Inflammatory remodeling of the HDL proteome impairs cholesterol efflux capacity[S]

Tomáš Vaisar,[1,*] Chongren Tang,[*] Ilona Babenko,[*] Patrick Hutchins,[*] Jake Wimberger,[*] Anthony F. Suffredini,[†] and Jay W. Heinecke[*]

Department of Medicine,[*] University of Washington, Seattle, WA 98105; and Critical Care Medicine Department,[†] National Institutes of Health, Bethesda, MD 20892

Abstract **Recent studies demonstrate that HDL's ability to promote cholesterol efflux from macrophages associates strongly with cardioprotection in humans independently of HDL-cholesterol (HDL-C) and apoA-I, HDL's major protein. However, the mechanisms that impair cholesterol efflux capacity during vascular disease are unclear. Inflammation, a well-established risk factor for cardiovascular disease, has been shown to impair HDL's cholesterol efflux capacity. We therefore tested the hypothesis that HDL's impaired efflux capacity is mediated by specific changes of its protein cargo. Humans with acute inflammation induced by low-level endotoxin had unchanged HDL-C levels, but their HDL-C efflux capacity was significantly impaired. Proteomic analyses demonstrated that HDL's cholesterol efflux capacity correlated inversely with HDL content of serum amyloid A (SAA)1 and SAA2. In mice, acute inflammation caused a marked impairment of HDL-C efflux capacity that correlated with a large increase in HDL SAA. In striking contrast, the efflux capacity of mouse inflammatory HDL was preserved with genetic ablation of SAA1 and SAA2. Our observations indicate that the inflammatory impairment of HDL-C efflux capacity is due in part to SAA-mediated remodeling of HDL's protein cargo.**—Vaisar, T., C. Tang, I. Babenko, P. Hutchins, J. Wimberger, A. F. Suffredini, and J. W. Heinecke. **Inflammatory remodeling of the HDL proteome impairs cholesterol efflux capacity.** ***J. Lipid Res.*** **2015.** 56: **1519–1530.**

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Epidemiological and clinical studies have reported a robust inverse association of HDL-cholesterol (HDL-C) levels with risk of coronary artery disease (CAD) (1). Moreover, mice with genetically engineered deficiencies in proteins involved in HDL metabolism have atherosclerotic phenotypes (2). These observations provide strong evidence that HDL plays a causal role in vascular disease and have triggered intense interest in targeting HDL for therapeutic intervention.

Several recent observations have cast doubt on the hypotheses that HDL-C levels relate to CAD risk in humans and that elevating HDL-C is therapeutic (3, 4). For example, genetic variations that alter levels of HDL-C do not always associate with CAD risk, and interventions that elevate HDL-C do not necessarily reduce cardiovascular events in humans with established CAD (5). Taken together, these observations indicate that HDL is complex and that simply quantifying HDL-C might be a poor way to assess HDL function (6, 7).

The ability of HDL (or serum HDL, serum depleted of apoB-containing lipoproteins) to promote sterol efflux from cultured macrophages incubated with radiolabeled cholesterol can vary markedly, despite similar levels of HDL-C and apoA-I (8). Therefore, HDL-C is not necessarily the major determinant of HDL's macrophage sterol efflux capacity in this system. Importantly, the efflux capacity of serum HDL is lower in individuals with prevalent CAD (9–11). A recent study of a large cohort, initially free of CAD, demonstrated that sterol efflux associates strongly and negatively with the risk of future cardiac events (12). This association was strengthened by multivariate adjustment, suggesting that impaired HDL function affects incident cardiovascular risk by processes distinct from those involving HDL-C, LDL-cholesterol (LDL-C), and other traditional lipid risk factors. Together, these observations indicate that the sterol efflux capacity of HDL might be a marker, and perhaps a mediator, of atherosclerotic

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Abbreviations: CAD, coronary artery disease; FDA, Food and Drug Administration; FDR, false discovery rate; HDL-C, HDL-cholesterol; HSD, honest significant difference; LDL-C, LDL-cholesterol; LXR, liver X receptor; NIH, National Institutes of Health; SAA, serum amyloid A; SAA1/2, serum amyloid A isoforms 1 and 2 (SAA1.1 and SAA2.1) because SAA1 and SAA2 are highly homologous and not readily distinguishable by proteomics.

[1] To whom correspondence should be addressed.
e-mail: tvaisar@u.washington.edu

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burden that is independent of HDL-C and apoA-I levels. However, the molecular factors controlling the sterol efflux capacity of serum HDL are poorly understood (8, 13). For example, a recent study found that the majority of radiolabeled cholesterol released from macrophages did not reside in HDL, suggesting that impaired sterol efflux capacity does not necessarily reflect alterations in HDL itself (11).

Inflammation and metabolic disorders have been proposed to convert HDL to a dysfunctional form lacking anti-atherogenic properties (14–19). For example, HDLs from mice and humans with acute inflammation are less able to promote sterol efflux from macrophages (20–23). Detailed metabolic studies demonstrate that multiple steps in reverse cholesterol transport from macrophages are inhibited in inflamed mice (21, 22). Furthermore, proteins cotransported with HDL in plasma, such as paraoxonase and clusterin, have been proposed to have antioxidant and anti-inflammatory properties, and their levels change in response to inflammation (15, 24–27). Loss of sterol efflux capacity and of anti-inflammatory and/or antioxidant proteins, perhaps in concert with gain of pro-inflammatory proteins, may thus be key factors in generating dysfunctional HDL (7).

In the current study, we investigated the protein cargo and function of HDL isolated from humans and mice with inflammation. Our observations indicate that inflammatory remodeling mediated by serum amyloid A (SAA) is one mechanism for generating HDL whose capacity to promote sterol efflux is impaired.

METHODS

Human studies

The study was approved by the institutional review board of the Clinical Research Center, New Orleans, and reviewed by the institutional review board of the National Institute of Allergy and Infectious Diseases (28). Twelve healthy male volunteers aged 22–49 years underwent a complete history and physical examination prior to entering the study. All subjects had normal physical exams and routine blood and urine chemistries, and none were taking medications or had known medical conditions. The 12 subjects were randomly assigned to the endotoxin injection groups. The subjects were injected iv with 1 ng/kg or 2 ng/kg National Institutes of Health (NIH) "equivalent" endotoxin (n = 4; Clinical Center Reference Endotoxin; *E. coli* O:113, Pharmacy Development Service, Clinical Center, NIH, Bethesda, MD) or 4 ng/kg (n = 4) of Food and Drug Administration (FDA) endotoxin (*E. coli* O:113, Lot EC-5; H. D. Hochstein, Center for Biologics Evaluation and Research, FDA, Bethesda, MD) (28). Consistent with previous studies with these endotoxin preparations, we found that high-sensitivity C-reactive protein levels in the group that received the 4 ng/kg dose of FDA endotoxin were comparable to those in the group that received the 1 ng/kg dose of NIH endotoxin. Because SAA and CRP levels were similar in the 1 ng/kg NIH endotoxin and 4 ng/kg FDA endotoxin groups, we combined the subjects into a 1 ng/kg endotoxin group in subsequent analyses.

Mouse studies

Experiments with mice lacking SAA1.1 and SAA2.1 (termed here as *Saa1/2*$^{-/-}$ mice) were performed at the University of Kentucky. *Saa1/2*$^{-/-}$ mice and littermate WT mice were in a 129SvEv/C57BL/6 background (29). Experiments with C57BL/6 mice were performed at the University of Washington. Animal experiments were approved by the Institutional Animal Care and Use Committees of each institution.

All mice were 8–12 weeks of age, fed a low-fat diet, and maintained in a pathogen-free facility with 12 h light-dark cycles and free access to food and water. Acute inflammation was induced in female mice by subcutaneous injection of silver nitrate (0.5 ml, 2% w/w) (30, 31). Control mice were injected with 0.5 ml of sterile normal saline. Blood anticoagulated with EDTA was collected 24 h after the injection, and plasma was prepared by centrifugation. Plasma samples from two mice were combined for HDL isolation.

HDL

HDL was isolated using sequential ultracentrifugation (d = 1.063–1.21 g/ml), as described for mouse (29, 32) and human HDL (33, 34). HDL was stored on ice in the dark and used within 1 week of preparation.

SAA enrichment of HDL

Human HDL (200 μg protein) was incubated alone or with recombinant SAA1 (PeproTech) [ratio 6:1 and 2:1 (w/w) HDL protein:SAA1 protein] for 3 h at 25°C (35, 36). HDL was then re-isolated by ultracentrifugation (d < 1.21 g/ml).

Cholesterol efflux assays

J774 macrophages were loaded with cholesterol for 24 h at 37°C in DMEM containing acetylated-LDL (50 μg protein/ml) and [^{3}H]cholesterol (1 μCi/ml). The cells were then washed with DMEM and incubated for an additional 24 h in DMEM containing cAMP (0.5 mM) and the liver X receptor (LXR) agonist, T0901317 (5 μM) (37). Efflux of [^{3}H]cholesterol was measured after a 6 h incubation with DMEM supplemented with 0.1% BSA without or with 30 μg/ml of HDL protein. Cholesterol efflux mediated by HDL was calculated as the percentage of total [^{3}H]cholesterol (medium plus cell) released into the medium after the value obtained with DMEM/BSA alone was subtracted.

LC-MS/MS analysis

Tryptic digests of mouse HDL (2 μg protein) were injected onto a C18 trap column (Paradigm Platinum Peptide Nanotrap, 0.15 × 50 mm; Michrom Bioresources, Inc.), desalted (50 μl/min) for 5 min with 1% acetonitrile/0.1% formic acid, eluted onto an analytical reverse-phase column (0.15 × 150 mm, Magic C18AQ, 5 μm, 200 Å; Michrom Bioresources, Inc.), and separated at a flow rate of 1 μl/min over 180 min, using a linear gradient of 5–35% buffer B (90% acetonitrile, 0.1% formic acid) in buffer A (5% acetonitrile, 0.1% formic acid) on a Paradigm M4B HPLC (Michrom Bioresources, Inc.). Positive ion mass spectra were acquired with ESI in a linear ion trap mass spectrometer (LTQ; Thermo Electron Corp., San Jose, CA) with data-dependent acquisition (one MS survey scan followed by MS/MS scans of the eight most abundant ions in the survey scan). An exclusion window of 45 s was used after two acquisitions of the same precursor ion. Alternatively, the HDL from WT mice, with and without inflammation, were analyzed on Thermo QE+ mass spectrometer using data-independent acquisition (*m/z* 20 window with *m/z* 10 overlap, 17,500 resolution, normalized collision energy 25) (38) and data for apoA-I native and oxidized methionine- and tryptophan-containing peptides were analyzed and quantified using Skyline (39).

Tryptic digests of human HDL (2 μg protein) were injected onto a C18 trap column (Magic AQ C18 200A, 5 μm, 0.1 × 20 mm;

Michrom Bioresources, Inc.), desalted for 15 min with water/0.1% formic acid (4 μl/min), eluted onto an analytical column (Magic AQ C18 90A, 5 μm, 0.1 × 200 mm; Michrom Bioresources, Inc.), and separated at a flow rate of 0.4 μl/min over 180 min, using a linear gradient of 5–35% buffer D (acetonitrile/0.1% formic acid) in buffer C (0.1% formic acid) on a NanoAquity HPLC (Waters, Milford, MA). Positive ion mass spectra were acquired with ESI in a hybrid linear ion trap-Orbitrap mass spectrometer (LTQ Orbitrap XL; Thermo Fisher, San Jose, CA) with data-dependent acquisition of MS/MS scans (linear ion trap) on the eight most abundant ions in the survey scan (Orbitrap, resolution 60,000). An exclusion window of 45 s was used after two repeated acquisitions of the same precursor ion.

Protein identification and quantification

HDL protein was digested and analyzed essentially as previously described (40). MS/MS spectra were matched against the human International Protein Index (IPI) database (mouse v.3.54, January 2009; human v.3.72, April 2010), using the SEQUEST (v2.7) search engine with fixed Cys carbamidomethylation and variable Met oxidation modifications. SEQUEST results were further validated with Trans-Proteomic Pipeline tools, using an adjusted probability of ⩾0.90 for peptides and ⩾0.95 for proteins. Proteins considered for analysis had to be detected in ⩾4 analyses with ⩾2 unique peptides. Relative protein quantification was performed using spectral counting (41). Significant differences in spectral counts were identified using the combination of *G*-test and *t*-test together with permutation analysis to estimate false discovery rate (FDR) (42). The abundance of SAA relative to apoA-I was estimated using extracted ion chromatograms and sum of peak areas for the three most abundant peptides from each protein according to the approach of Silva et al. (43).

Biochemical and immunochemical assays

HDL protein levels were quantified by Bradford assay and corrected to set of standards quantified by Lowry assay. HDL and plasma lipids were quantified biochemically [cholesterol and cholesteryl ester (Amplex Red, Invitrogen), triglycerides (Cayman Chemical, Ann Arbor, MI), phospholipids (Wako Chemical, Richmond, VA)]. CRP (Invitrogen, Carlsbad, CA) and SAA were measured by ELISA (Invitrogen, Camarillo, CA). HDL particle concentration was measured by calibrated ion mobility analysis (44).

Statistical analyses

Analyses were performed with the Statistical Package for the Social Sciences (SPSS v.19) and R statistical package (v.2.14). Results represent means and SEMs. The statistical significance of differences between groups was evaluated by the two-tailed Student's *t*-test or ANOVA with Tukey's honest significant difference (HSD) post hoc test.

RESULTS

The human study involved 12 male subjects (40 ± 7 years old; fasting plasma levels of lipids and inflammatory proteins: HDL-C, 39.9 ± 3.8 mg/dl; LDL-C, 107 ± 4.9 mg/dl; CRP, 2.0 ± 0.6 mg/l; SAA, 14.3 ± 5.3 μg/ml). All subjects were apparently healthy, normolipidemic, not using medications, and had no symptoms or signs of inflammation. To induce inflammation, subjects were injected with the equivalent of 1 ng/kg (n = 8) or 2 ng/kg (n = 4) of reference endotoxin (28). All of the subjects developed the hallmarks of acute inflammation, as indicated by: *i*) symptoms (chills, headache) and signs (elevated body temperature); *ii*) a rapid transient elevation of inflammatory cytokines; and *iii*) a dose-dependent increase in blood levels of CRP and SAA after the injection (28).

Acute inflammation induced by low doses of endotoxin remodel the human HDL proteome and impair cholesterol efflux from macrophages

To investigate the ability of HDL and inflammatory HDL to promote sterol efflux from macrophages, we used ultracentrifugation (d = 1.063–1.21 g/ml) to isolate HDL from blood collected from each subject 30 min prior to (control HDL) and 24 h after (inflammatory HDL) the injection of saline or low doses of endotoxin. At these doses, endotoxin induced only a mild inflammation. The efflux capacity of inflammatory HDL was reduced (**Fig. 1A**) by ∼10% at 1 ng/kg of endotoxin and by ∼20% at 2 ng/kg (P = 0.009, ANOVA with Tukey's HSD). These observations are consistent with previous reports that the inflammatory response impairs HDL's ability to remove sterol from macrophages in mice and rabbits, as well as in humans (20–23).

Acute inflammation induced by low doses of endotoxin selectively increases SAA levels in HDL

To assess how changing the relative abundance of proteins in HDL might affect the lipoprotein's function, we analyzed control and inflammatory HDL with MS. LC-ESI-MS/MS of tryptic digests of the HDL identified with high confidence (estimated FDR 2%) 82 proteins associated with HDL (supplementary Table 1). This approach identified all of the proteins found in our earlier studies of HDL (33, 34). We also identified a number of proteins that were not known to reside in HDL (45). The fact that we identified more proteins in this analysis than in our previous studies likely reflects improvements in LC and mass spectrometers.

To determine which HDL proteins changed in relative abundance when humans were challenged with endotoxin, we used the *G*-test and *t*-test to find significant differences in spectral counts, a measure of relative protein abundance (42). We estimated the FDR by using the same statistical tests with all possible permutations of the data (42). Permutation analysis revealed that $G > 3.5$ (*G*-test) and $P < 0.001$ (*t*-test) yielded the most true-positive changes in protein abundance after endotoxin challenge, with an estimated FDR of 4% (data not shown).

Using these stringent statistical criteria (Fig. 1D, supplementary Table 1), the only proteins that changed in relative abundance in inflammatory HDL were the acute-phase proteins, SAA1 and SAA2. The relative concentration of SAA1/2 (SAA1 and SAA2 are quantified together because the proteins share 95% sequence homology) increased 6-fold in the subjects who received the 1 ng/kg dose of endotoxin and 9-fold in those who received the 2 ng/kg dose.

We used two independent approaches to confirm that levels of SAA1 and SAA2 were elevated. First, we used extracted ion chromatograms to quantify the relative

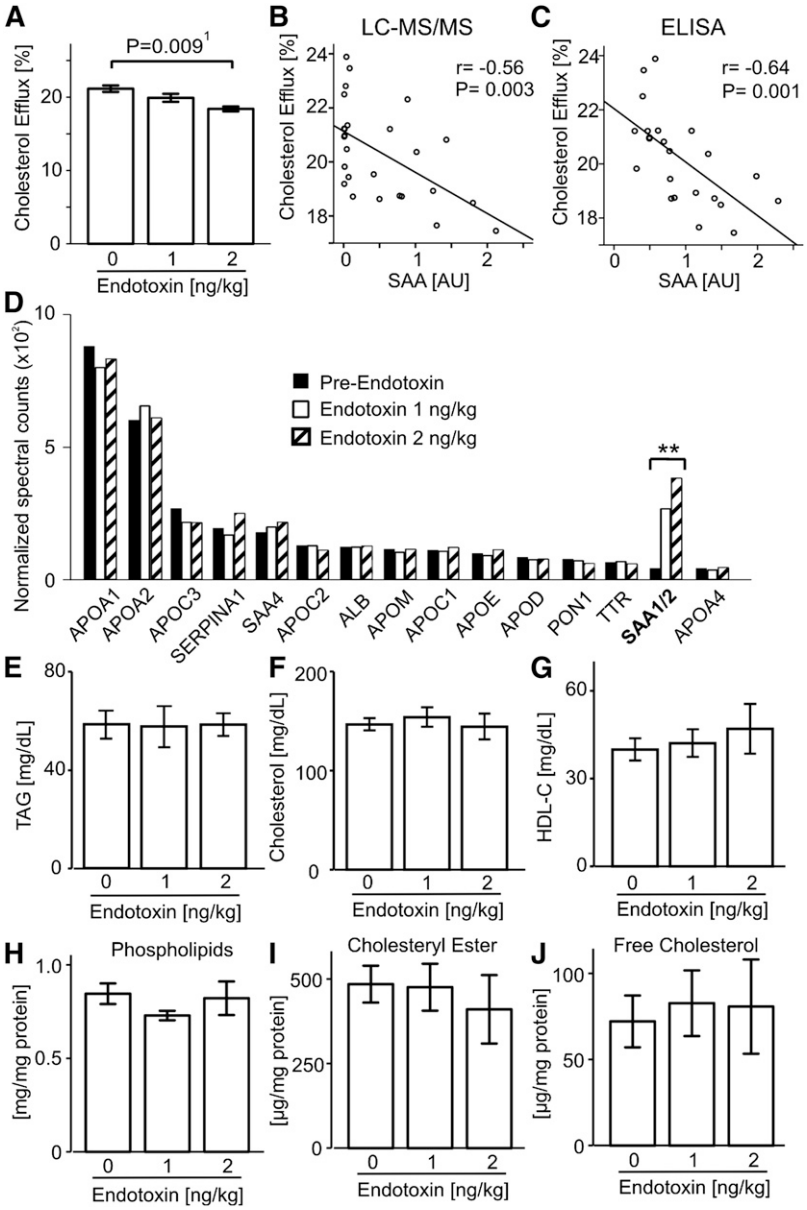

Fig. 1. HDL composition and function in control and endotoxin-treated subjects. A: HDL was isolated by ultracentrifugation (d = 1.063–1.210 g/ml) from EDTA plasma of subjects before (endotoxin 0, n = 12) and 24 h after an endotoxin injection [1 ng/kg (n = 8) or 2 ng/kg (n = 4)]. Efflux of [^{3}H]cholesterol from macrophages to cell medium was measured in cholesterol-loaded cAMP and LXR agonist-treated J774 macrophages incubated with 30 µg/ml of HDL in serum-free medium for 6 h. B: Relative abundance of SAA in HDL was determined by the extracted ion chromatogram of peptide SFFSFLGEAFDGAR common to both SAA1 and SAA2 determined by LC-ESI-MS/MS. C: Relative abundance of SAA in HDL was determined by ELISA, using an antibody that reacts with SAA1, SAA2, and SAA4. Correlation in (B) and (C) was calculated using Spearman's ranked-order method. D: LC-ESI-MS/MS analysis of control and inflammatory HDL. Only the 15 most abundant proteins are shown. E–G: Plasma lipids and HDL-C. H–J: HDL lipid composition. [1]ANOVA with Tukey's HSD. AU, arbitrary units. **Significant change in protein abundance as assessed by $G > 3.5$ (G-test) and $P < 0.001$ (t-test), with an estimated FDR of 4%.

abundance of peptides unique to SAA1 and SAA2, because this approach quantifies the individual isoforms and estimates protein ratios more accurately than spectral counting. This approach demonstrated 19- and 30-fold increases in ion current for peptides specific for SAA1 (FFGHGAEDSLADQAANEWGR) and SAA2 (GPGGAWAAEVISNAR) in the subjects treated with 1 ng/kg and 2 ng/kg of endotoxin, respectively. Quantification of SAA with an ELISA (which detects all the SAA isoforms) similarly indicated 15- and 17-fold increases of SAA in HDL with 1 and 2 ng/kg of endotoxin. Based on extracted ion chromatograms (see Methods), we calculated that SAA represented approximately 13 and 20% of apoA-I with 1 and 2 ng/kg doses of endotoxin, but less than 1% of apoA-I in HDL from the same subjects prior to endotoxin challenge.

To assess how changing the relative abundance of SAA in HDL might affect the lipoprotein's function, we measured the abilities of control HDL and inflammatory HDL to promote cholesterol efflux from macrophages (Fig. 1B, C). There was a strong inverse linear correlation between HDL sterol efflux ability and levels of SAA in HDL, as assessed by MS ($r = -0.56$, $P = 0.003$) and biochemically ($r = -0.64$, $P = 0.001$). These observations are consistent with the hypothesis that SAA impairs HDL's ability to promote sterol efflux from macrophages. Because regression analysis indicates that SAA levels only explain approximately 36% of the variance in efflux capacity, other factors also likely contribute to altered efflux capacity, including interindividual differences in sterol efflux capacity without inflammation.

Inflammation with low doses of endotoxin fails to affect plasma lipids in humans

At the time point and low endotoxin doses used in our study, we did not observe significant changes in HDL-C or other plasma lipids (Fig. 1E–G), the lipid composition of isolated HDL (Fig. 1H–J), or plasma LCAT activity (data not shown). High-resolution size-exclusion chromatography also failed to reveal alterations in the distribution of plasma cholesterol in the endotoxin-treated subjects (data not shown). These observations, in concert with our demonstration of increased levels of SAA1/2 in inflammatory HDL of humans treated with endotoxin, suggest that alterations in the lipoprotein's protein cargo impair its efflux capacity.

SAA1 generates dysfunctional HDL in a model system

To determine whether SAA1 alters sterol efflux from macrophages in vitro, human HDL was first incubated with recombinant SAA1 at various ratios for 3 h at 25°C, and the lipoproteins were then re-isolated by ultracentrifugation (d = 1.21 g/ml). SDS-PAGE and densitometric quantification of Imperial Blue-stained proteins (**Fig. 2A, B**) indicated that SAA1 accounted for ~10–30% of the protein in re-isolated HDL, levels markedly higher than those observed with mild inflammation in humans (see above) and similar to that observed in inflammatory HDL from mice (see below). The molar fraction of SAA1 incorporated into HDL increased in concert with the loss of the ability of the lipoprotein to promote sterol efflux from macrophages (Fig. 2C). ESI-MS/MS of tryptic digests demonstrated that apoA-I and SAA1 were the major proteins that changed in relative abundance when HDL was enriched with SAA1 in vitro (Fig. 2D). These observations support the proposal that SAA enrichment of HDL in vitro impairs its sterol efflux capacity with macrophages.

Acute inflammation alters plasma lipids and remodels the HDL proteome in mice

The human HDL proteome has been extensively investigated (26, 33, 46, 47), but much less is known about the protein composition of mouse HDL. Previous studies have shown differences in the apoA-I and apoA-II content and apparent sizes of mouse and human HDL (48). We therefore first characterized the HDL proteome of C57BL/6J mice isolated by ultracentrifugation (d = 1.063–1.21 g/ml) and compared it to human HDL. Of the 75 and 82 proteins identified in mouse and human HDL, respectively, 17 were identified only in human HDL, while 8 proteins were detected only in mouse HDL (supplementary Fig. 1). Nine of the proteins identified in only mice or human HDL lacked orthologs in the other species. However, gene ontology analysis of the identified proteins revealed that the HDLs of both species contain the same functional categories of proteins. These data demonstrate that the HDL proteomes of mice and humans share many proteins, but also are distinct.

To examine the impact of inflammation on the protein cargo of mouse HDL, we injected saline or silver nitrate subcutaneously into WT C57BL/6 mice, and collected blood 24 h later. We used silver nitrate because this model of inflammation, while unsuitable for human studies, has been widely studied in animal models (30, 31). It also avoids the possibility that any endotoxin adsorbed by HDL would affect cell-based assays. With acute inflammation, HDL isolated from the WT animals exhibited ~20% less efflux capacity in J774 macrophages (**Fig. 3A**; $P = 0.0002$) and exhibited 40% less ability to decrease cholesterol mass with macrophage foam cells isolated from the peritoneum of cholesterol-fed $Ldlr^{-/-}$ mice (supplementary Fig. 2).

Proteomic analysis identified 81 proteins with high confidence in HDL isolated from inflamed mice. As expected, based on higher level of inflammation in mice in contrast to HDL isolated from endotoxin-treated humans, one-third of the proteins in inflamed mouse HDL had changed their relative abundance (Fig. 3E, supplementary Table 2). Peptides for SAA1 and SAA2 were virtually undetectable in HDL of mice injected with saline (Fig. 3B–D). In inflamed mice, SAA1 and SAA2 were among the most abundant HDL proteins (Fig. 3E). Extracted ion chromatograms of peptides specific to each SAA isoform demonstrated a marked increase in levels of both SAA1 and SAA2 in inflammatory HDL (Fig. 3B–D). Based on extracted ion chromatograms (see Methods), we calculated that SAA1/2 increased from less than 1% of apoA-I in control HDL to approximately 70% of apoA-I in inflammatory HDL.

With acute inflammation, 18 proteins in HDL increased and 11 decreased in relative abundance (supplementary Table 2). Many of those proteins have previously been shown to change in abundance in acute-phase HDL (17, 49), including apoJ, apoE, apoA-V, phospholipid transfer protein, lipopolysaccharide binding protein, multiple apoCs, apoM, LCAT, and PON1. Haptoglobin was detected in inflammatory HDL but not control HDL. Inflammation did not significantly affect levels of the α and β chains of hemoglobin, which have been proposed as markers of dysfunctional HDL (50). While inflammation induced profound changes in the HDL proteome, it only modestly altered plasma phospholipid and total cholesterol levels (Fig. 3F, G). In contrast, inflammation failed to alter HDL-C or HDL particle concentration (Fig. 3H, I).

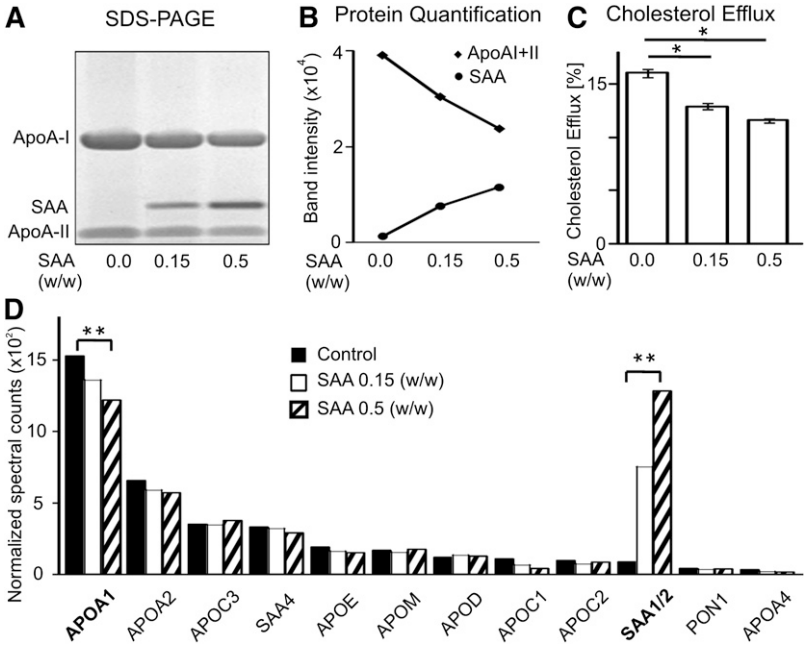

Fig. 2. Cholesterol efflux capacity and composition of human HDL enriched with SAA in vitro. Human HDL was incubated with buffer or recombinant SAA1 (SAA1:HDL protein, 1:6, 1:2, w:w). Following a 3 h incubation at 25°C, SAA-enriched HDL was isolated from the reaction mixture by ultracentrifugation (d = 1.121 g/ml). A: SDS-PAGE with Coomassie blue staining of re-isolated HDLs. B: Abundance of SAA, apoA-I, and apoA-II in HDL quantified by densitometry. C: Cholesterol efflux from radiolabeled macrophages to re-isolated HDLs. D: LC-ESI-MS/MS analysis of re-isolated HDLs. Only the 12 most abundant proteins are shown. $*P < 0.05$. $**G > 3.5$ (*G*-test) and $P < 0.001$ (*t*-test), FDR 4%.

Acute inflammation does not alter oxidation state of HDL

In addition to altering HDL, proteome inflammation may also increase HDL and apoA-I oxidation, a modification known to impair HDL sterol efflux capacity (10, 51–53). We therefore interrogated the proteomics data of WT mice with and without inflammation to determine whether acute inflammation increased oxidation of apoA-I methionine or tryptophan residues (10, 54). Extracted ion chromatograms of tryptic peptides showed no difference in oxidized peptides containing Met91, Met180, or Trp77 between HDL isolated from control and inflamed mice (data not shown), demonstrating that oxidation is not contributing to impaired sterol efflux of inflammatory HDL.

HDL of inflamed SAA-deficient mice is protected from loss of cholesterol efflux capacity

We next compared the abilities of HDLs isolated from WT and *Saa1/2*$^{-/-}$ mice on the same genetic background, without and with inflammation, to promote sterol efflux from macrophages. In the absence of inflammation, there was no difference between the two strains (**Fig. 4A**). The efflux capacity of HDL from inflamed WT animals exhibited ~40% loss of efflux capacity compared with HDL of WT animals without inflammation ($P < 0.001$, n = 6). In striking contrast, HDL from inflamed *Saa1/2*$^{-/-}$ mice exhibited no impairment in the ability to promote sterol efflux (Fig. 4A). The efflux capacity of HDL from inflamed WT mice and inflamed *Saa1/2*$^{-/-}$ mice were significantly different ($P = 0.008$, n = 6). HDL isolated from inflamed WT mice was significantly enriched in protein content compared with HDL from inflamed *Saa1/2*$^{-/-}$ mice (Fig. 4B, supplementary Table 3). SDS-PAGE of Coomassie-stained proteins indicated that SAA content of HDL of inflamed *Saa1/2*$^{-/-}$ mice was markedly reduced (Fig. 4C); MS/MS analysis indicated that SAA1/2 was undetectable in these HDLs (see below).

Inflammation-induced alterations in plasma and HDL lipid composition are similar in WT and *Saa1/2*$^{-/-}$ mice

Previous studies demonstrate that inflammation markedly alters the lipid composition of plasma and HDL, and that lipids can contribute to the sterol efflux capacity of HDL by certain pathways (e.g., ABCG1) (49, 55, 56). We therefore quantified levels of specific lipid classes in plasma and in HDL of WT and *Saa1/2*$^{-/-}$ mice, with and without inflammation.

Plasma levels of HDL-C, cholesterol, and phospholipids were altered significantly 24 h after injection of silver nitrate (Fig. 4E–G). However, the patterns of change in plasma lipids and HDL-C during inflammation were similar in WT and *Saa1/2*$^{-/-}$ mice (Fig. 4E–G). Moreover, there were no significant differences in the inflammation-induced pattern of changes in phospholipid or cholesteryl ester of HDL isolated from WT and *Saa1/2*$^{-/-}$ mice (Fig. 4H, I). The free cholesterol content of HDL increased significantly with inflammation in both strains. The enrichment of HDL from inflamed *Saa1/2*$^{-/-}$ mice was significantly greater than that of inflamed WT mice (Fig. 4J). Together with the data on humans and on HDL enriched in vitro with SAA1, these observations suggest that changes induced by inflammation in the proportions of lipid classes

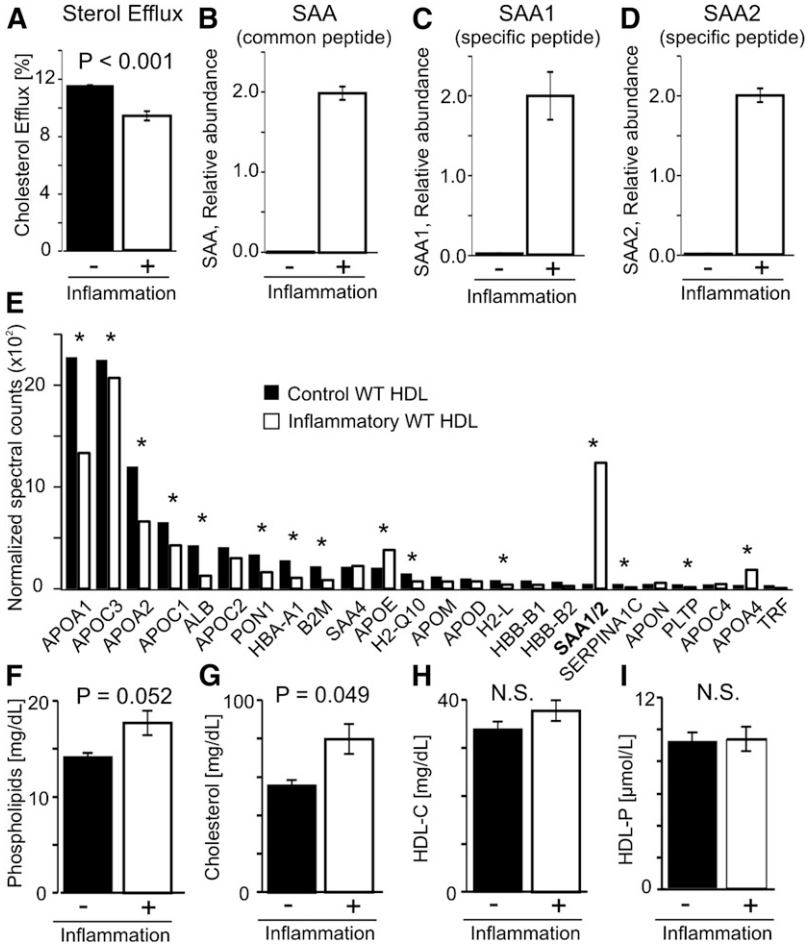

Fig. 3. Macrophage cholesterol efflux capacity of HDL isolated from WT mice, with and without inflammation. HDL was isolated from plasma harvested 24 h after subcutaneous injection of normal saline (n = 4) or silver nitrate (n = 4) in C57BL/6 mice. A: Cholesterol efflux from radiolabeled cholesterol-loaded macrophages to mouse HDL. B–D: Relative abundance of SAA in HDL was determined by LC-ESI-MS/MS and extracted ion chromatograms of a peptide, GPGGVWAAEK, common to both SAA1 and SAA2 or peptides unique to SAA1 (EAFQEFFGR) and SAA2 (ESFQEFFGR). E: Relative abundance of proteins in control and inflammatory HDL. Only the 24 most abundant proteins are shown. F–I: Plasma lipid levels and HDL particle concentration. *$G > 3.5$ (G-test) and $P < 0.001$ (t-test), FDR 4%.

in HDL are unlikely to explain the normal efflux capacity of HDL isolated from inflamed *Saa1/2*$^{-/-}$ mice. However, it is possible that inflammation-induced alterations in a specific species of lipids [such as phosphatidylserine (57)] contribute to impairment of sterol efflux capacity.

SAA deficiency reduces HDL protein remodeling during inflammation

These observations suggest that increased levels of SAA1/2 and/or lower levels of other HDL proteins might contribute to the loss of efflux capacity of HDL of inflamed WT mice. We therefore compared the protein composition of HDL isolated from WT and *Saa1/2*$^{-/-}$ mice, with and without inflammation (supplementary Table 4). In the absence of inflammation, only two proteins (SAA4 and APOA2) met our dual statistical criteria for differential protein abundance between HDL isolated from WT and *Saa1/2*$^{-/-}$ mice (supplementary Fig. 3). In contrast, markedly more proteins differed in relative abundance between control and inflamed WT mice (n = 8) than in inflamed *Saa1/2*$^{-/-}$ mice (n = 8), strongly suggesting that increases in SAA1 and SAA2 remodel the HDL proteome under inflammatory conditions. As expected, SAA1 and SAA2 were undetectable in the inflammatory HDL of *Saa1/2*$^{-/-}$ mice (Fig. 4D).

Loss from HDL of proteins with proposed cardioprotective effects is mediated by SAA

We next compared levels of proposed anti-atherogenic proteins in WT and *Saa1/2*$^{-/-}$ mice. The molar fractions of apoA-I, apoE, phospholipid transfer protein, and apoA-II were all increased in inflammatory HDL isolated from *Saa1/2*$^{-/-}$ mice relative to HDL from WT mice (Fig. 4D, inset). Moreover, levels of PON1 ($r = 0.65$, $P = 0.007$) and apoA-II ($r = 0.78$, $P = 0.0004$), but not apoA-I ($r = 0.37$, $P > 0.05$) or apoE ($r = -0.34$, $P > 0.05$), correlated with the sterol efflux capacity of HDL (supplementary Fig. 4). These observations support the proposal that SAA modulates the abundance in HDL of proteins that affect atherosclerosis in mice, and that this in turn may alter the

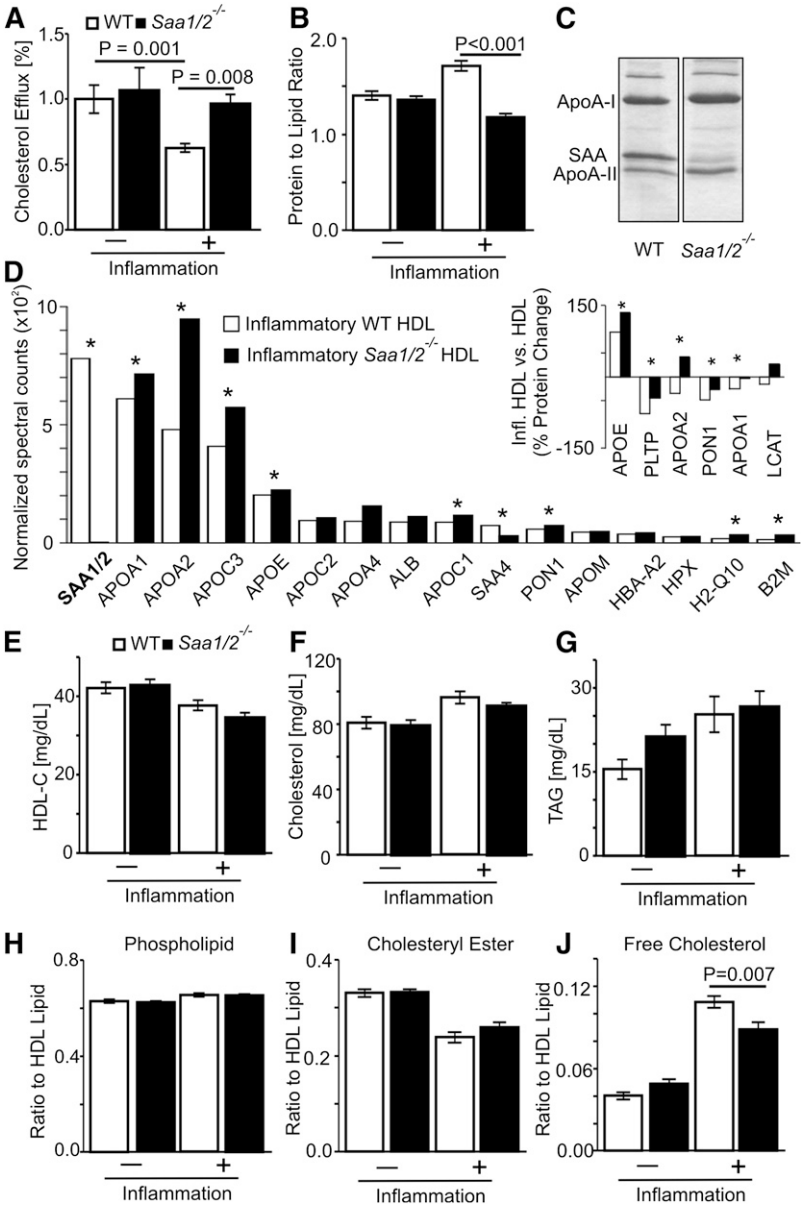

Fig. 4. Macrophage cholesterol efflux capacity of HDL isolated from WT and *Saa1/2*$^{-/-}$ mice, with and without inflammation. HDL was isolated from plasma of mice 24 h after injection with silver nitrate. A: Cholesterol efflux from radiolabeled cholesterol-loaded macrophages to HDL of WT (n = 6) and *Saa1/2*$^{-/-}$ mice (n = 6). B: Biochemical analysis of HDL (n = 8). C: Relative abundance of proteins in HDL assessed by SDS-PAGE with Imperial protein stain staining of proteins. D: LC-ESI-MS/MS analysis of inflammatory HDL (n = 8). E–G: Plasma lipids and HDL-C (n = 8). H–J: HDL lipid composition (n = 8). *$G > 3.5$ (G-test) and $P < 0.001$ (t-test), FDR 4%; A, B, E–J: Significance, ANOVA with Tukey's HSD.

cardioprotective effects of HDL by mechanisms independent of HDL-C.

DISCUSSION

HDL's cardioprotective effect is attributed, in part, to its ability to mobilize excess cholesterol from artery wall macrophages (2–4, 6, 7). Consistent with this proposal, recent studies demonstrate that impaired sterol efflux capacity of serum HDL from J774 macrophages strongly associates with prevalent and incident CAD status, but is independent of HDL-C and apoA-I levels (9, 11, 12). It is therefore critical to uncover the molecular mechanisms that modulate efflux capacity. Because acute inflammation markedly impairs the sterol efflux capacity of HDL in mice and in humans (20–23), we used MS to investigate the role of HDL proteins in modulating cholesterol efflux from macrophages. In humans challenged with a low dose of endotoxin [~1/2 dose previously published (21)], stringent statistical analysis demonstrated that only two proteins, SAA1 and SAA2, were differentially abundant in inflammatory HDL. Moreover, the ability of HDL isolated from the inflamed subjects to accept cholesterol from macrophages correlated inversely with the HDL's SAA1/2 content. Importantly, inflammation did not significantly change HDL's lipid composition or plasma levels of HDL-C, LDL-C, and triglycerides, raising the possibility that the increased SAA1/2 content was the main factor impairing cholesterol efflux from macrophages.

Using WT mice and mice deficient in SAA1/2, we directly determined whether those proteins alter HDL's cholesterol efflux capacity. In the absence of inflammation, the protein composition of HDL isolated from WT mice was essentially identical to that of HDL from the *Saa1/2*$^{-/-}$ mice. However, acute inflammation induced with silver nitrate markedly remodeled the HDL proteome of the WT mice. Indeed, the relative abundance of

one-third of the proteins we detected in WT mouse HDL changed significantly. These observations suggest that the level of inflammation induced by silver nitrate injection in mice was much greater than the level we observed in humans treated with low doses of endotoxin. Indeed, we estimate that the relative level of SAA was approximately 70% that of apoA-I in mouse inflammatory HDL compared with ~20% that of apoA-I in inflammatory HDL in humans exposed to the highest dose of endotoxin. Consistent with previous studies, levels of SAA1/2 were markedly higher in inflammatory HDL of the WT mice, representing ~30% of the protein mass. Moreover, the ability of inflammatory HDL isolated from WT mice to promote sterol efflux from J774 macrophages was markedly impaired, as previously reported by other investigators (21, 22). In contrast, HDL isolated from inflamed *Saa1/2*$^{-/-}$ mice was completely protected from the loss of cholesterol efflux capacity. Plasma lipids, HDL-C levels, and HDL lipid composition changed to the same extent in inflamed WT and *Saa1/2*$^{-/-}$ mice. Collectively, our observations suggest that SAA enrichment impairs the sterol efflux capacity of HDL in both humans and mice by mechanisms independent of altered lipid composition. However, changes in specific lipid subclasses, such as phosphatidylserine, might also contribute to impaired sterol efflux capacity during inflammation. In vivo, it is likely that factors other than SAA1/2 associated with inflammation also reduce HDL's ability to inhibit atherogenesis (49, 58, 59). For example, inflammation downregulates expression of apoA-I in the liver, lowering its circulating levels. It can also alter HDL's lipid composition by changing levels of plasma triglycerides, CETP, secretory phospholipase A2, and other lipid-metabolizing enzymes (36, 49, 58, 60, 61). Such enzymes are important for regulating HDL's ability to accept cholesterol by both the ABCA1 and ABCG1 pathways. Altered levels of lipids and anti-inflammatory and antioxidant proteins, in concert with the gain of SAA and perhaps other inflammatory proteins, may thus be key factors that deprive HDL of its cardioprotective functions. In addition to altered lipid composition, other factors such as HDL protein and lipid oxidation may also contribute to impaired sterol efflux capacity during inflammation (10, 62).

The impact of SAA on sterol efflux capacity is controversial. Early studies showed that lipid-free SAA2 promotes sterol efflux by the ABCA1 pathway (63). However, virtually all circulating SAA1/2 is associated with HDL in humans and mice (32, 64, 65), and there is no convincing evidence that free SAA1 or SAA2 exists in plasma or blood. A recent study of WT and *Saa1/2*$^{-/-}$ mice suggested that SAA does not contribute to impaired reverse cholesterol transport during inflammation in vivo (66). This study used macrophages containing radiolabeled cholesterol to assess the impact of SAA on HDL function. Because the radiolabeled macrophages were injected into the animals' peritoneum only 4 h after endotoxin treatment, while circulating SAA levels in HDL generally peak at approximately 24 h after treatment, it is possible that the ability of HDL to promote efflux from macrophages, the initial step of reverse cholesterol transport, was not affected by elevated SAA content during the time points used for the study. A recent study of type 2 diabetics found negative association of SAA levels with SRBI-mediated cholesterol efflux capacity and no association with ABCG1-mediated cholesterol efflux capacity in 500 diabetic and nondiabetic subjects, although clinical relevance of these assays of cholesterol efflux capacity is unknown (67). In contrast, another study suggested that inflammation-induced increase in the phospholipid content of HDL modestly improved the ability of HDL to promote sterol efflux by the ABCG1 pathway (55). However, efflux by this pathway only contributes about one-fourth of the efflux from macrophages to HDL (68). Moreover, while the major determinant of sterol efflux by the ABCG1 pathway to lipoproteins is their phospholipid content (69), we observed similar levels of the phospholipid in HDL isolated from inflamed WT and *Saa1/2*$^{-/-}$ mice, and the phospholipid as well as the cholesteryl ester content of HDL isolated from control and inflamed *Saa1/2*$^{-/-}$ mice were similar to those of control and inflamed WT mice. In contrast to lipids, protein content significantly increased only in the HDL of inflamed WT mice, with this increase largely accounted for by the marked increase in SAA1/2 content of the lipoprotein by addition to, rather than by displacement of, apoA-I and other HDL proteins (70). Our model system studies further strengthen the hypothesis that SAA is important for generating HDL with impaired sterol efflux capacity from J774 macrophages. Taken together, our observations provide strong evidence that SAA1 and SAA2 play critical roles in rendering HDL dysfunctional during inflammation (71).

Recent studies of hypercholesterolemic mice have reached different conclusions regarding the role of SAA in atherosclerosis. apoE-deficient mice deficient in SAA1/2 fed a Western-type diet were not protected from atherosclerosis (72). In contrast, a study using lentiviral-induced expression of SAA in apoE-deficient mice fed a Western-type diet (73), as well as a study using adenoviral transfection of SAA in apoE-deficient mice on chow diet, found significantly increased atherosclerosis when SAA was expressed (74).

A key issue then becomes whether the modestly elevated level of SAA found in HDL isolated from hypercholesterolemic animal models of atherosclerosis (75) is likely to affect the efflux capacity of HDL. Those levels appear to be lower than the levels of SAA1/2 in HDL we observed in our human and mouse studies. The levels of SAA1/2 in humans injected with low doses of endotoxin were significantly lower than those observed in mice injected with silver nitrate (approximately 13 and 20% of apoA-I in HDL in humans compared with 70% in mice), and we observed a more modest impairment of the sterol efflux capacity of inflammatory HDL isolated from humans than from mice. Our model system studies also demonstrated that impaired efflux capacity of HDL was quantitatively linked to HDL's content of SAA. Collectively, these studies indicate that significant levels of SAA in HDL are necessary to cause significant impairment of HDL sterol efflux capacity. Such levels may be relevant in chronic inflammatory diseases

associated with elevated risk of cardiovascular disease (e.g., systemic lupus erythematosus, rheumatoid arthritis) (76) or acute coronary syndrome (77).

Inflammatory HDL that is enriched in SAA1 and SAA2 is also depleted in specific proteins, including several that may be cardioprotective including apoA-I (78). Moreover, we found that levels of PON1 and apoA-II associated with HDL's cholesterol efflux capacity. Collectively, our observations provide strong support for the proposal that SAA alters HDL's biological effects by replacing cardioprotective proteins.

In summary, our observations have identified a specific mechanism, enrichment of the HDL proteome with SAA1/2, which alters HDL's functionality. It is noteworthy that both inflammation and elevated levels of SAA strongly associate with an increased risk of cardiovascular disease in humans (79, 80). These findings may have important implications for understanding how HDL helps prevent CAD and for developing HDL therapeutics that increase its cardioprotective effects.

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REFERENCES

1. Wilson, P. W., R. B. D'Agostino, D. Levy, A. M. Belanger, H. Silbershatz, and W. B. Kannel. 1998. Prediction of coronary heart disease using risk factor categories. *Circulation.* **97:** 1837–1847.
2. Tall, A. R., L. Yvan-Charvet, N. Terasaka, T. Pagler, and N. Wang. 2008. HDL, ABC transporters, and cholesterol efflux: implications for the treatment of atherosclerosis. *Cell Metab.* **7:** 365–375.
3. Rader, D. J., and A. R. Tall. 2012. The not-so-simple HDL story: is it time to revise the HDL cholesterol hypothesis? *Nat. Med.* **18:** 1344–1346.
4. Heinecke, J. W. 2012. The not-so-simple HDL story: a new era for quantifying HDL and cardiovascular risk? *Nat. Med.* **18:** 1346–1347.
5. Rader, D. J., and G. K. Hovingh. 2014. HDL and cardiovascular disease. *Lancet.* **384:** 618–625.
6. Francis, G. A. 2010. The complexity of HDL. *Biochim. Biophys. Acta.* **1801:** 1286–1293.
7. Rosenson, R. S., H. B. Brewer, W. S. Davidson, Z. A. Fayad, V. Fuster, J. Goldstein, M. Hellerstein, X-C. Jiang, M. C. Phillips, D. J. Rader, et al. 2012. Cholesterol efflux and atheroprotection: advancing the concept of reverse cholesterol transport. *Circulation.* **125:** 1905–1919.
8. de la Llera-Moya, M., D. Drazul-Schrader, B. F. Asztalos, M. Cuchel, D. J. Rader, and G. H. Rothblat. 2010. The ability to promote efflux via ABCA1 determines the capacity of serum specimens with similar high-density lipoprotein cholesterol to remove cholesterol from macrophages. *Arterioscler. Thromb. Vasc. Biol.* **30:** 796–801.
9. Khera, A. V., M. Cuchel, M. de la Llera-Moya, A. Rodrigues, M. F. Burke, K. Jafri, B. C. French, J. A. Phillips, M. L. Mucksavage, R. L. Wilensky, et al. 2011. Cholesterol efflux capacity, high-density lipoprotein function, and atherosclerosis. *N. Engl. J. Med.* **364:** 127–135.
10. Shao, B., C. Tang, A. Sinha, P. S. Mayer, G. D. Davenport, N. Brot, M. N. Oda, X. Q. Zhao, and J. W. Heinecke. 2014. Humans with atherosclerosis have impaired ABCA1 cholesterol efflux and enhanced high-density lipoprotein oxidation by myeloperoxidase. *Circ. Res.* **114:** 1733–1742.
11. Li, X. M., W. H. Tang, M. K. Mosior, Y. Huang, Y. Wu, W. Matter, V. Gao, D. Schmitt, J. A. Didonato, E. A. Fisher, et al. 2013. Paradoxical association of enhanced cholesterol efflux with increased incident cardiovascular risks. *Arterioscler. Thromb. Vasc. Biol.* **33:** 1696–1705.
12. Rohatgi, A., A. Khera, J. D. Berry, E. G. Givens, C. R. Ayers, K. E. Wedin, I. J. Neeland, I. S. Yuhanna, D. R. Rader, J. A. de Lemos, et al. 2014. HDL cholesterol efflux capacity and incident cardiovascular events. *N. Engl. J. Med.* **371:** 2383–2393.
13. Lewis, G. F., and D. J. Rader. 2005. New insights into the regulation of HDL metabolism and reverse cholesterol transport. *Circ. Res.* **96:** 1221–1232.
14. Van Lenten, B. J., S. Y. Hama, F. C. de Beer, D. M. Stafforini, T. M. McIntyre, S. M. Prescott, B. N. La Du, A. M. Fogelman, and M. Navab. 1995. Anti-inflammatory HDL becomes pro-inflammatory during the acute phase response. Loss of protective effect of HDL against LDL oxidation in aortic wall cell cocultures. *J. Clin. Invest.* **96:** 2758–2767.
15. Barter, P. J., S. Nicholls, K. A. Rye, G. M. Anantharamaiah, M. Navab, and A. M. Fogelman. 2004. Antiinflammatory properties of HDL. *Circ. Res.* **95:** 764–772.
16. Vaisar, T., B. Shao, P. S. Green, M. N. Oda, J. F. Oram, and J. W. Heinecke. 2007. Myeloperoxidase and inflammatory proteins: pathways for generating dysfunctional high-density lipoprotein in humans. *Curr. Atheroscler. Rep.* **9:** 417–424.
17. Feingold, K. R., and C. Grunfeld. 2010. The acute phase response inhibits reverse cholesterol transport. *J. Lipid Res.* **51:** 682–684.
18. Sorci-Thomas, M. G., and M. J. Thomas. 2012. High density lipoprotein biogenesis, cholesterol efflux, and immune cell function. *Arterioscler. Thromb. Vasc. Biol.* **32:** 2561–2565.
19. van der Westhuyzen, D. R., F. C. de Beer, and N. R. Webb. 2007. HDL cholesterol transport during inflammation. *Curr. Opin. Lipidol.* **18:** 147–151.
20. Banka, C. L., T. Yuan, M. C. de Beer, M. Kindy, L. K. Curtiss, and F. C. de Beer. 1995. Serum amyloid A (SAA): influence on HDL-mediated cellular cholesterol efflux. *J. Lipid Res.* **36:** 1058–1065.
21. McGillicuddy, F. C., M. de la Llera Moya, C. C. Hinkle, M. R. Joshi, E. H. Chiquoine, J. T. Billheimer, G. H. Rothblat, and M. P. Reilly. 2009. Inflammation impairs reverse cholesterol transport in vivo. *Circulation.* **119:** 1135–1145.
22. Annema, W., N. Nijstad, M. Tolle, J. F. de Boer, R. V. Buijs, P. Heeringa, M. van der Giet, and U. J. Tietge. 2010. Myeloperoxidase and serum amyloid A contribute to impaired in vivo reverse cholesterol transport during the acute phase response but not group IIA secretory phospholipase A(2). *J. Lipid Res.* **51:** 743–754.
23. Artl, A., G. Marsche, S. Lestavel, W. Sattler, and E. Malle. 2000. Role of serum amyloid A during metabolism of acute-phase HDL by macrophages. *Arterioscler. Thromb. Vasc. Biol.* **20:** 763–772.
24. Navab, M., S. Hama-Levy, B. J. Van Lenten, G. C. Fonarow, C. J. Cardinez, L. W. Castellani, M. L. Brennan, A. J. Lusis, A. M. Fogelman, and B. N. La Du. 1997. Mildly oxidized LDL induces an increased apolipoprotein J/paraoxonase ratio. *J. Clin. Invest.* **99:** 2005–2019.
25. Getz, G. S., and C. A. Reardon. 2004. Paraoxonase, a cardioprotective enzyme: continuing issues. *Curr. Opin. Lipidol.* **15:** 261–267.
26. Davidson, W. S., R. A. Silva, S. Chantepie, W. R. Lagor, M. J. Chapman, and A. Kontush. 2009. Proteomic analysis of defined HDL subpopulations reveals particle-specific protein clusters: relevance to antioxidative function. *Arterioscler. Thromb. Vasc. Biol.* **29:** 870–876.
27. Tchoua, U., C. Rosales, D. Tang, B. K. Gillard, A. Vaughan, H. Y. Lin, H. S. Courtney, and H. J. Pownall. 2010. Serum opacity factor enhances HDL-mediated cholesterol efflux, esterification and anti-inflammatory effects. *Lipids.* **45:** 1117–1126.
28. Suffredini, A. F., H. D. Hochstein, and F. G. McMahon. 1999. Dose-related inflammatory effects of intravenous endotoxin in humans: evaluation of a new clinical lot of Escherichia coli O:113 endotoxin. *J. Infect. Dis.* **179:** 1278–1282.
29. de Beer, M. C., N. R. Webb, J. M. Wroblewski, V. P. Noffsinger, D. L. Rateri, A. Ji, D. R. van der Westhuyzen, and F. C. de Beer. 2010. Impact of serum amyloid A on high density lipoprotein composition and levels. *J. Lipid Res.* **51:** 3117–3125.
30. Kisilevsky, R., M. D. Benson, M. A. Axelrad, and L. Boudreau. 1979. The effect of a liver protein synthesis inhibitor on plasma SAA levels in a model of accelerated amyloid deposition. *Lab. Invest.* **41:** 206–210.
31. Sipe, J. D., I. Carreras, W. A. Gonnerman, E. S. Cathcart, M. C. de Beer, and F. C. de Beer. 1993. Characterization of the in-bred CE/J mouse strain as amyloid resistant. *Am. J. Pathol.* **143:** 1480–1485.
32. Coetzee, G. A., A. F. Strachan, D. R. van der Westhuyzen, H. C. Hoppe, M. S. Jeenah, and F. C. de Beer. 1986. Serum amyloid A-containing human high density lipoprotein 3. Density, size, and apolipoprotein composition. *J. Biol. Chem.* **261:** 9644–9651.

33. Vaisar, T., S. Pennathur, P. S. Green, S. A. Gharib, A. N. Hoofnagle, M. C. Cheung, J. Byun, S. Vuletic, S. Kassim, P. Singh, et al. 2007. Shotgun proteomics implicates protease inhibition and complement activation in the antiinflammatory properties of HDL. *J. Clin. Invest.* **117:** 746–756.
34. Green, P. S., T. Vaisar, S. Pennathur, J. J. Kulstad, A. B. Moore, S. Marcovina, J. Brunzell, R. H. Knopp, X-Q. Zhao, and J. W. Heinecke. 2008. Combined statin and niacin therapy remodels the high-density lipoprotein proteome. *Circulation.* **118:** 1259–1267.
35. Cabana, V. G., N. Feng, C. A. Reardon, J. Lukens, N. R. Webb, F. C. de Beer, and G. S. Getz. 2004. Influence of apoA-I and apoE on the formation of serum amyloid A-containing lipoproteins in vivo and in vitro. *J. Lipid Res.* **45:** 317–325.
36. Jahangiri, A., M. C. de Beer, V. Noffsinger, L. R. Tannock, C. Ramaiah, N. R. Webb, D. R. van der Westhuyzen, and F. C. de Beer. 2009. HDL remodeling during the acute phase response. *Arterioscler. Thromb. Vasc. Biol.* **29:** 261–267.
37. Tang, C., Y. Liu, P. S. Kessler, A. M. Vaughan, and J. F. Oram. 2009. The macrophage cholesterol exporter ABCA1 functions as an anti-inflammatory receptor. *J. Biol. Chem.* **284:** 32336–32343.
38. Egertson, J. D., A. Kuehn, G. E. Merrihew, N. W. Bateman, B. X. MacLean, Y. S. Ting, J. D. Canterbury, D. M. Marsh, M. Kellmann, V. Zabrouskov, et al. 2013. Multiplexed MS/MS for improved data-independent acquisition. *Nat. Methods.* **10:** 744–746.
39. MacLean, B., D. M. Tomazela, N. Shulman, M. Chambers, G. L. Finney, B. Frewen, R. Kern, D. L. Tabb, D. C. Liebler, and M. J. MacCoss. 2010. Skyline: an open source document editor for creating and analyzing targeted proteomics experiments. *Bioinformatics.* **26:** 966–968.
40. Hoofnagle, A. N., J. O. Becker, M. N. Oda, G. Cavigiolio, P. Mayer, and T. Vaisar. 2012. Multiple-reaction monitoring-mass spectrometric assays can accurately measure the relative protein abundance in complex mixtures. *Clin. Chem.* **58:** 777–781.
41. Liu, H., R. G. Sadygov, and J. R. Yates III. 2004. A model for random sampling and estimation of relative protein abundance in shotgun proteomics. *Anal. Chem.* **76:** 4193–4201.
42. Becker, L., S. A. Gharib, A. D. Irwin, E. Wijsman, T. Vaisar, J. F. Oram, and J. W. Heinecke. 2010. A macrophage sterol-responsive network linked to atherogenesis. *Cell Metab.* **11:** 125–135.
43. Silva, J. C., M. V. Gorenstein, G-z. Li, J. P. C. Vissers, and S. J. Geromanos. 2006. Absolute quantification of proteins by LCMSE: a virtue of parallel MS acquisition. *Molecular & cellular proteomics.* **5:** 144–156.
44. Hutchins, P. M., G. E. Ronsein, J. S. Monette, N. Pamir, J. Wimberger, Y. He, G. M. Anantharamaiah, D. S. Kim, J. E. Ranchalis, G. P. Jarvik, et al. 2014. Quantification of HDL particle concentration by calibrated ion mobility analysis. *Clin. Chem.* **60:** 1393–1401.
45. Davidson, W. S. 2014. HDL Proteome Watch. Accessed January 30, 2015, at http://homepages.uc.edu/~davidswm/HDLproteome.html.
46. Alaupovic, P. 1996. Significance of apolipoproteins for structure, function, and classification of plasma lipoproteins. *Methods Enzymol.* **263:** 32–60.
47. Hoofnagle, A. N., and J. W. Heinecke. 2009. Lipoproteomics: using mass spectrometry-based proteomics to explore the assembly, structure, and function of lipoproteins. *J. Lipid Res.* **50:** 1967–1975.
48. Cheung, M. C., and J. J. Albers. 1984. Characterization of lipoprotein particles isolated by immunoaffinity chromatography. Particles containing A-I and A-II and particles containing A-I but no A-II. *J. Biol. Chem.* **259:** 12201–12209.
49. Khovidhunkit, W., M. S. Kim, R. A. Memon, J. K. Shigenaga, A. H. Moser, K. R. Feingold, and C. Grunfeld. 2004. Effects of infection and inflammation on lipid and lipoprotein metabolism: mechanisms and consequences to the host. *J. Lipid Res.* **45:** 1169–1196.
50. Watanabe, J., V. Grijalva, S. Hama, K. Barbour, F. Berger, M. Navab, A. M. Fogelman, and S. T. Reddy. 2009. Hemoglobin and its scavenger protein haptoglobin associate with apoA-I containing particles and influence the inflammatory properties and function of high density lipoprotein. *J. Biol. Chem.* **284:** 18292–18301.
51. Shao, B., C. Tang, J. W. Heinecke, and J. F. Oram. 2010. Oxidation of apolipoprotein A-I by myeloperoxidase impairs the initial interactions with ABCA1 required for signaling and cholesterol export. *J. Lipid Res.* **51:** 1849–1858.
52. Shao, B., G. Cavigiolio, N. Brot, M. N. Oda, and J. W. Heinecke. 2008. Methionine oxidation impairs reverse cholesterol transport by apolipoprotein A-I. *Proc. Natl. Acad. Sci. USA.* **105:** 12224–12229.
53. Shao, B., M. N. Oda, C. Bergt, X. Fu, P. S. Green, N. Brot, J. F. Oram, and J. W. Heinecke. 2006. Myeloperoxidase impairs ABCA1-dependent cholesterol efflux through methionine oxidation and site-specific tyrosine chlorination of apolipoprotein A-I. *J. Biol. Chem.* **281:** 9001–9004.
54. Huang, Y., J. A. DiDonato, B. S. Levison, D. Schmitt, L. Li, Y. Wu, J. Buffa, T. Kim, G. S. Gerstenecker, X. Gu, et al. 2014. An abundant dysfunctional apolipoprotein A1 in human atheroma. *Nat. Med.* **20:** 193–203.
55. de Beer, M. C., A. Ji, A. Jahangiri, A. M. Vaughan, F. C. de Beer, D. R. van der Westhuyzen, and N. R. Webb. 2011. ATP binding cassette G1-dependent cholesterol efflux during inflammation. *J. Lipid Res.* **52:** 345–353.
56. Davidson, W. S., K. L. Gillotte, S. Lund-Katz, W. J. Johnson, G. H. Rothblat, and M. C. Phillips. 1995. The effect of high density lipoprotein phospholipid acyl chain composition on the efflux of cellular free cholesterol. *J. Biol. Chem.* **270:** 5882–5890.
57. Camont, L., M. Lhomme, F. Rached, W. Le Goff, A. Nègre-Salvayre, R. Salvayre, C. Calzada, M. Lagarde, M. J. Chapman, and A. Kontush. 2013. Small, dense high-density lipoprotein-3 particles are enriched in negatively charged phospholipids: relevance to cellular cholesterol efflux, antioxidative, antithrombotic, anti-inflammatory, and antiapoptotic functionalities. *Arterioscler. Thromb. Vasc. Biol.* **33:** 2715–2723.
58. de Beer, F. C., M. C. de Beer, D. R. van der Westhuyzen, L. W. Castellani, A. J. Lusis, M. E. Swanson, and D. S. Grass. 1997. Secretory non-pancreatic phospholipase A2: influence on lipoprotein metabolism. *J. Lipid Res.* **38:** 2232–2239.
59. Libby, P. 2002. Inflammation in atherosclerosis. *Nature.* **420:** 868–874.
60. Pruzanski, W., E. Stefanski, F. C. de Beer, M. C. de Beer, A. Ravandi, and A. Kuksis. 2000. Comparative analysis of lipid composition of normal and acute-phase high density lipoproteins. *J. Lipid Res.* **41:** 1035–1047.
61. Hudgins, L. C., T. S. Parker, D. M. Levine, B. R. Gordon, S. D. Saal, X. C. Jiang, C. E. Seidman, J. D. Tremaroli, J. Lai, and A. L. Rubin. 2003. A single intravenous dose of endotoxin rapidly alters serum lipoproteins and lipid transfer proteins in normal volunteers. *J. Lipid Res.* **44:** 1489–1498.
62. Shao, B., S. Pennathur, I. Pagani, M. N. Oda, J. L. Witztum, J. F. Oram, and J. W. Heinecke. 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.
63. Tam, S. P., A. Flexman, J. Hulme, and R. Kisilevsky. 2002. Promoting export of macrophage cholesterol: the physiological role of a major acute-phase protein, serum amyloid A 2.1. *J. Lipid Res.* **43:** 1410–1420.
64. Husebekk, A., B. Skogen, and G. Husby. 1987. Characterization of amyloid proteins AA and SAA as apolipoproteins of high density lipoprotein (HDL). Displacement of SAA from the HDL-SAA complex by apo AI and apo AII. *Scand. J. Immunol.* **25:** 375–381.
65. Cabana, V. G., C. A. Reardon, B. Wei, J. R. Lukens, and G. S. Getz. 1999. SAA-only HDL formed during the acute phase response in apoA-I+/+ and apoA-I−/− mice. *J. Lipid Res.* **40:** 1090–1103.
66. de Beer, M. C., J. M. Wroblewski, V. P. Noffsinger, A. Ji, J. M. Meyer, D. R. V. D. Westhuyzen, F. C. de Beer, and N. R. Webb. 2013. The impairment of macrophage-to-feces reverse cholesterol transport during inflammation does not depend on serum amyloid A. *J. Lipids.* **2013:** 283486.
67. Tsun, J. G., S. W. Shiu, Y. Wong, S. Yung, T. M. Chan, and K. C. Tan. 2013. Impact of serum amyloid A on cellular cholesterol efflux to serum in type 2 diabetes mellitus. *Atherosclerosis.* **231:** 405–410.
68. Adorni, M. P., F. Zimetti, J. T. Billheimer, N. Wang, D. J. Rader, M. C. Phillips, and G. H. Rothblat. 2007. The roles of different pathways in the release of cholesterol from macrophages. *J. Lipid Res.* **48:** 2453–2462.
69. Sankaranarayanan, S., J. F. Oram, B. F. Asztalos, A. M. Vaughan, S. Lund-Katz, M. Adorni, M. C. Phillips, and G. H. Rothblat. 2009. Effects of acceptor composition and mechanism of ABCG1-mediated cellular free cholesterol efflux. *J. Lipid Res.* **50:** 275–284.
70. Hosoai, H., N. R. Webb, J. M. Glick, U. J. Tietge, M. S. Purdom, F. C. de Beer, and D. J. Rader. 1999. Expression of serum amyloid A protein in the absence of the acute phase response does not reduce HDL cholesterol or apoA-I levels in human apoA-I transgenic mice. *J. Lipid Res.* **40:** 648–653.

71. de la Llera Moya, M., F. C. McGillicuddy, C. C. Hinkle, M. Byrne, M. R. Joshi, V. Nguyen, J. Tabita-Martinez, M. L. Wolfe, K. Badellino, L. Pruscino, et al. 2012. Inflammation modulates human HDL composition and function in vivo. *Atherosclerosis.* **222:** 390–394.
72. De Beer, M. C., J. M. Wroblewski, V. P. Noffsinger, D. L. Rateri, D. A. Howatt, A. Balakrishnan, A. Ji, P. Shridas, J. C. Thompson, D. R. van der Westhuyzen, et al. 2014. Deficiency of endogenous acute phase serum amyloid A does not affect atherosclerotic lesions in apolipoprotein E-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* **34:** 255–261.
73. Dong, Z., T. Wu, W. Qin, C. An, Z. Wang, M. Zhang, Y. Zhang, C. Zhang, and F. An. 2011. Serum amyloid A directly accelerates the progression of atherosclerosis in apolipoprotein E-deficient mice. *Mol. Med.* **17:** 1357–1364.
74. Thompson, J. C., C. Jayne, J. Thompson, P. G. Wilson, M. H. Yoder, N. Webb, and L. R. Tannock. 2015. A brief elevation of serum amyloid A is sufficient to increase atherosclerosis. *J. Lipid Res.* **56:** 286–293.
75. Subramanian, S., C. Y. Han, T. Chiba, T. S. McMillen, S. A. Wang, A. Haw, E. A. Kirk, K. D. O'Brien, and A. Chait. 2008. Dietary cholesterol worsens adipose tissue macrophage accumulation and atherosclerosis in obese LDL receptor-deficient mice. *Arterioscler. Thromb. Vasc. Biol.* **28:** 685–691.
76. Vivekanandan-Giri, A., J. L. Slocum, J. Byun, C. Tang, R. L. Sands, B. W. Gillespie, J. W. Heinecke, R. Saran, M. J. Kaplan, and S. Pennathur. 2013. High density lipoprotein is targeted for oxidation by myeloperoxidase in rheumatoid arthritis. *Ann. Rheum. Dis.* **72:** 1725–1731.
77. Hafiane, A., B. Jabor, I. Ruel, J. Ling, and J. Genest. 2014. High-density lipoprotein mediated cellular cholesterol efflux in acute coronary syndromes. *Am. J. Cardiol.* **113:** 249–255.
78. Zabalawi, M., M. Bharadwaj, H. Horton, M. Cline, M. Willingham, M. J. Thomas, and M. G. Sorci-Thomas. 2007. Inflammation and skin cholesterol in LDLr-/-, apoA-I-/- mice: link between cholesterol homeostasis and self-tolerance? *J. Lipid Res.* **48:** 52–65.
79. Ridker, P. M., N. Rifai, M. A. Pfeffer, F. M. Sacks, L. A. Moye, S. Goldman, G. C. Flaker, and E. Braunwald. 1998. Inflammation, pravastatin, and the risk of coronary events after myocardial infarction in patients with average cholesterol levels. Cholesterol and Recurrent Events (CARE) Investigators. *Circulation.* **98:** 839–844.
80. Danesh, J., P. Whincup, M. Walker, L. Lennon, A. Thomson, P. Appleby, J. R. Gallimore, and M. B. Pepys. 2000. Low grade inflammation and coronary heart disease: prospective study and updated meta-analyses. *BMJ.* **321:** 199–204.