High-density lipoprotein reduces inflammation from cholesterol crystals by inhibiting inflammasome activation

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Summary
Interleukin-1β (IL-1β), a potent pro-inflammatory cytokine, has been implicated in many diseases, including atherosclerosis. Activation of IL-1β is controlled by a multi-protein complex, the inflammasome. The exact initiating event in atherosclerosis is unknown, but recent work has demonstrated that cholesterol crystals (CC) may promote atherosclerosis development by activation of the inflammasome. High-density lipoprotein (HDL) has consistently been shown to be anti-atherogenic and to have anti-inflammatory effects, but its mechanism of action is unclear. We demonstrate here that HDL is able to suppress IL-1β secretion in response to cholesterol crystals in THP-1 cells and in human-monocyte-derived macrophages. HDL is able to blunt inflammatory monocyte cell recruitment in vivo following intraperitoneal CC injection in mice. HDL appears to modulate inflammasome activation in several ways. It reduces the loss of lysosomal membrane integrity following the phagocytosis of CC, but the major mechanism for the suppression of inflammasome activation by HDL is decreased expression of pro-IL-1β and NLRP3, and reducing caspase-1 activation. In summary, we have described a novel anti-inflammatory effect of HDL, namely its ability to suppress inflammasome activation by CC by modulating the expression of several key components of the inflammasome.

Keywords: apolipoprotein A I; high-density lipoprotein; inflammasome; interleukin-1β.

Introduction
The cholesterol content of high-density lipoprotein (HDL), commonly referred to as HDL-C, has consistently been shown to be inversely related to atherosclerosis disease risk.1,2 Clinical drug trials aimed at raising levels of HDL-C, however, have failed in reducing cardiovascular events.3,4 These discordant results may be partially explained by the fact that HDL is a heterogeneous family of particles, with a wide variety of both protein and lipid cargo.5 Indeed, HDL has been shown to be a multifunctional collection of lipoprotein particles that mediate a diverse set of functions, which include cholesterol efflux, anti-oxidation, anti-inflammation and vasoprotection.6 In fact, recent work has demonstrated that some of the functional characteristics of HDL, such as the cholesterol efflux capacity, may more accurately predict HDL’s ability to prevent cardiovascular disease than its cholesterol content.7,8 The role of inflammation as a major driver of atherosclerosis initiation and development is now widely recognized.1,3 One important inflammatory pathway involved in atherosclerosis is the interleukin-1β (IL-1β) pathway.9–11 Interleukin-1β has been shown to be produced by endothelial cells, vascular smooth muscles cells...
and macrophages, but macrophages appear to be the most important producers of IL-1β in atherosclerosis development.11,12 We focused our studies on the response of macrophages to CC as Duewell et al.11 have previously demonstrated that cholesterol crystals (CC) associate with macrophages in vivo.

Transcription of pro-IL-1β is induced in response to pro-inflammatory signals that activate nuclear factor-κB (NF-κB).13 Pro-IL-1β and the related protein pro-IL-18 are then activated by a macromolecular complex termed the inflammasome. Inflammasome activation is a multi-step process that ultimately results in the activation of caspase-1, which then cleaves pro-IL-1β or pro-IL-18, allowing the mature form to be secreted.14 The mature inflammasome consists of a NOD-like receptor (NLR), pro-caspase-1, and usually the adaptor protein Apoptosis-associated speck-like protein containing caspase-associated recruitment domain (ASC). The NLR domain is the portion that confers ligand specificity upon the inflammasome. A diverse range of ligands have been identified, such as damage-associated molecular patterns, which are recognized by NLR proteins. ASC links the NLR via its PYD domain to the pro-caspase-1 via its caspase-associated recruitment domain (CARD), which leads to caspase-1 auto-activation.15 Inflammasome activation by diverse damage-associated molecular patterns appears to converge on common molecular mechanisms, such as lysosomal membrane destabilization, reactive oxygen species production, and mitochondrial function.16

CC, which are abundantly found in atherosclerotic lesions, like other crystals such as uric acid, which are classical inflammasome activators, have recently been shown to activate the NLRP3 inflammasome.11,17 CC were once thought to be a late manifestation of plaque development, but Duewell et al.11 recently demonstrated that small CC can form in early plaques. Indeed, CC have been proposed as one of the initiating inflammatory insults in atherosclerosis.

Although HDL is known to have many anti-inflammatory effects, its effects on the inflammasome are poorly studied. In our current study, we demonstrate that HDL through multiple pathways is able, both in vitro and in vivo, to potently suppress inflammasome activation in response to CC, so identifying a novel anti-atherogenic mechanism for HDL.

Materials and methods

Reagents

Reconstituted HDL (rHDL) was prepared as described previously,18 with a final molar ratio of apolipoprotein A I (apoA-I) to soybean phosphatidylcholine of 1 : 150. The following viability dye and antibodies were obtained from BioLegend (San Diego, CA) CD45 (30-F11, Peridinin chlorophyll protein/Cy5.5), CD3 [17A2, allophycocyanin (APC)], CD19 [6D5, phycoerythrin (PE)/Cy5], CD11b (M1/70, PE), Gr-1 (RB6-8C5, FITC), Ly6C (HK1.4, PE/Cy7), CD115 (AF598, APC), F4/80 (BM8, APC/Cy7), and Zombie Violet.19 Fixable Viability Kit (San Diego, CA). Polyclonal goat anti-human apoA-I was obtained from Abcam (Cambridge, MA). Unless otherwise noted, all other chemicals were obtained from Sigma (St Louis, MO).

Preparation of cholesterol crystals

Cholesterol monohydrate crystals (CC) were produced as described previously.11,17 Briefly, tissue-grade cholesterol (Sigma-Aldrich) was dissolved in hot acetone and cooled to allow crystallization. Acetone/cholesterol mixture was heated and cooled for a total of five cycles and 10% endotoxin-free water was added during the final crystallization. The solution containing CC was sonicated with a probe tip for 1 hr to generate a diverse size range of crystals. Crystals were resuspended in endotoxin-free PBS at a concentration of 50 mg/ml. Concentrated CC were stored at −20°C until used. Concentrated CC were diluted into fresh media and then added to cells. The average size of CC was found to be 1-028 ± 0.268 μm, as measured using a Delsa Nano (Beckman Coulter, Inc., Indianapolis, IN). For some experiments fluorescent CC were generated by adding a trace amount of BODIPY-labelled cholesterol (Avanti Polar Lipids, Inc., Alabaster, AL), during the crystallization process.

Cholesterol crystal dissolution and characterization by scanning electron microscopy

The CC (1 mg) were incubated with HDL (1 mg) or BSA (1 mg) for 18 hr at 37°C. Following incubation, crystal solution was filtered using a 0.22-μm filter to remove undissolved crystals. Filters were rinsed with clean PBS. Hot methanol was passed over the washed filters to dissolve the CC captured on the filter. Two hundred microlitres of either the supernatant or dissolved crystals was transferred to a 96-well microplate in triplicate and fluorescence was measured using the VICTOR Multilabel Plate Reader (PerkinElmer, Waltham, MA). Total fluorescence of each fraction was determined and the degree of efflux was obtained using the following equation: fluorescence\textsubscript{supernatant}/(fluorescence\textsubscript{supernatant} + fluorescence\textsubscript{crystals}) × 100. CC with or without HDL pretreatment were dried and gold coated using an EMS 575X sputter coater (Electron Microscopy Sciences, Hatfield, PA). CC were imaged using a Hitachi S-3400N1 scanning electron microscope (Hitachi High Technologies America, Inc., Schaumburg, IL).

Isolation and preparation of native HDL and apoA-I

HDL was isolated from healthy human plasma, using sequential KBr density gradient ultracentrifugation

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Lipid vesicles were generated in the following manner. Extracted lipids obtained during apoA-I purification were dried under an N₂ stream. Dried lipids were resuspended in PBS and sonicated with a probe tip for 15 seconds on ice for total time of 30 min to generate lipid vesicles.

**In vivo peritonitis mouse model**

C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were housed under controlled conditions, with a 12 hr/12 hr light/dark cycle, and fed a standard rodent autoclaved chow diet, containing 4-0% fat (NIH31 chow diet; Zeigler Brothers Inc., Gardners, PA). Eight-week-old female mice were used in this study. Immediately before injection, CC and rHDL were mixed and mice were injected intraperitoneally with 1 mg CC with or without 1 mg of rHDL, and 18 hr later the inflammatory infiltrate was isolated by peritoneal lavage. All animal procedures were approved by a National Institutes of Health Institutional Animal Care and Use Committee (protocol H-0050R2).

**Cell culture**

THP-1 cells, a monocytic cell line, (ATCC, Manassas, VA) were activated for 3 hr with 0.5 μM PMA (Sigma-Aldrich). Cells were washed and plated at a concentration of 1 x 10⁶ cells/cm² in a 24-multiwell plate (Corning Inc., Corning, NY). THP-1 cells were then treated with CC (1 mg/ml) for 6 hr. Native HDL (nHDL), rHDL or methyl-β-cyclodextrin (mβCD) were given to cells at the same time as CC addition. As indicated, in some experiments cells were pretreated with nHDL, rHDL or mβCD for 18 hr after which the cells were washed and CC was added for 6 hr and supernatants or RNA were harvested for further analysis. Additionally, in some experiments CC (1 mg/ml) were pretreated with nHDL or rHDL (1 mg/ml) for 18 hr, and then, following incubation, CC were washed extensively to remove unbound nHDL. In some experiments poly(dAdT)/LyoVec (InvivoGen, San Diego, CA) was added to cells overnight following manufacturer’s recommendations.

Monocytes from healthy controls were obtained by mononuclear cell apheresis followed by elutriation of the monocytes. Monocytes were differentiated into macrophages, using 25 ng granulocyte–macrophage colony-stimulating factor/ml (Peprotech, Rocky Hill, NJ) for 7 days. Monocyte-derived macrophages were treated in a similar manner to THP-1 cells with the exception of a 1-hr priming step with 100 ng lipopolysaccharide (LPS) followed by extensive washing.

**ELISA for cytokines**

Supernatants from THP-1 and monocyte-derived macrophages were harvested after 6 hr of incubation in the presence of CC. ELISAs for human IL-1β and mouse IL-6 were both obtained from eBioscience (San Diego, CA). ELISAs were carried out following the manufacturer’s recommendations.

**Peritoneal lavage cell characterization**

Dead cells were excluded from the analysis and the following cell populations were quantified with the following markers: polymorphonuclear leucocytes (CD45⁺ Gr-1⁺ CD115⁻), macrophages (CD45⁺ F4/80⁺), T cells (CD45⁺ CD3⁺ CD19⁻) B cells (CD45⁺ CD3⁻ CD19⁺) and monocytes (CD45⁺ CD115⁺ CD11b⁻). The monocyte population was further divided into Ly6Ch⁺ (inflammatory) and Ly6Cmed⁻ (conventional); see Supplementary material (Fig. S1) for complete gating strategy. All flow cytometry was carried using an LSR II (BD Biosciences, San Jose, CA).

**Immunoblot analysis**

The CC (1 mg/ml) were co-incubated with an equal amount of nHDL (1 mg/ml), rHDL (1 mg/ml), or lipid-free apoA-I (1 mg/ml) for 18 hr. CC crystals were extensively washed with PBS by centrifugation five times. Following the final wash, CC were resuspended in 1 x loading buffer (ThermoFisher Scientific Inc, Grand Island, NY) containing 2-mercaptoethanol and heated for 10 min at 95°C. For immunoblotting, equal volumes of sample were loaded onto a 4-12% Bis-Tris gel (Thermo Fisher Scientific Inc.) and electrophoresed at 200 V in MOPS buffer. Proteins were transferred to PVDF membrane (ThermoFisher Scientific Inc.), using a Pierce G2 Fast Blotter (ThermoFisher Scientific Inc.) for 15 min at 25 V and 3-2 A. Following the protein transfer, membranes were blocked with 5% milk and 3% BSA. To detect if HDL or apoA-I was binding CC, membranes were stained with goat anti-human apoA-I (Meridian Life Science, Inc., Memphis, TN). Cell lysates, following stimulation with nHDL (1 mg/ml) or rHDL (1 mg/ml), were transferred to PVDF membranes as above and stained with rabbit anti-NLRP3 (Cell Signaling Technology, Danvers, MA), rabbit anti-caspase-1 (Cell Signaling Technology), and goat anti-GAPDH (Santa Cruz Biotechnology, Inc., Dallas, TX). Primary antibodies were detected with the appropriate secondary antibody conjugated to
horseradish peroxidase (Abcam). Staining was visualized using the WesternBright Quantum detection kit (Advansta Inc., Menlo Park, CA) on an LAS-3000 (Fujifilm Medical Systems U.S.A., Inc., Stamford, CT).

**Cholesterol content of cells**

After activation, THP-1 cells were cultured overnight in the presence of 1 mg/ml HDL or 200 μg mβCD for 18 hr. Cell monolayers were washed extensively with PBS and cholesterol content of cells was determined using the amplex red cholesterol assay (Thermo Fisher Scientific Inc.) following the manufacturer’s recommendations. Amount of protein in cell monolayers was quantified using the micro BCA kit (Thermo Fisher Scientific Inc.) following the manufacturer’s recommendations.

**Phagocytosis assays**

The amount of phagocytosis of CC was assessed by flow cytometry as an increase in the side scatter characteristics of THP-1 cells or an increase in the fluorescence of cells if using BODIPY-labelled CC. To detect phagocytosis of *Escherichia coli* bioparticles (Life Technologies, Inc., Grand Island, NY) THP-1 cells were treated according to the manufacturer’s recommendations and the increase in fluorescence by flow cytometry was used to determine the amount of phagocytosis. All flow cytometry was carried out using an LSR II (BD Biosciences).

**Lysosomal and mitochondrial stability**

To detect the loss of lysosomal integrity THP-1 cells were incubated with LysoSensor (Life Technologies, Inc.), following the manufacturer’s recommendations. The loss of fluorescence as detected by flow cytometry was used to assess loss of lysosomal integrity. Mitochondrial health was checked by loading cells with Mitotracker green (mitochondrial mass) and Mitotracker red (mitochondrial membrane potential) (Life Technologies, Inc.) to quantify the amount and respiratory capacity of the mitochondria as described before. All flow cytometry was carried out using an LSR II (BD Biosciences).

**Intracellular staining for IκB-α**

THP-1 cells were cultured in serum-free media overnight. THP-1 cells were stimulated with 1 mg/ml of nHDL for 15 or 30 min. Following stimulation cells were fixed using the Intracellular Fixation & Permeabilization Buffer Set (eBioscience) following the manufacturer’s recommendation. Fixed and permeabilized cells were stained with antibody recognizing IκB-α (PE; eBioscience). All flow cytometry was carried out using an LSR II (BD Biosciences).

**RNA isolation and gene expression analysis**

All reagents used in real-time PCR experiments were obtained from Applied Biosystems (Foster City, CA). RNA was isolated from cell monolayers following treatment with HDL. RNA was extracted using Trizol, and reverse transcription was carried out using Moloney murine leukaemia virus reverse transcriptase oligo(dT) primers, and 1 μg of total RNA. Gene expression analysis was performed with TaqMan® Universal PCR Master Mix and commercially available primers were obtained from Applied Biosystems, unless otherwise noted, for human *ATF3* (Hs00231069_m1), *CASP1* (Hs00354836_m1), *IL1B* (Hs01555410_m1), *NLRP3* (Hs00918082_m1), *ACTB*, and *PYCARD* (Hs00203118_m1) on an ABI 7900. Values were normalized to human *ACTB* and then normalized to expression of each gene in the untreated cells. Values are expressed as log(2−ΔΔCt).

**RNA-seq data processing and pathway analysis**

RNA-seq transcriptome profiling was carried out for six samples (three samples from each of the PBS-treated and CSL111-treated groups). On average, 33 million reads are obtained. The RNA-seq reads are mapped to the hg19 with UCSC annotation. The average mapping rate is 88%.

The differential expression analysis was performed using the Bioconductor tool EDGER (Bioconductor EDGER is available at https://bioconductor.org/packages/release/bioc/html/edgeR.html). To integrate the differential expressed genes with biological pathways, we perform signalling pathway impact analysis (SPIA), which measures the significance of the observed total pathway perturbation.

**Statistical analysis for non-RNAseq data**

Results represent the mean ± standard error of the mean (SEM) and all statistics were calculated using PRISM 6 (GraphPad Software, Inc., La Jolla, CA). Group means were compared using a Student’s t-test or one-way analysis of variance as appropriate.

**Results**

**HDL blocks inflammasome activation**

We first investigated the ability of HDL to modulate inflammasome activation in a human monocyte/macrophage cell line THP-1, which secrete IL-1β in response to CC without any prior priming with LPS. We found that cells treated with HDL at the same time as CC had a profound reduction in the levels of IL-1β secreted into the media (Fig. 1a). To confirm these findings, we
possibly account for our findings. We further wished to significantly reduce the increase in inflammatory monocytes without rHDL and found that rHDL was able to significa-

tion of inflammasome activation and IL-1β production by rHDL treatment in LPS primed monocyte-derived macrophages following stimulation with CC (Fig. 1b).

To further explore the anti-inflammatory effect of HDL on CC in an animal model, we utilised a mouse model of peritoneal inflammation, which has been shown to be dependent on IL-1β.24,25 We injected CC with or without rHDL and found that rHDL was able to signifi-

cantly reduce the increase in inflammatory monocytes (CD45+ CD115+ CD11b+ Ly6CHi) recruited into the peritoneal cavity by over 2.5-fold (Fig. 1c). We also observed a trend toward decreased numbers of polymor-

phonuclear leucocytes following rHDL treatment (Fig. 1c). As the peritoneal cells were isolated 18 hr after injection, the peak of polymorphonuclear leucocyte infiltration was probably earlier and could explain why the difference was not larger for this particular cell population. The rHDL co-administration was also able to block the rise in serum IL-6 levels after injection of CC, so providing a possible explanation for the reduced recruitment of inflammatory monocytes (Fig. 1c). We were unable to detect IL-1β in either serum or peritoneal lavage fluid following treatment with CC at the 18-hr time-point (data not shown).

HDL interacts with cholesterol crystals

It has been previously reported that HDL can bind and partially dissolve CC,24,25 hence dissolution of CC could possibly account for our findings. We further wished to compare the ability of rHDL, which contains only apoA-I and soybean phospholipid, to nHDL, which contains the many different types of endogenous proteins, as well as different types of lipids known to comprise native HDL. Differences in either the protein or lipid content between the rHDL and nHDL could potentially result in differing effects for the two types of HDL particles. Using an immunoblot for detecting apoA-I, we found evidence for apoA-I binding to CC if either free apoA-I, nHDL, or rHDL were incubated with the crystals (Fig. 2a) as assessed by the presence of apoA-I protein bound to the CC after overnight incubation and extensive washing to remove unbound HDL or apoA-I. We did not observe, however, any appreciable loss of cholesterol from the crystals after incubation with nHDL or rHDL, as assessed by liberation of fluorescent cholesterol from crystals (Fig. 2b), or by a decrease in cholesterol crystal size, as determined by dynamic light scatter (Fig. 2c). When CC morphology following nHDL co-incubation was exam-

ined by scanning electron microscopy there were also no gross changes seen in CC morphology with the nHDL treatment (Fig. 2d). Our findings are similar to recent work by Niyonzima et al.,26 who was also unable to see significant dissolution of CC by HDL, despite earlier reports of HDL being able to mediate CC dissolution.24,25 We did observe a modest but non-significant decrease in inflammasome activation when nHDL or rHDL pre-
treated CC were given to THP-1 cells, as assessed by IL-

β release (Fig. 2e). We chose to focus mainly on nHDL as both forms of HDL (nHDL and rHDL) resulted in damping of IL-1β secretion, but nHDL would be more representative of the majority of HDL found in vivo in circulation.
HDL's inhibitory effect on inflammasomes requires cell interaction but not cholesterol efflux

Because pretreatment of CC with HDL did not explain its anti-inflammatory effect, we tested the pretreatment of THP-1 cells with nHDL. We found that when nHDL was added to cells at the same time as CC there was a significant 2-fold reduction in IL-1β release, but when the cells were first pretreated with nHDL overnight and nHDL was removed before addition of CC there was a striking 25-fold reduction in IL-1β release (Fig. 3a). HDL is a complex mixture of particles with multiple proteins and lipid species present, and many of these components have been described to have bioactive roles. To better understand which HDL component may be mediating the observed effects, we treated THP-1 cells with nHDL isolated from plasma, rHDL (phospholipid and apoA-I only), purified lipid-poor apoA-I, and lipid vesicles produced from the lipid portion of HDL. Interestingly, we found that purified apoA-I and the lipid vesicles when given alone had no significant effect in suppressing IL-1β secretion in response to CC (Fig. 3b). In contrast, both nHDL and rHDL demonstrated a strong suppressive effect (60 ± 15.4% and 70 ± 0.02%, respectively), although the rHDL effect was greater (Fig. 3b). These findings suggest the possible importance for the conformation of apoA-I in mediating these anti-inflammatory effects.

One of the main anti-atherogenic functions of HDL is believed to be its role in promoting the efflux of excess cellular cholesterol. In fact, recent work has demonstrated that the degree of cholesterol in cell membranes has been shown to modulate the immune responses in some cells. To determine the potential contribution of cholesterol efflux to inflammasome activation, THP-1 macrophages were treated with mβCD, a cyclical polymer of glucose that, similar to HDL, also removes cholesterol from cells. Treatment of THP-1 cells with nHDL caused no net change in total cholesterol content of the cells; however, treatment with mβCD removed about 45% of total cholesterol from the cells (Fig. 3c). Despite the much greater efflux of cholesterol from cells treated with mβCD, it did not significantly alter IL-1β secretion after stimulation with CC, whereas we detected, as before, a marked decrease in IL-1β secretion with nHDL treatment (Fig. 3d).

HDL alters CC cellular uptake and processing

To further explore the mechanism by which HDL is inhibiting inflammasome activation, we examined whether nHDL could decrease the association of CC with cells. Unexpectedly, we found that either pretreating cells or giving nHDL at the same time as the CC actually resulted in a small but significant 1.4-fold increase in the...
binding/phagocytosis of CC with THP-1 cells (Fig. 4a), as assessed by increased side scatter (SSC), but no change when using a CC that was BODIPY labelled by flow cytometry (Fig. 4b). Cytocholasin D, a phagocytosis inhibitor, significantly reduced the amount of CC phagocytosis but did not fully disrupt binding of the CC to cells as seen by the increase in SSC over cells not treated with CC (Fig. 4a). In contrast, we observed the opposite effect when we tested the uptake of *E. coli* particles labelled with pHrodo/C226 green. The pHrodo/C226 fluorophore is only fluorescent in the acidic environment of the lysosome and should only fluoresce when the *E. coli* labelled particles are internalized and trafficked to the lysosome. We found that nHDL pretreatment of cells resulted in an approximate 25% reduction of internalized *E. coli* particles (Fig. 4c). Similar results were also seen with *E. coli* particles labelled with Alexa Fluor® 488-labelled *E. coli* particles (Fig. 4c). As expected, cytochalasin D was able to completely block the phagocytosis of pHrodo-labelled particles.

Downstream activation of the NLRP3 inflammasome in response to the uptake of particulates has been shown to be dependent on lysosomal membrane rupture.\(^\text{11,32-34}\) To examine the effects of nHDL on CC-mediated lysosomal rupture, we monitored the decrease of LysoSensor staining in THP-1 cells following treatment with CC. Loss of LysoSensor signal has been previously shown to correlate with lysosome rupture after treatment with CC.\(^\text{11}\) We found that nHDL was able to stabilize the lysosome in response to CC cell uptake when it was given at the same time (2.6-fold reduction) as CC or if the cells were pretreated with nHDL (2.17-fold reduction, Fig. 5a).

As mitochondrial damage has been shown to play an important role in the activation of the NLRP3 inflammasome,\(^\text{20}\) we next determined if HDL could modulate this process. THP-1 cells were pretreated with nHDL and the degree with which CC were able to alter mitochondrial function was assessed by staining with MitoTracker green (labels all mitochondria) and MitoTracker red (labels only respiring mitochondria). We observed that CC were

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**Figure 3.** Pretreating cells with high-density lipoprotein (HDL) results in stronger interleukin-1β (IL-1β) suppression and is not dependent on cholesterol efflux. (a) THP-1 macrophages were treated with 1 mg/ml native HDL (nHDL) 18 hr before or with 1 mg/ml nHDL simultaneously with the addition of 1 mg/ml cholesterol crystals (CC). (b) THP-1 macrophages were treated for 18 hr with 1 mg/ml nHDL, reconstituted HDL (rHDL), apolipoprotein A-I (apoA-I), or lipid portion of HDL matched by phospholipid content to nHDL. (c) THP-1 macrophages were treated with 1 mg/ml nHDL or 200 μg/ml methyl-β-cyclodextrin (mβCD) followed by treatment with 1 mg/ml CC. (d) Amount of cholesterol (μg cholesterol/mg protein) recovered from THP-1 cells after overnight incubation with 1 mg/ml nHDL or 200 μg/ml mβCD. All data represent the mean ± SEM graph representative finding from at least three experiments, n = 3. *P < 0.05, ***P < 0.001.
HDL suppresses inflammasome activation

Figure 4. High-density lipoprotein (HDL) modulates phagocytosis of cholesterol crystals (CC). (a) CC phagocytosis as assessed by an increase in SSC was determined using CC with BODIPY-cholesterol incorporated. (b) Representative histograms of the increase in fluorescence of THP-1 following phagocytosis. (c) Phagocytosis of *Escherichia coli* particles was determined using a pH-sensitive and pH-insensitive fluorophore following HDL treatment of cells. (d, e) Representative histograms of THP-1 cells following incubation of *E. coli* particles using a pH-sensitive and pH-insensitive fluorophore, respectively. Data represent the mean ± SEM from at least three experiments, n = 3. *** P < 0.001.

able to induce a significant 2.5-fold increase in the number of cells with dysfunctional (non-respiring) mitochondria, but nHDL treatment had minimal effect on mitochondrial function after CC treatment (Fig. 5c).

HDL modifies expression of key components of the inflammasome

Before cells can rapidly respond to stimuli that activate the inflammasome, they typically need to be primed, which induces the expression of key structural and catalytic components of the inflammasome. To better understand the mechanism by which HDL is blocking inflammasome activation, we performed an RNA-seq experiment, using monocyte-derived macrophages primed with LPS and treated with rHDL. We used SPIA (http://www.bioconductor.org/packages/release/bioc/html/SPIA.html)\(^21\) to examine signalling pathways impacted by HDL treatment, which identifies pathways most relevant to the conditions of interest by considering two facts: (i) the over-representation of differentially expressed genes in a given pathway, and (ii) perturbation to signalling pathways topology by the differentially expressed genes. Perturbations that affect signalling propagation, such as the perturbations occurring upstream of the signalling pathway, are highly weighted as they are more sensitive to external signals. Following SPIA we observed a significant change in 2292 genes with cut-off being false discovery rate (fdr) \(< 0.05\) (Fig. 6a). The top 20 significantly altered pathways as identified by SPIA are reported in the Supplementary material (Table S1). Of particular note are alterations in the cytokine–cytokine receptor interaction, NF-κB signalling and NOD-like receptor signalling pathways, ranking first, second and seventh, respectively. rHDL treatment resulted in strong down-regulation of many of the genes in all of these pathways (see Supplementary material, Figs S2–S4).
We, therefore, performed a similar experiment in THP-1 cells with rHDL and examined the expression of key inflammasome genes that are known to be regulated by the NF-κB pathway by RT-PCR analysis. We observed inhibition of the expression of several key components of the inflammasome pathway (Fig. 6b). We did not observe any significant change in CASP1 mRNA after treatment with rHDL, but transcripts for both IL1β and NLRP3 were significantly decreased after preincubation of THP-1 cells with rHDL. This nicely mirrors the data we obtained from the RNA-seq experiment in monocyte-derived macrophages. Similar to what was recently reported by De Nardo et al., we also observed an increase in the level of ATF3, a transcription factor that has been demonstrated to mediate many of HDL’s anti-inflammatory effects to Toll-like receptor agonists.

In Fig. 6c, we also examined the effect of rHDL treatment on the protein expression of key components of the inflammasome. Although there was no effect of rHDL on CASP1 mRNA, we observed a decrease in both unactivated caspase-1 protein and the cleaved active form of caspase after incubation with either rHDL or nHDL but saw no effect on NLRP3 protein levels.

We also found that 15–30 min after addition of nHDL, an increase in the levels of IκB-α, indicating the attenuation of NF-κB signalling by nHDL (Fig. 6e). These findings coupled with the expression results indicated that modulation of the NF-κB pathway by HDL could be contributing to the suppression of inflammasome activation.

**HDL’s anti-inflammasome effects are broad and not limited to CC**

Given that the expression of several key components of the NLRP3 inflammasome were down-regulated in response to rHDL treatment, we next determined if HDL could also affect the activation of the inflammasome by other particulate stimuli besides CC. We observed that nHDL did indeed inhibit inflammasome activation by a wide variety of particles known to activate the NLRP3 inflammasome, such as silica, titanium dioxide and alum (Fig. 7a), as well as several soluble activators of the inflammasome, namely ATP and nigericin (Fig. 7b). We additionally treated THP-1 cells with poly(dA:dT), a synthetic activator of the AIM2 inflammasome, an inflammasome known to be activated by cytosolic dsDNA. Although the magnitude was not as great as seen for the NLRP3 inflammasome, nHDL was also able to suppress the activation of AIM2 inflammasomes in response to poly(dA:dT) (Fig. 7c).

**Discussion**

The main finding from this study is that HDL can potently suppress NLRP3 inflammasome activation, so identifying
potential new anti-inflammatory function of HDL. We investigated the different steps in activation of the inflammasome pathway by which HDL could alter this process (Fig. 8). As described below, it appears that several steps in the inflammasome activation pathway are potentially affected by HDL but the largest effect appears to be related to the decreased expression of several key inflammasome components, such as IL-1β and NLRP3, as well as decreased activation of caspase-1. SPIA analysis implicates a profound role of NF-κB signalling in down-regulation of many key components of the inflammasome (Supplementary Fig 4). We further found evidence that these anti-inflammatory effects of HDL extend beyond stimulation with CC but also apply to other particulate material and that not only NLRP3 inflammasome but other types of inflammasomes, such as AIM2, may also be affected by HDL.

Figure 6. High-density lipoprotein (HDL) suppresses expression of key components of the inflammasome. HDL’s ability to affect gene transcription in primary monocytes and THP-1 was examined. (a) Volcano plot showing genes alternatively expressed in monocyte-derived macrophages (DM) cells following treatment with lipopolysaccharide (LPS) and HDL. Genes that were significantly altered and play a role in the inflammasome are labelled. THP-1 macrophages were treated overnight with 1 mg/ml native (nHDL) or reconstituted (rHDL) HDL. (b) The expression level of genes playing a role in inflammasome activation was measured. (c) Western blot analysis of the protein level of caspase-1 and NLRP3 was measured (d) and quantified by densitometry and standardized to levels of GAPDH. (e) THP-1 cells were incubated with 1 mg/ml rHDL for the 15 or 30 min and the intracellular levels of IκB-α were assessed by flow cytometry. All data represent the mean ± SEM graph representative finding from at least three experiments, n = 3. ** P < 0.01, *** P < 0.001.
HDL was shown to interact with CC but this interaction did not result in the dissolution of CC (Fig. 2) or prevent its cellular uptake (Fig. 2). Niyonzima et al.²⁶ has also recently shown that HDL treatment does not dissolve CC; however, in their study they showed that HDL blocks complement deposition to the surface of CC and this resulted in the suppression of markers of activation in monocytes. They suggest that HDL’s anti-inflammatory effects are due mainly to HDL binding of crystals and modulation of complement activation, but we here demonstrate that HDL independent of complement inhibition has potent anti-inflammatory effects on the cells themselves. Differences between our two studies are probably explained by the lack of active complement in our study, and their use of a whole blood assay.

HDL pretreatment with CC resulted in a slight increase in CC cellular uptake or binding (Fig. 4). One early step in which HDL did appear to potentially block inflammasome activation was at the level of lysosomal integrity (Fig. 5). Loss of lysosomal integrity is a key step in inflammasome activation, because activated cathepsins are thought to generate a secondary messenger that activates NLRP3, which then results in activation of caspase-1 and

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**Figure 7.** High-density lipoprotein (HDL) suppresses inflammasome activation in response to a wide range of activators. Pretreatment of cells with 1 mg/ml native HDL (nHDL) was able to suppress activation of the inflammasome to (a) diverse particulate activators of the NLRP3 inflammasome, and (b) unrelated soluble NLRP3 activators. (c) Pretreatment with 1 mg/ml of nHDL blocks activation of the AIM2 inflammasome. All data represent the mean ± SEM graph representative finding from at least three experiments, n = 3. * indicated P < 0.05, ** P < 0.01, *** P < 0.001.

**Figure 8.** Model of high-density lipoprotein (HDL) suppression of the inflammasome. HDL exerts many different effects on cholesterol crystals (CC) induced inflammasome activation. HDL binds directly to CC (Step 1) but does not dissolve the CC or prevent phagocytosis. HDL treatment of cells results in decreased lysosomal rupture (Step 2). HDL signaling results in a decrease in nuclear factor-kB (NF-kB) signalling (Step 3) and reduces the expression of pro-interleukin-1β and NLRP3, and increases the expression of ATF3 (Step 4). Caspase-1 protein levels are decreased following HDL treatment (Step 5).
the cleavage of pro-IL-1β, allowing for its secretion. Cholesterol is well known to alter membrane stability and fluidity, and it has also been shown that cholesterol enrichment of the lysosomal membrane interferes with acidification by type V vacuolar ATPase. It is, therefore, likely that the enrichment of CC in lysosomes could alter cholesterol membrane content directly, leading to lysosomal instability, but the fact that other insoluble crystals do the same suggests that it may occur by some sort of mechanical disruptive process. Based on the lack of correlation between cholesterol efflux and inflammasome activation (Fig. 3), HDL’s beneficial effect on lysosomal integrity may be related to some other property of HDL. HDL pretreatment of CC, for example, has been shown to limit the amount of complement 3b deposited on the surface of the crystals, which could potentially alter how cells process these particles. Further work is needed to elucidate the exact mechanism by which HDL is modulating the stability of the lysosomal membrane.

Although it has been previously reported that HDL can mediate the down-regulation of the IL-1β transcript, other stimuli besides CC were previously tested and the mechanism was not well understood. Down-regulation by HDL of either mRNA or protein for IL-1β, NLRP3 and caspase-1 (Fig. 6), which are all core components of the inflammasome, is presumably why HDL is able to suppress inflammasome activation to a wide range of particles both those recognized by the NLRP3 inflammasome, such as alum, silica and TiO2, as well as poly(dA:dT) (Fig. 7). It was recently shown that some of HDL’s anti-inflammatory effects in response to TLR signalling were mediated through activation of ATF3, though the exact signalling pathway for ATF3 activation was not elucidated. In our study, we found mixed results regarding the importance of ATF3. In the THP-1 cells, we did observe an increase in ATF3 following treatment with HDL, but we saw no evidence for increased ATF3 when we examined monocyte-derived macrophages.

Although it is known that treatment of cells with HDL results in distinct intracellular signalling pathways, it is not clear which exact receptor or signalling cascade HDL is activating through to exert its anti-inflammatory effects. Our results here provide further evidence that the NF-κB pathway is playing a vital role in the mediation of anti-inflammatory effects of HDL (see Supplementary material, Fig. S3). Some of this signalling has been shown to be intrinsic to the protein cargo of HDL, such as apoA-I. HDL also transports a wide variety of bioactive lipids, such as sphingosine-1-phosphate and lysophosphatidylcholines, which are also potent signalling molecules. Our findings in Fig. 3 demonstrate that the suppressive effects of HDL are not being mediated by bioactive lipids on nHDL as similar results were found with rHDL, which is only composed of soybean phosphatidylcholine and apoA-I. Interestingly, apoA-I alone is also not able to mediate suppression of the inflammasome. This would suggest that the conformation of apoA-I may be important in mediating the suppression of the inflammasome. It is well known that the tertiary structure of apoA-I is quite dynamic and can change dramatically depending on lipid content and size of the HDL particle. Our results here suggest that lipid-free/poor apoA-I may not have the correct structure to suppress activation of the inflammasome. Also the results in Fig. 3(b) suggest that different subtypes of HDL may have differing capacities to block activation of inflammasome. Further research is needed to explore this observation, and proteomics and lipidomics could provide further elucidation of the key components that play a role in the anti-inflammatory effects of HDL. The NF-κB pathway has been shown to control the expression of IL-1β and NLRP3. Our current study provides further evidence of NF-κB’s role in mediating HDL’s anti-inflammatory effects, but more work is needed to link how HDL affects signalling by this pathway.

One limitation of our study is that the THP-1 cells used in our study do not need to be primed to up-regulate their expression of IL-1β or NLRP3, which may result in differences from what is observed in primary cells. It is difficult, however, to test HDL’s effects on the priming stage in inflammasome activation as HDL has long been known to adsorb LPS and changes observed could be due solely to neutralization of LPS in the culture. We have demonstrated, however, that HDL treatment also affected inflammasome activation of primary monocyte-derived macrophages (Fig. 1). HDL also blocked cell activation and recruitment in response to inflammation induced by injecting CC into the peritoneal cavity of mice (Fig. 1), showing the likely in vivo relevance of our findings.

In conclusion, we have described here a novel anti-inflammatory effect of HDL on inflammasome activation. Whereas further studies are needed to better understand the exact molecular mechanism and integral components of HDL that are required, we have described a new pathway through which HDL could suppress inflammation caused by CC in atherosclerotic plaque. Results from this study may also help to explain the beneficial effects of HDL that have been described in other crystalline diseases, such as gout and silicosis.

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Author contributions
SGT designed, performed experiments, and wrote the manuscript. AZ, MSA, LF, DOS and SD performed experiments. YC analysed the RNAseq data. ATR designed and wrote the manuscript.

Disclosure
None of the authors have any conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

**Figure S1.** Inflammatory cell immunophenotyping gating strategy.

**Figure S2.** Alterations in cytokine signalling pathways.

**Figure S3.** Nuclear factor-κB signalling pathway.

**Figure S4.** Alterations in NOD-like receptor pathways.

**Table S1.** Top 20 pathways altered by reconstituted high-density lipoprotein treatment of primary monocytes.

HDL suppresses inflammasome activation