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Modification of HDL by Reactive Aldehydes Alters Select Cardio-Protective Functions of HDL in Macrophages

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Nonstandard Abbreviations

Acro	acrolein
ApoA-I	apolipoprotein A-I
CBA	coumarin-7-boronic acid
COH	7-hydroxycoumarin
CVD	cardiovascular disease
HDL	high density lipoprotein
HNE	4-hydroxynonenal
IL	interleukin
LDL	low density lipoprotein
LPS	lipopolysaccharide
MDA	malondialdehyde
NF- κ B	Nuclear factor-kappa B

oxLDL	oxidized low density lipoprotein
PCR	polymerase chain reaction
PEG	polyethylene glycol
ROS	reactive oxygen species
SFM	serum-free media

Abstract

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, 4-hydroxynonenol (HNE), 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, acrolein- and MDA-modified HDL have impaired abilities to promote migration of primary peritoneal macrophages isolated from C57BL/6/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 NF- κ B signaling pathways. Consistent with this finding, treatment of either non-cholesterol-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

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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.

Key Words

HDL, reactive aldehydes, macrophages, inflammation, reactive oxygen species

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 including scavenger receptor BI (SR-BI) and CD36, the primary receptors for HDL[4] and oxidized LDL (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 anti-atherogenic 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]. Further, 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 cardio-protective 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]. Further, 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 impairs SR-BI-mediated cholesterol transport[23]. These findings led us to hypothesize that acrolein-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 (acrolein, HNE, and MDA) can impair some of HDL's cardio-protective 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 cardio-protective functions, we first confirmed *in vitro* oxidative modification of HDL, similar to what we had shown in our previous studies with acrolein-modified HDL[23]. As shown in **Figure 1**, immunoblot analyses demonstrated that aldehyde adduct formation promoted crosslinking of apoA-I. Further, using antibodies that recognize specific adducts

formed by these aldehydes, immunoblot analyses confirmed the presence of acrolein, 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 acrolein- and MDA-modified HDL failed to promote macrophage migration (**Figure 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 SFM alone (**Figure 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 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- κ B signaling.

When oxLDL interacts with macrophages, several downstream signaling cascades are activated, including pathways related to NF- κ B signaling[26]. Additionally, others have reported that HDL modified by myeloperoxidase can activate NF- κ B signaling[27]. To determine if aldehyde-modification alters HDL function by activating NF- κ B signaling pathways, we measured the phosphorylation status of p65 in peritoneal macrophages treated with 50 μ g/mL of oxLDL, native HDL or aldehyde-modified HDL for 0 (SFM), 5, 10, or 30 min incubations (**Figure 4A and 4B**). Treatment with lipopolysaccharide (LPS) or TNF α 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- κ B activation. These data confirmed that, unlike oxLDL, none of the aldehyde-modified forms of HDL activated the reporter line (**Figure 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 μ g/mL of oxLDL, HDL, acro-HDL, HNE-HDL, or MDA-HDL for 6 h. Transcript levels of interleukin (IL)-1 β , inducible nitric oxide synthase (iNOS), IL-10, IL-6, and TNF α were measured using qRT-PCR. Our results showed that treatment of non-cholesterol 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 (**Figures 5A-5E**). 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.

HDL 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 μ g/mL oxLDL for 24 h, followed by incubation with SFM or SFM containing 50 μ g/mL oxLDL, HDL, acro-HDL, HNE-HDL, or MDA-HDL for 6 h and transcript levels of IL-1 β , iNOS, IL-10, IL-6, and TNF α were measured as previously described (**Figures 5F-5J**). Similar to the results seen in non-cholesterol loaded macrophages, modification of HDL with reactive aldehydes does not impact inflammatory cytokine expression. Additionally, secreted cytokine levels of IL-1 β , TNF α , and IL-6 were measured by ELISA following 24 h incubation of foam cells with SFM or SFM containing 50 μ g/mL oxLDL, HDL, acro-HDL, HNE-HDL, or MDA-HDL (**Figure 6**). There were no significant changes in secretion of IL-1 β , TNF α , or IL-6 levels with any of the modified forms of HDL. To further determine the if modified HDL impacts inflammatory gene expression in the presence of a pro-inflammatory stimuli, we co-incubated primary peritoneal macrophages with 50 μ g/mL oxLDL and 50 μ g/mL native or modified HDL for 2, 6, or 24 h. Our results demonstrated that co-incubation with native HDL or the

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modified forms of HDL with oxLDL did not lead to major changes in inflammatory gene expression at any of the time points tested (**Figure 7**).

Discussion

Recent studies suggest that enhancing HDL's cardio-protective *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 (acrolein, 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 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 acrolein is a major component of cigarette smoke and other environmental atmospheric emissions, exposure to acrolein is considered a CVD risk factor[36]. In mice, acrolein consumption can induce dyslipidemia and modify lipoproteins[17]. In humans, acrolein co-localizes 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 crosslinking on whole particle HDL. *Shao et al.* demonstrated that the specific lysine residues on apoA-I modified by acrolein are similar between lipid-free apoA-I and whole-particle HDL[23]. Modification of apoA-I with MDA also predominantly leads to modification of lysine residues 56 and 118[40]. While HNE can modify lysines on other proteins, *Shao et al.* demonstrated that HNE preferentially targets all 5 histidine residues present on apoA-I[40]. 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 acrolein[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 a PEG-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 acrolein, 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 Figure 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- κ B signaling is activated[26]. It has also been suggested that HDL, too, can activate NF- κ B signaling in macrophages[27], although others have reported that HDL inhibits inflammation by suppressing NF- κ B signaling[46]. Interestingly, HDL modified by myeloperoxidase can activate NF- κ B signaling in endothelial cells[27]. One group has reported that while HDL induced expression of NF- κ B reporter genes, HDL did not lead to nuclear localization of p65[47], suggesting that HDL-dependent regulation of NF- κ B responses potentially implicates another subunit. Our data suggests that aldehyde-modified HDL does not activate NF- κ B 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- κ B signaling and increase expression of IL-8, IL-1 β , and TNF α 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 co-incubated with a pro-inflammatory stimuli (oxLDL), modified forms of HDL showed no significant changes in

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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 non-protein components of HDL. Additionally, the majority of our studies were performed using primary peritoneal macrophages that were isolated from mice four days post-injection 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 cardio-protective 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 cardio-protective functions in the fight against CVD.

Materials and Methods

1,1,3,3-Tetramethoxypropane for MDA generation, lipopolysaccharide (LPS), and catalase-polyethylene glycol (PEG) were purchased from Sigma-Aldrich (Saint Louis, MO). Boyden chambers (6.5mm insert, 5.0 μ m polycarbonate membrane) were from Costar (3421). 4-hydroxynonenal was purchased from Cayman Chemicals (Ann Arbor, MI). Acrolein was from Ultra Scientific (North Kingstown, RI). Aminoguanidine hydrochloride was from Acros Organics (Morris Plains, NJ). The coumarin-7-boronic acid (CBA) probe was generously provided by Dr. Kalyanaraman (Free Radical Research Center, Medical College of Wisconsin). ELISA kits for IL-1 β , IL-6, and TNF α were from Invitrogen (Carlsbad, CA). Human LDL (1 mg/mL) was purchased from Lee BioSolutions (Maryland Heights, MO). Human HDL (10 mg/mL), purified via sequential high-speed ultracentrifugation (density = 1.063-1.21 g/mL), was purchased from Alfa Aesar (Ward Hill, MA). It consists of

60-70% apoA-I, 20% apoA-II and 7-8% (or less) apoE. HDL preps contain approximately 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), anti-4-hydroxynonenal Michael adducts (Fisher Scientific), anti-malondialdehyde (Abcam), anti-acrolein (Abcam), anti-rabbit IgG (Amersham-GE Healthcare), anti-mouse IgG (Amersham-GE Healthcare), phospho-p65 at residue Ser536 (Cell Signaling) and total p65 (Cell Signaling). All other reagents were of analytical grade.

Lipoproteins and their chemical modification

Native HDL (1 mg/mL) was incubated with 500 μ M HNE, 250 μ M acrolein, or 15 μ M 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 μ M CuSO₄ 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 CO₂ 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 CO₂ inhalation followed by cervical dislocation, and macrophages were isolated in PBS, centrifuged at 400g for 10 min at 4 °C, and resuspended in complete RPMI media (RPMI 1640 containing 10% fetal bovine serum, 2 mM L-glutamine, 50 units/mL penicillin, 50 μ g/mL streptomycin, and 1 mM sodium pyruvate), with the exception of macrophages used for migration studies, which were resuspended in RPMI lacking serum and antibiotics and directly plated in to chamber wells. All experiments were performed within 7 days post-isolation of macrophages.

Immunoblot analysis

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Modification of HDL

HDL or HDL modified *in vitro* with HNE, MDA, or acrolein 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 acrolein (Abcam) modification.

p65 Expression

Primary peritoneal macrophages were treated with serum-free RPMI medium (SFM) or 50 µg/mL 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 pepstatin, 0.2 mM phenylmethylsulfonyl fluoride, 1 µg/mL leupeptin, and 10 µg/mL aprotinin) and phosphatase inhibitor cocktail (Sigma). Proteins were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane at 100V 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 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/wash. Membranes were stained with DAPI and the number of migrated cells was blindly quantified by fluorescence microscopy (7-8 images/condition).

ROS generation

Primary peritoneal macrophages were cholesterol-loaded with 50 µg/mL oxLDL for 1 h at 37°C in SFM. Cells were then stimulated with SFM or SFM containing 50 µg/mL oxLDL, HDL, acro-HDL, HNE-HDL, or MDA-HDL for 1 h at 37°C in the presence or absence of 500 U/mL PEG-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

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NF- κ B-reporter THP-1 cells were treated with SFM or SFM containing 50 μ g/mL 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).

Inflammatory cytokine analysis

RNA isolation and quantitative RT-PCR: Primary peritoneal macrophages (some of which were pre-treated with 50 μ g/mL 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 μ g/mL) 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 μ g total RNA using the reverse transcriptase MMLV and random primers (Invitrogen). Quantitative RT-PCR was performed using an iCycler (Biorad). Relative gene expression was determined using the $2^{-\Delta\Delta C_t}$ 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 μ g/mL oxLDL for 24 h and then incubated again with either RPMI 1640 SFM, SFM containing 50 μ g/mL oxLDL, or SFM containing 50 μ g/mL 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. Significance was set at $p < 0.05$.

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

Author Contributions

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

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Figure Legends

Figure 1: Immunoblot analysis verifies apoA-I protein crosslinking and modification of HDL with reactive aldehydes. HDL was incubated with either 250 μ M acrolein, 500 μ M HNE or 15 μ M MDA for 18 h. The reaction was stopped with a 20-fold excess of aminoguanidine-HCl. HDL proteins (20 μ g 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 acrolein, HNE or MDA. Reactive aldehyde structures are shown below each immunoblot. Data are representative of 4 independent experiments.

Figure 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 μ g/mL) 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 4 independent experiments (2 in the case of MDA-HDL), each performed in duplicate, with 3-5 fields quantified from each sample. Values are shown as mean \pm 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.

Figure 3: MDA-HDL increases ROS generation. Cholesterol-loaded primary peritoneal macrophages were treated with SFM or SFM containing 50 $\mu\text{g}/\text{mL}$ oxLDL, HDL, acro-HDL, HNE-HDL, and MDA-HDL for 1 h at $37^\circ\text{C} \pm 500$ U/mL of PEG-catalase. Peroxynitrite and/or hydrogen peroxide generation were quantified by measuring the conversion of coumarin-7-boronic acid to 7-hydroxycoumarin (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 8 experiments (4 in the case of \pm PEG-catalase addition), each performed in duplicate (each dot represents the average of the duplicates). Values are shown as mean \pm SEM.

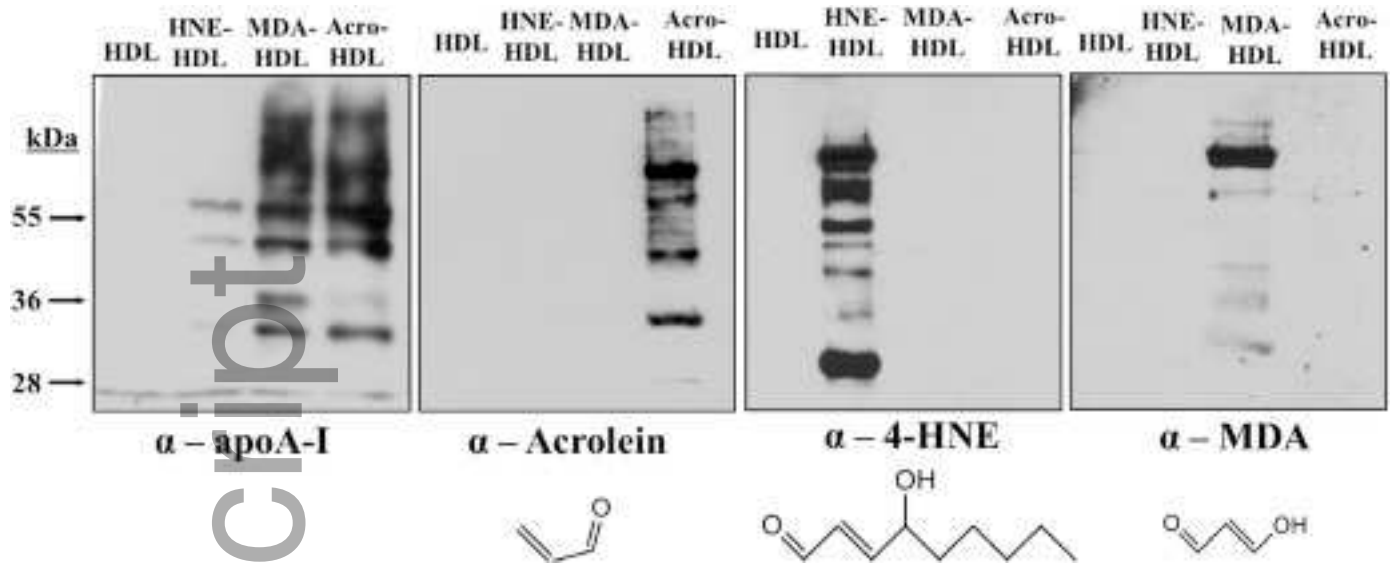
Figure 4: Aldehyde-modified HDL does not activate NF- κ B signaling. (A) Primary peritoneal macrophages were treated with SFM (0 time points) or SFM containing 50 $\mu\text{g}/\text{mL}$ HDL, acro-HDL, HNE-HDL, or MDA-HDL for 5, 15, or 30 min at 37°C . Cells were lysed and the level of phosphorylated p65 was determined by immunoblot analysis. Total p65 levels were measured as a loading control for phosphorylation. Treatment with oxLDL (50 $\mu\text{g}/\text{mL}$), LPS (100 nM), or TNF α (10 nM) served as positive controls for phosphorylation. Data shown are representative of 4 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 \pm SEM of 4 independent experiments. Statistical analyses were determined by two-way ANOVA. $*p < 0.05$, $***p < 0.001$. (C) NF- κ B-reporter THP-1 cells were treated for 1 h with SFM or SFM containing 50 $\mu\text{g}/\text{mL}$ 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 6 independent experiments and data are shown as mean \pm SEM. Statistical analyses were determined by one-way ANOVA. $***p < 0.001$ vs HDL.

Figure 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 $\mu\text{g}/\text{mL}$ 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 4 independent experiments. (F-J) Primary peritoneal macrophages were pre-treated with 50 $\mu\text{g}/\text{mL}$ oxLDL for 24 h to induce foam cell expression. Next, foam cells were incubated with SFM alone or SFM containing 50 $\mu\text{g}/\text{mL}$ 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 3 independent experiments and values are shown as mean \pm SEM. All statistical analyses were determined by one-way ANOVA. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ vs HDL.

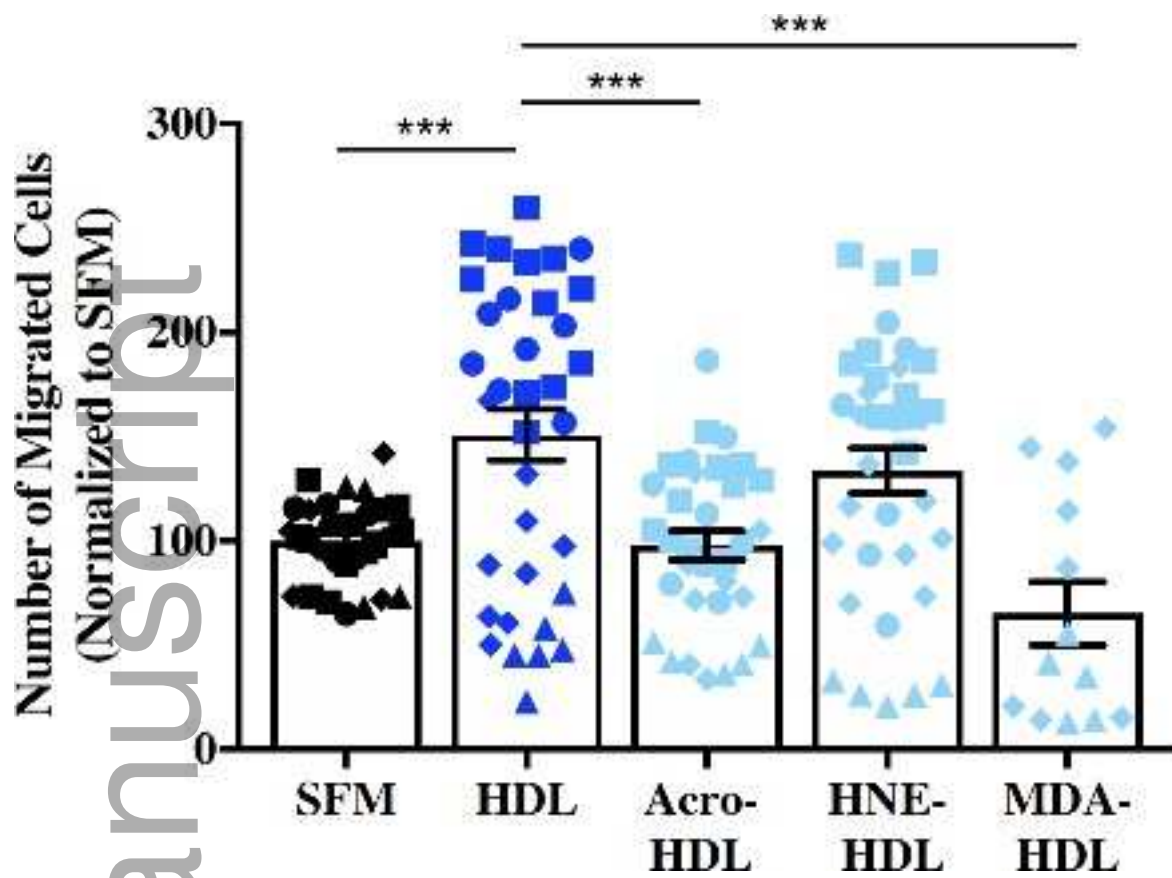
Figure 6: Modification of HDL with reactive aldehydes does not alter inflammatory cytokine secretion in foam cells. Primary peritoneal macrophages, pre-treated with 50 µg/mL oxLDL for 24 h to induce foam cell expression. Next, foam cells were incubated with SFM alone or SFM containing 50 µg/mL HDL, acro-HDL, HNE-HDL, or MDA-HDL for 24 h at 37°C. Foam cells treated with a second incubation of 50 µg/mL 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 3 independent experiments and values are shown as mean ± SEM. All statistical analyses were determined by one-way ANOVA. **p<0.01 vs HDL.

Figure 7: Co-incubation 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 oxLDL in combination with 50 µg/mL 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 3 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.

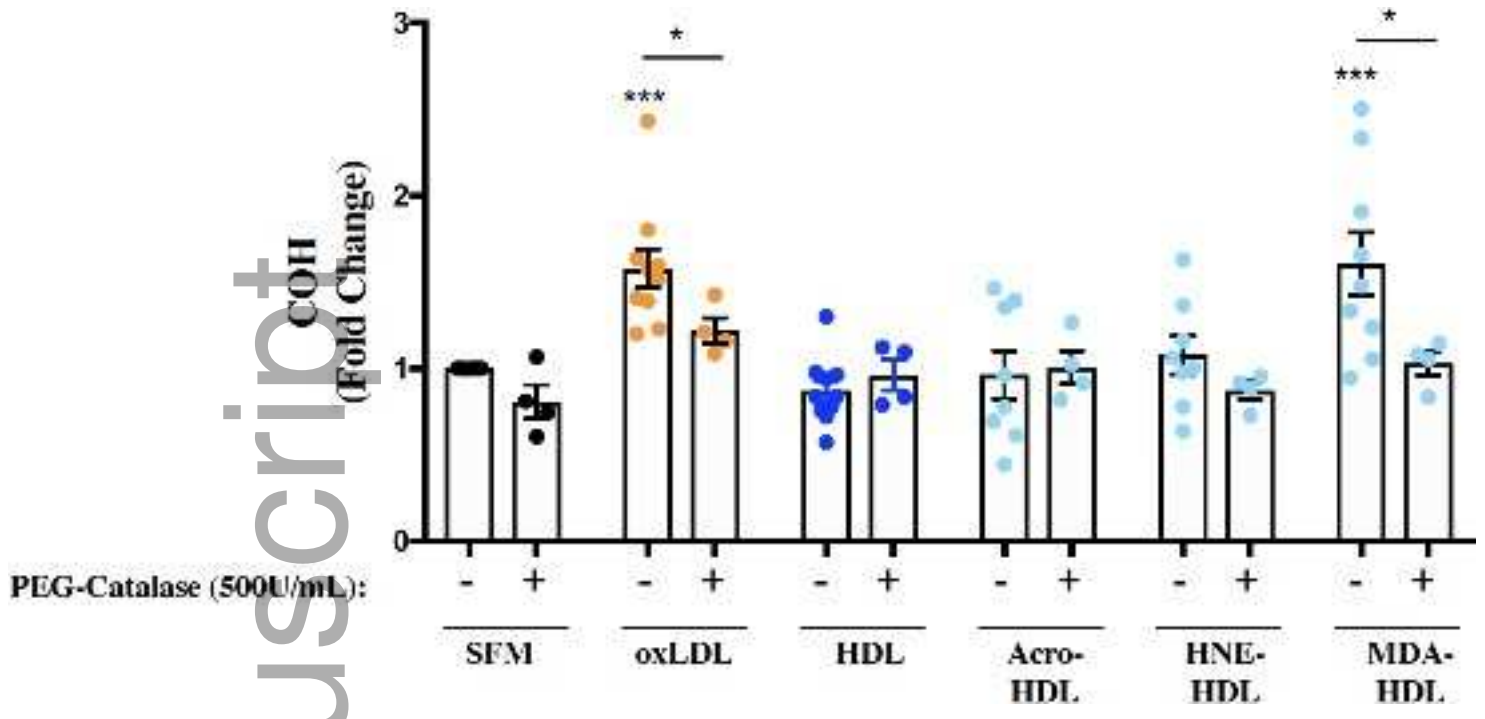
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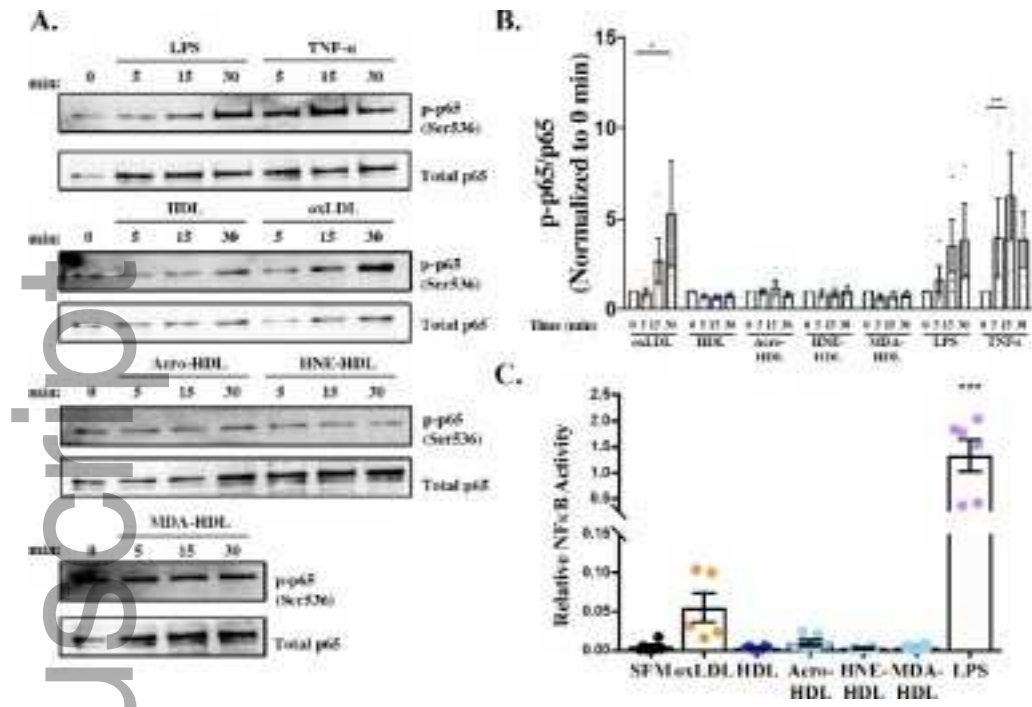
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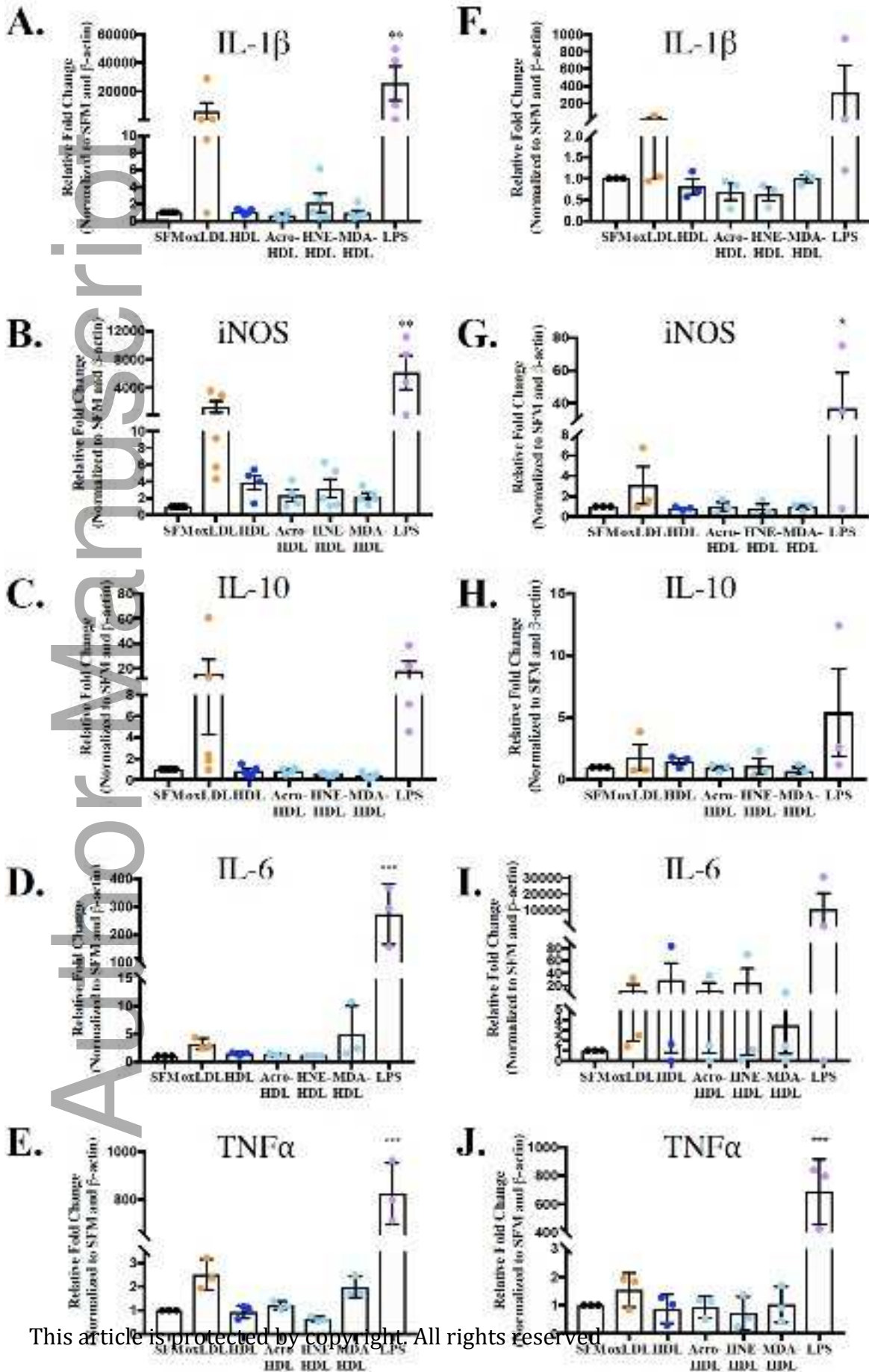
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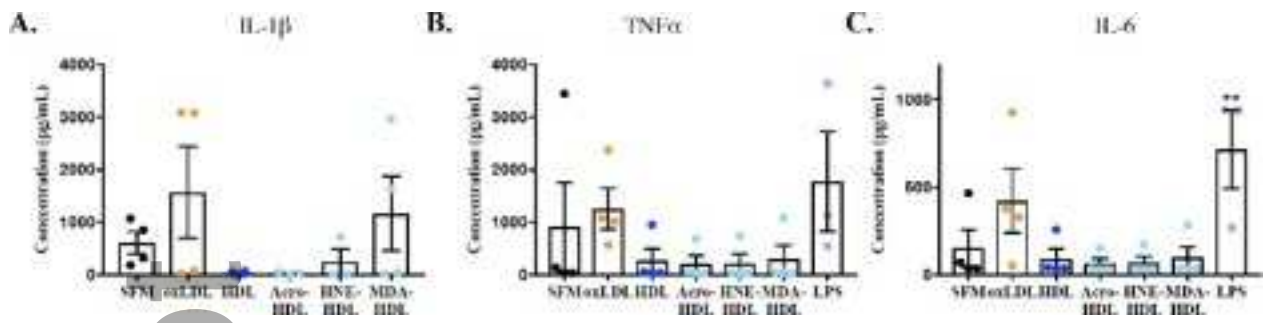
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Non-Cholesterol Loaded

Cholesterol Loaded

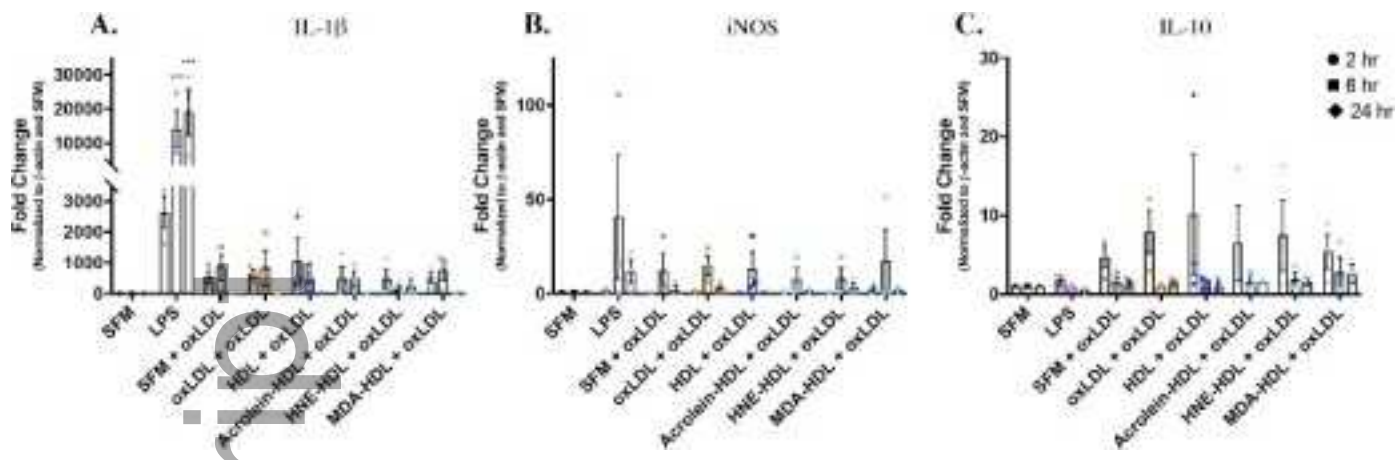


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