cardiol* **46**, 1792–1798.

4 Acton S, Rigotti A, Landschulz KT, Xu S, Hobbs HH & Krieger M (1996) Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science* **271**, 518–520.

5 Endemann G, Stanton LW, Madden KS, Bryant CM, White RT & Protter AA (1993) CD36 is a receptor for oxidized low density lipoprotein. *J Biol Chem* **268**, 11811–11816.

6 Yu XH, Fu YC, Zhang DW, Yin K & Tang CK (2013) Foam cells in atherosclerosis. *Clin Chim Acta* **424**, 245–252.

7 Berliner JA & Heinecke JW (1996) The role of oxidized lipoproteins in atherogenesis. *Free Radic Biol Med* **20**, 707–727.

8 Ji Y, Wang N, Ramakrishnan R, Sehayek E, Huszar D, Breslow JL & Tall AR (1999) Hepatic scavenger receptor BI promotes rapid clearance of high density lipoprotein free cholesterol and its transport into bile. *J Biol Chem* **274**, 33398–33402.

9 Terasaka N, Wang N, Yvan-Charvet L & Tall AR (2007) High-density lipoprotein protects macrophages from oxidized low-density lipoprotein-induced apoptosis by promoting efflux of 7-ketocholesterol via ABCG1. *Proc Natl Acad Sci USA* **104**, 15093–15098.

10 Yvan-Charvet L, Kling J, Pagler T, Li H, Hubbard B, Fisher T, Sparrow CP, Taggart AK & Tall AR (2010) Cholesterol efflux potential and antiinflammatory properties of high-density lipoprotein after treatment with niacin or anacetrapib. *Arterioscler Thromb Vasc Biol* **30**, 1430–1438.

11 Al-Jarallah A, Chen X, Gonzalez L & Trigatti BL (2014) High density lipoprotein stimulated migration of macrophages depends on the scavenger receptor class B, type I, PDZK1 and Akt1 and is blocked by sphingosine 1 phosphate receptor antagonists. *PLoS ONE* **9**, e106487.

12 Besler C, Heinrich K, Rohrer L, Doerries C, Riwanto M, Shih DM, Chroni A, Yonekawa K, Stein S, Schaefer N *et al.* (2011) Mechanisms underlying adverse effects of HDL on eNOS-activating pathways in patients with coronary artery disease. *J Clin Invest* **121**, 2693–2708.

13 Kaseda R, Jabs K, Hunley TE, Jones D, Bian A, Allen RM, Vickers KC, Yancey PG, Linton MF, Fazio S *et al.* (2014) Dysfunctional high-density lipoproteins in children with chronic kidney disease. *Metabolism* **64**, 263–273.

14 Smith CK, Seto NL, Vivekanandan-Giri A, Yuan W, Playford MP, Manna Z, Hasni SA, Kuai R, Mehta

NN, Schwendeman A *et al*. (2017) Lupus high-density lipoprotein induces proinflammatory responses in macrophages by binding lectin-like oxidised low-density lipoprotein receptor 1 and failing to promote activating transcription factor 3 activity. *Ann Rheum Dis.* **76**, 602–611.

15 McMahon M, Grossman J, FitzGerald J, Dahlin-Lee E, Wallace DJ, Thong BY, Badsha H, Kalunian K, Charles C, Navab M *et al*. (2006) Proinflammatory high-density lipoprotein as a biomarker for atherosclerosis in patients with systemic lupus erythematosus and rheumatoid arthritis. *Arthritis Rheum* **54**, 2541–2549.

16 Ellis EM (2007) Reactive carbonyls and oxidative stress: potential for therapeutic intervention. *Pharmacol Ther* **115**, 13–24.

17 Conklin DJ, Barski OA, Lesgards JF, Juvan P, Rezen T, Rozman D, Prough RA, Vladykovskaya E, Liu S, Srivastava S *et al*. (2010) Acrolein consumption induces systemic dyslipidemia and lipoprotein modification. *Toxicol Appl Pharmacol* **243**, 1–12.

18 Srivastava S, Conklin DJ, Liu SQ, Prakash N, Boor PJ, Srivastava SK & Bhatnagar A (2001) Identification of biochemical pathways for the metabolism of oxidized low-density lipoprotein derived aldehyde-4-hydroxy trans-2-nonenal in vascular smooth muscle cells. *Atherosclerosis* **158**, 339–350.

19 Tanaga K, Bujo H, Inoue M, Mikami K, Kotani K, Takahashi K, Kanno T & Saito Y (2002) Increased circulating malondialdehyde-modified LDL levels in patients with coronary artery diseases and their association with peak sizes of LDL particles. *Arterioscler Thromb Vasc Biol* **22**, 662–666.

20 Carnuta MG, Stancu CS, Toma L, Sanda GM, Niculescu LS, Deleanu M, Popescu AC, Popescu MR, Vlad A, Dimulescu DR *et al*. (2017) Dysfunctional high-density lipoproteins have distinct composition, diminished anti-inflammatory potential and discriminate acute coronary syndrome from stable coronary artery disease patients. *Sci Rep* **7**, 7295.

21 Huang Y, DiDonato JA, Levison BS, Schmitt D, Li L, Wu Y, Buffa J, Kim T, Gerstenecker GS, Gu X *et al*. (2014) An abundant dysfunctional apolipoprotein A1 in human atheroma. *Nat Med* **20**, 193–203.

22 Holvoet P, Perez G, Zhao Z, Brouwers E, Bernar H & Collen D (1995) Malondialdehyde-modified low density lipoproteins in patients with atherosclerotic disease. *J Clin Invest* **95**, 2611–2619.

23 Chadwick AC, Holme RL, Chen Y, Thomas MJ, Sorci-Thomas MG, Silverstein RL, Pritchard KA Jr & Sahoo D (2015) Acrolein impairs the cholesterol transport functions of high density lipoproteins. *PLoS ONE* **10**, e0123138.

24 Park YM, Febbraio M & Silverstein RL (2009) CD36 modulates migration of mouse and human macrophages

in response to oxidized LDL and may contribute to macrophage trapping in the arterial intima. *J Clin Invest* **119**, 136–145.

25 Zielonka J, Zielonka M, Sikora A, Adamus J, Joseph J, Hardy M, Ouari O, Dranka BP & Kalyanaraman B (2012) Global profiling of reactive oxygen and nitrogen species in biological systems: high-throughput real-time analyses. *J Biol Chem* **287**, 2984–2995.

26 Silverstein RL, Li W, Park YM & Rahaman SO (2010) Mechanisms of cell signaling by the scavenger receptor CD36: implications in atherosclerosis and thrombosis. *Trans Am Clin Climatol Assoc* **121**, 206–220.

27 Undurti A, Huang Y, Lupica JA, Smith JD, DiDonato JA & Hazen SL (2009) Modification of high density lipoprotein by myeloperoxidase generates a pro-inflammatory particle. *J Biol Chem* **284**, 30825–30835.

28 McLaren JE & Ramji DP (2009) Interferon gamma: a master regulator of atherosclerosis. *Cytokine Growth Factor Rev* **20**, 125–135.

29 Ramji DP & Davies TS (2015) Cytokines in atherosclerosis: key players in all stages of disease and promising therapeutic targets. *Cytokine Growth Factor Rev* **26**, 673–685.

30 Singh NN & Ramji DP (2006) The role of transforming growth factor-beta in atherosclerosis. *Cytokine Growth Factor Rev* **17**, 487–499.

31 Liu W, Yin Y, Zhou Z, He M & Dai Y (2014) OxLDL-induced IL-1 beta secretion promoting foam cells formation was mainly via CD36 mediated ROS production leading to NLRP3 inflammasome activation. *Inflamm Res* **63**, 33–43.

32 Rios FJ, Ferracini M, Pecenin M, Koga MM, Wang Y, Ketelhuth DF & Jancar S (2013) Uptake of oxLDL and IL-10 production by macrophages requires PAFR and CD36 recruitment into the same lipid rafts. *PLoS ONE* **8**, e76893.

33 Huang H, Koelle P, Fendler M, Schrottle A, Czihal M, Hoffmann U, Conrad M & Kuhlencordt PJ (2014) Induction of inducible nitric oxide synthase (iNOS) expression by oxLDL inhibits macrophage derived foam cell migration. *Atherosclerosis* **235**, 213–222.

34 Suzuki M, Pritchard DK, Becker L, Hoofnagle AN, Tanimura N, Bammler TK, Beyer RP, Bumgarner R, Vaisar T, de Beer MC *et al*. (2010) High-density lipoprotein suppresses the type I interferon response, a family of potent antiviral immunoregulators, in macrophages challenged with lipopolysaccharide. *Circulation* **122**, 1919–1927.

35 Robbesyn F, Auge N, Vindis C, Cantero AV, Barbaras R, Negre-Salvayre A & Salvayre R (2005) High-density lipoproteins prevent the oxidized low-density lipoprotein-induced epidermal [corrected] growth factor receptor activation and subsequent matrix metalloproteinase-2 upregulation. *Arterioscler Thromb Vasc Biol* **25**, 1206–1212.

36 DeJarnett N, Conklin DJ, Riggs DW, Myers JA, O'Toole TE, Hamzeh I, Wagner S, Chugh A, Ramos KS, Srivastava S *et al.* (2014) Acrolein exposure is associated with increased cardiovascular disease risk. *J Am Heart Assoc* **3**, e000934.

37 Shao B, O'brien KD, McDonald TO, Fu X, Oram JF, Uchida K & Heinecke JW (2005) Acrolein modifies apolipoprotein A-I in the human artery wall. *Ann NY Acad Sci* **1043**, 396–403.

38 McCall MR, Tang JY, Bielicki JK & Forte TM (1995) Inhibition of lecithin-cholesterol acyltransferase and modification of HDL apolipoproteins by aldehydes. *Arterioscler Thromb Vasc Biol* **15**, 1599–1606.

39 Esterbauer H, Schaur RJ & Zollner H (1991) Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med* **11**, 81–128.

40 Shao B, Pennathur S, Pagani I, Oda MN, Witztum JL, Oram JF & Heinecke JW (2010) Modifying apolipoprotein A-I by malondialdehyde, but not by an array of other reactive carbonyls, blocks cholesterol efflux by the ABCA1 pathway. *J Biol Chem* **285**, 18473–18484.

41 Weber D, Milkovic L, Bennett SJ, Griffiths HR, Zarkovic N & Grune T (2013) Measurement of HNE-protein adducts in human plasma and serum by ELISA-Comparison of two primary antibodies. *Redox Biol* **1**, 226–233.

42 Sima A & Stancu C (2002) Modified lipoproteins accumulate in human coronary atheroma. *J Cell Mol Med* **6**, 110–111.

43 Shao B, Fu X, McDonald TO, Green PS, Uchida K, O'Brien KD, Oram JF & Heinecke JW (2005) Acrolein impairs ATP binding cassette transporter A1-dependent cholesterol export from cells through site-specific modification of apolipoprotein A-I. *J Biol Chem* **280**, 36386–36396.

44 Virella G, Derrick MB, Pate V, Chassereau C, Thorpe SR & Lopes-Virella MF (2005) Development of capture assays for different modifications of human low-density lipoprotein. *Clin Diagn Lab Immunol* **12**, 68–75.

45 Wagner P & Heinecke JW (1997) Copper ions promote peroxidation of low density lipoprotein lipid by binding to histidine residues of apolipoprotein B100, but they are reduced at other sites on LDL. *Arterioscler Thromb Vasc Biol* **17**, 3338–3346.

46 Gerster R, Eloranta JJ, Hausmann M, Ruiz PA, Cosin-Roger J, Terhalle A, Ziegler U, Kullak-Ublick GA, von Eckardstein A & Rogler G (2015) Anti-inflammatory function of high-density lipoproteins via autophagy of IkappaB kinase. *Cell Mol Gastroenterol Hepatol* **1**, 171–187.e1.

47 van der Vorst EPC, Theodorou K, Wu Y, Hoeksema MA, Goossens P, Bursill CA, Aliyev T, Huitema LFA, Tas SW, Wolfs IMJ *et al.* (2017) High-density lipoproteins exert pro-inflammatory effects on macrophages via passive cholesterol depletion and PKC-NF-kappaB/STAT1-IRF1 signaling. *Cell Metab* **25**, 197–207.

48 McLaren JE, Michael DR, Ashlin TG & Ramji DP (2011) Cytokines, macrophage lipid metabolism and foam cells: implications for cardiovascular disease therapy. *Prog Lipid Res* **50**, 331–347.

49 Gargiulo S, Gamba P, Testa G, Rossin D, Biasi F, Poli G & Leonarduzzi G (2015) Relation between TLR4/NF-kappaB signaling pathway activation by 27-hydroxycholesterol and 4-hydroxynonenal, and atherosclerotic plaque instability. *Aging Cell* **14**, 569–581.

50 Lou Y, Liu S, Zhang C, Zhang G, Li J, Ni M, An G, Dong M, Liu X, Zhu F *et al.* (2013) Enhanced atherosclerosis in TIPE2-deficient mice is associated with increased macrophage responses to oxidized low-density lipoprotein. *J Immunol* **191**, 4849–4857.

51 Bird DA, Gillotte KL, Horkko S, Friedman P, Dennis EA, Witztum JL & Steinberg D (1999) Receptors for oxidized low-density lipoprotein on elicited mouse peritoneal macrophages can recognize both the modified lipid moieties and the modified protein moieties: implications with respect to macrophage recognition of apoptotic cells. *Proc Natl Acad Sci USA* **96**, 6347–6352.

52 Fujioka Y, Cooper AD & Fong LG (1998) Multiple processes are involved in the uptake of chylomicron remnants by mouse peritoneal macrophages. *J Lipid Res* **39**, 2339–2349.

53 Brundert M, Heeren J, Bahar-Bayansar M, Ewert A, Moore KJ & Rinninger F (2006) Selective uptake of HDL cholesteryl esters and cholesterol efflux from mouse peritoneal macrophages independent of SR-BI. *J Lipid Res* **47**, 2408–2421.

54 Durham KK, Chathely KM & Trigatti BL (2018) High-density lipoprotein protects cardiomyocytes against necrosis induced by oxygen and glucose deprivation through SR-B1, PI3K, and AKT1 and 2. *Biochem J* **475**, 1253–1265.

55 Murch AR & Papadimitriou JM (1981) The kinetics of murine peritoneal macrophage replication. *J Pathol* **133**, 177–183.

56 Marinkovic E, Djokic R, Lukic I, Filipovic A, Inic-Kanada A, Kosanovic D, Gavrovic-Jankulovic M & Stojanovic M (2017) Modulation of functional characteristics of resident and thioglycollate-elicited peritoneal murine macrophages by a recombinant banana lectin. *PLoS ONE* **12**, e0172469.

57 Pavlou S, Wang L, Xu H & Chen M (2017) Higher phagocytic activity of thioglycollate-elicited peritoneal macrophages is related to metabolic status of the cells. *J Inflamm (Lond)* **14**, 4.