Neutrophil Activation Is Attenuated by High-Density Lipoprotein and Apolipoprotein A-I in In Vitro and In Vivo Models of Inflammation

Andrew J. Murphy, Kevin J. Woollard, Andreas Suhartoyo, Roslynn A. Stirzaker, James Shaw, Dmitri Sviridov, Jaye P.F. Chin-Dusting

Objective—Neutrophils play a key role in the immune response but can undesirably exacerbate inflammation. High-density lipoproteins (HDL) are antiinflammatory particles, exerting beneficial cardiovascular influences. We determined whether HDL exerts antiinflammatory effects on neutrophils and explored the mechanisms by which these occur.

Methods and Results—CD11b on activated human neutrophils was significantly attenuated by apolipoprotein A-I (apoA-I) and HDL. The effects of apoA-I were mediated via ABCA1, whereas the effects of HDL were via scavenger receptor BI. Both were associated with a reduction in the abundance of lipid rafts, and a strong correlation between raft abundance and CD11b activation was observed. ApoA-I and HDL reduced neutrophil adhesion to a platelet monolayer under shear flow, as well as neutrophil spreading and migration. ApoA-I also inhibited leukocyte recruitment to the endothelium in an acute in vivo model of inflammation. Finally, infusion of reconstituted HDL in patients with peripheral vascular disease was demonstrated to significantly attenuate neutrophil activation.

Conclusion—We describe here a novel role for HDL and apoA-I in regulating neutrophil activation using in vitro, in vivo, and clinical approaches. We also show that these effects of HDL and apoA-I involve a mechanism requiring changes in membrane domain content rather than in cholesterol efflux per se. (Arterioscler Thromb Vasc Biol. 2011;31:1333-1341.)

Key Words: apolipoprotein A-I ■ cell adhesion ■ high density lipoprotein ■ inflammation ■ neutrophils

There is increasing evidence of a causative role for neutrophils in atherosclerosis. Neutrophil abundance is positively correlated with atherosclerosis and acute coronary events,1,2 and neutrophils have been identified in atherosclerotic lesions.3 They become primed in hyperlipidemia, where severity directly correlates with CD11b expression and superoxide release.4–6 The effects may be directly linked with increases in membrane cholesterol, as cholesterol loading of neutrophils causes activation.7 Furthermore, the recent findings that hypercholesterolemia induces rapid neutrophilia that infiltrates aortic lesions8 and that depletion of neutrophils can lead to a decrease in plaque size9 demonstrate a clear and important role for neutrophils in atherogenesis.

The cardioprotective effects of high-density lipoprotein (HDL) and its principal protein component, apolipoprotein A-I (apoA-I), are well described.10,11 These effects include the ability of HDL to facilitate reverse cholesterol transport, regulate vascular tone through nitric oxide (NO), to act as an antiinflammatory and antithrombotic agent,10,11 and regulate hematopoiesis.12 HDL has been reported to exert antiinflammatory effects not only on the endothelium but also on leukocytes.13,14 It has also become apparent that different constituents of HDL are required to mediate these events on different cell types. Phospholipids, for example, have been shown to be key in inhibiting adhesion molecule expression on endothelial cells in a process linked to NO production.15 On the other hand, a potent and rapid antiinflammatory role for both HDL and lipid-free apoA-I on monocytes has been reported.14 In the latter, the common pathway for both HDL and apoA-I appears to involve modulation of lipid rafts despite these molecules acting via different receptors. Interestingly, macrophages from mice deficient in the 2 major HDL receptors, ATP-binding cassette transporter (ABC) A1 and ABCG1, show increased inflammatory features which are associated with increases in lipid rafts.16 Thus lipid rafts appear to be an important determinant of the inflammatory status of leukocytes. Interestingly, ABCA1/ABCG1 double-knockout mice develop dramatic neutrophilia and monocytosis, resulting in a dramatic increase in atherosclerosis12 that is reversible by overexpression of apoA-I.

With the growing body of evidence that neutrophils are directly activated by hypercholesterolemia and that they are a key mediator of atherogenesis, we examined whether, and how, modulation of cholesterol efflux pathways by HDL and
apoA-I attenuates neutrophil activation. To do this, in vitro, in vivo and clinical approaches were used.

Methods
An expanded Methods section is given in the supplemental materials, available online at http://atvb.ahajournals.org.

Blood Collection
Blood was collected from healthy volunteers by venipuncture. This study was approved by the Alfred Hospital Human Ethics Committee, and animal components of this work was approved by the Alfred Medical Research and Educational Precinct Animal Ethics Committee.

Neutrophil Isolation
Human neutrophils were isolated from whole blood by density centrifugation as previously described. Cell number and purity was determined using an automated hematology analyzer (KX-21N, Sysmex). Neutrophil preparations were used only if the purity of the sample was greater than 95%.

Platelet Isolation
Platelets were isolated from whole blood by centrifugation as previously described and resuspended in Tyrode buffer (10 mmol/L HEPES, 12 mmol/L NaHCO₃, pH 7.4, 137 mmol/L NaCl, 2.7 mmol/L KCl, 5 mmol/L glucose) containing Ca²⁺/Mg²⁺ at 2.8×10⁵ platelets/mL.

Cholesterol Acceptors
HDL was isolated from pooled normolipidemic plasma supplied by the Red Cross using sequential density ultracentrifugation with KBr (density, 1.085 to 1.21 g/mL). Human plasma apoA-I was isolated as previously described, as was HDL. Human rHDL Clinical Study was approved by the Alfred Hospital Human Ethics Committee.

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Neutrophil Activation
CD11b expression and activation was measured as previously described. Experiments in whole blood used the following gating strategy. Neutrophils were gated from viable cells, removing doublets and then selecting CD45hiCD115loLy6-C/Ghi (Gr-1), and the expression of CD11b or CD62L was quantified using FlowJo analysis software. Expression was controlled using the appropriate fluorescently tagged rat IgG2b isotype control antibodies.

Receptor Detection: Flow Cytometry
For ABCA1 detection, neutrophils were incubated with an anti-ABCA1 antibody and a fluorescein isothiocyanate (FITC)-conjugated secondary antibody. Scavenger receptor BI (SR-BI) was detected with an anti-SR-BI antibody (Novus) and a Texas Red–conjugated secondary antibody. To assess nonspecific binding, neutrophils were first incubated with isotype-matched control antibodies before the addition of the respective fluorescently tagged secondary antibody. Expression was quantified by flow cytometry.

Immunofluorescent Microscopy
Isolated neutrophils were allowed to adhere onto glass cover-slips before incubation with the respective antibodies as described above. 4',6-Diamidino-2-phenylindole (DAPI; 5 μg/mL) was added during the final 30 minutes of incubation. The cells were then washed, fixed, mounted onto glass slides, and viewed using an Olympus microscope.

Cholesterol Efflux
Neutrophils were loaded with ³H-cholesterol for 4 hours at 37°C. The neutrophils were then washed 3 times in PBS by centrifugation. Efflux to the respective cholesterol acceptors was measured in triplicate and allowed to occur for 15, 30, and 60 minutes. Cells were then pelleted, and the radioactivity in cells and supernatant were used to quantify the cholesterol efflux. Results were expressed as percentage efflux compared with control (PBS).

Lipid Rafts
For quantification of lipid rafts, isolated neutrophils were incubated at 37°C with the various lipid acceptors for 15, 30, or 60 minutes. The neutrophils were washed in PBS and incubated with FITC-cholera toxin B for 1 hour at room temperature. The cells were fixed, and lipid rafts were measured by flow cytometry. Results were normalized to control.

Shear-Flow Cell Adhesion
Shear-flow experiments were conducted in glass capillaries coated with a monolayer of spread platelets. Isolated neutrophils were treated with phorbol 12-myristate 13-acetate (PMA) in the presence or absence of HDL or apoA-I for 15 or 60 minutes at 37°C before perfusion. Neutrophil-platelet interactions were visualized, recorded, and analyzed as previously described.

Migration Assay
Neutrophil migration was assessed as previously described. Neutrophils were preincubated in PBS in the presence or absence of HDL or apoA-I for either 15 or 60 minutes before seeding into the upper chamber, and formyl-Met-Leu-Phε (fMLP) was used as the chemoattractant in the lower chamber. Migration was allowed to occur for 30 minutes at 37°C. Migration was expressed as a percentage of control (PBS).

Intravital Microscopy
Leukocyte-endothelial interactions were studied in the cremaster vessels of male C57Bl/6 mice using intravital microscopy as previously described. Mice received an intraperitoneal injection with either vehicle (PBS) or tumor necrosis factor-α (TNF-α) (500 ng) 4 hours before imaging. This amount of TNF-α has previously been shown to induce systemic inflammation and significant increases in leukocyte recruitment to the inflamed endothelium. At time 0 of imaging, either vehicle (PBS) or apoA-I (20 mg/kg) was delivered via the jugular vein, and 30-second recordings were captured at 0, 2.5, 5, 10, 15, 30, 45, and 60 minutes. The captured videos were analyzed off-line for stationary adhesion, rolling flux, and rolling velocity as previously described. Neutrophil activation was also measured in a parallel set of mice subjected to the same treatment, except that apoA-I was administered via tail vein infusion and blood was drawn via tail bleeding. Neutrophil activation was measured via flow cytometry.

Human rHDL Clinical Study
For patient criteria, study design and methods please refer to the online methods.

Statistics
Values are presented as mean±SEM or percentage of control±SEM. All results were analyzed for statistical significance using 1-way ANOVA followed by the Bonferroni post hoc test, except the results of the perfusion study, which were analyzed using a 2-way ANOVA followed by Bonferroni post hoc test, and CD11b expression data from the reconstituted (r)HDL infusion study, which were analyzed using the unpaired Student t test. Statistical significance was set at P<0.05.

Results
Time-Dependent Modulation of CD11b Expression/Activation
CD11b expression and activation was measured by flow cytometry using specific antibodies that either recognize total CD11b surface expression or only the activated form respectively. When stimulated with PMA or lipopolysaccharide,
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neutrophils displayed increased expression and activation of CD11b. Although PMA is not a physiological activator of neutrophils, it provides a maximal and robust activation of neutrophils. Both were potently inhibited by apoA-I, but not by HDL, at 15 minutes (Figure 1A to ID). An inhibitory effect of HDL became apparent only at 60 minutes (Figure 1E). Cyclohextrin exhibited a profile similar to that of apoA-I. Incubation of neutrophils with dimyristoylphosphatidylcholine liposomes had no effect on CD11b expression or activation. Importantly, bovine serum albumin had no effect on reducing neutrophil activation (Supplemental Figure IA), confirming that apoA-I and HDL do indeed suppress neutrophil activation and that PMA is not merely being scavenged by HDL, at 15 minutes (Figure 1A to 1D). An inhibitory effect of HDL became apparent only at 60 minutes (Figure 1E).

CD11b Expression Is Attenuated Via Specific Receptor Interactions With apoA-I and HDL

Using real-time polymerase chain reaction to screen for HDL receptors, it was established that nonactivated primary human neutrophils isolated from healthy donors express both SR-BI and ABCA1 but extremely low amounts of ABCG1 (Supplemental Figure IIIA and IIIB). Cell surface expression of SR-BI and ABCA1 was confirmed via flow cytometry (Figure 2A and 2C) and immunofluorescent microscopy (Figure 2B and 2D and Supplemental Figure IIIIC and IIID, respectively). ApoA-I binding to neutrophils was also detected after a 15 minutes incubation by flow cytometry. Blockade of SR-BI significantly attenuated the ability of HDL to inhibit CD11b activation (Figure 2E), whereas ABCA1 blockade reduced the effects of apoA-I (Figure 2F). We have previously shown in monocytes that SR-BI does not inhibit the function of lipid-free apoA-I and that ABCA1 blockade does not attenuate the antiinflammatory effects of HDL, which is consistent with numerous studies reporting no interaction of lipid-free apoA-I with SR-BI or HDL with ABCA1.

Disruption of Lipid Rafts Correlates With Reduced CD11b Activation

HDL but not apoA-I induces a time-dependent removal of cholesterol from neutrophils preloaded with [3H]cholesterol (Figure 3A). ApoA-I and cyclohextrin but not HDL rapidly disrupted lipid rafts (Figure 3B). A similar result was also seen with apoA-I and HDL when neutrophils were stained with filipin to quantify unesterified cholesterol in the cell membrane (Supplemental Figure IV). Although no correlation was observed between cholesterol efflux and CD11b activation (Figure 3C), a significant correlation was evident between lipid raft abundance and CD11b activation (Figure 3D). No correlation between lipid raft abundance and cholesterol efflux was observed (Supplemental Figure V).

Cell Adhesion and Spreading Under Shear Flow

To examine the effects of apoA-I and HDL on neutrophil adhesion and spreading under flow conditions, isolated neutrophils were incubated with PMA to cause maximal activation and tested for adhesion to platelet monolayers in a flow chamber. Coincubation of neutrophils for 15 minutes with apoA-I and PMA before perfusion resulted in a significant reduction of adhesion and spreading (Figure 4A to 4C). Consistent with our findings for CD11b expression, incubation with apoA-I or HDL for 60 minutes before perfusion significantly attenuated both neutrophil adhesion and spreading to platelets (Figure 4D to 4F).

Figure 1. CD11b expression and activation. Total CD11b membrane surface expression (A and C) and the active ligand binding form of CD11b (B and D) were measured using flow cytometry to determine the inflammatory status of neutrophils. Neutrophils were stimulated with PMA (0.1 μmol/L) with or without HDL (50 μg/mL) or apoA-I (40 μg/mL) (A and B) or lipopolysaccharide (LPS) (1 μg/mL) with or without HDL or apoA-I (C and D) for 15 minutes. "P<0.05, **P<0.01, n=4. In the time-dependent studies (E), neutrophils were incubated with PMA (⊗) with or without HDL (⊗), apoA-I (♀), cyclodextrin (△), or dimyristoylphosphatidylcholine liposomes (■), and CD11b activation was measured using flow cytometry accompanied by an FITC-isotype control. #P<0.05, HDL vs PMA; ∗P<0.05, **P<0.01, apoA-I vs PMA; †P<0.01, cyclodextrin vs PMA; n=4 to 5.
Neutrophil Migration

Neutrophils showed a significant migratory response to fMLP compared with control (PBS). Incubation of the neutrophils with apoA-I but not HDL for 15 minutes significantly inhibited neutrophil migration to fMLP (Figure 4G). Both apoA-I and HDL significantly inhibited neutrophil migration after 60 minutes of incubation (Figure 4H).

ApoA-I Reduces Leukocyte Adhesion in an In Vivo Mouse Model of Inflammation

To investigate whether the effects of apoA-I observed in vitro are true in an in vivo situation, we tested them on mice using intravital microscopy. Mice were administered either saline (control) or TNF-α by intraperitoneal injection to induce an inflammatory response, upregulating vascular adhesion molecules and stimulating leukocyte recruitment. Leukocytes in mice injected with TNF-α displayed a significant increase in stationary adhesion over a 60-minute imaging period compared with mice injected with saline (control). Although there appeared to be more adherent leukocytes at time 0 in the mice stimulated with TNF-α and treated with apoA-I, there was a clear and potent antiinflammatory effect of apoA-I (Figure 5A). This became evident after 15 minutes of incubation compared with time 0. The inhibition was complete after 60 minutes, approaching the level of adhesion in control mice. At time 0, there was a significantly higher rolling flux in the TNF-α/apoA-I group, reflecting the increased adhesion before apoA-I infusion. Importantly, this was reduced to near basal levels after 1 hour of apoA-I infusion (Figure 5B). The rolling flux in TNF-α treated mice remained constant over time. We also examined the tethering and rolling velocity of the leukocytes (Figure 5C). As expected, leukocytes in control mice rolled on the endothelium with a consistently high velocity. Leukocytes of mice treated with TNF-α rolled with a significantly reduced velocity. The rolling flux of leukocytes after infusion of TNF-α/apoA-I was comparable to the levels of rolling flux in mice infused with TNF-α at time 0; however, it significantly increased over time to reach a velocity similar to the control mice, suggesting a downregulation of adhesion molecules. Interestingly, we observed a marked decrease in neutrophil activation, as assessed by CD11b levels, in TNF-α treated mice infused with apoA-I; the effect was evident after 30 minutes (Supplemental Figure VIA).
The effect of apoA-I infusion on size distribution of host HDL particles after 60 minutes was examined by native polyacrylamide gel electrophoresis following by immunoblotting for human apoA-I. There was a significant increase in poorly lipidated apoA-I in both the apoA-I-only and the TNF-α/H9251/apoA-I-treated groups (Figure 5D), and most of the apoA-I infused remained poorly lipidated (Supplemental Figure VIB). Furthermore, a significant increase in intensity of pre-H9252/HDL particles was observed in the apoA-I infused mice compared with control. This was accompanied by an increase in α-HDL particles, suggesting that some of the lipid-free apoA-I had acquired lipid (Figure 5E).

Neutrophils From Peripheral Vascular Disease Patients Treated With rHDL Have a Decrease in CD11b Expression
Peripheral vascular disease patients were infused with rHDL (80 mg/kg body weight) or placebo for 4 hours in a trial described previously. Blood was collected preinfusion or 5 days postinfusion. Plasma HDL cholesterol remained significantly raised 5 to 7 days postinfusion in patients receiving rHDL. This was accompanied by a significant decrease in neutrophil activation as determined by total CD11b membrane expression in the rHDL group pre- versus postinfusion (Figure 6B). No change was observed in patients receiving placebo (Figure 6A). Although CD11b expression tended to be higher in the preinfusion samples from patients receiving rHDL therapy, this was not significantly different from the baseline values in patients receiving placebo. A significant decrease in neutrophil activation was also observed when directly comparing change in CD11b expression postinfusion between rHDL and placebo (Figure 6C, *P<0.05*).

Discussion
A causative role for neutrophils in atherogenesis has been described. In this study, we demonstrated that apoA-I and HDL could inhibit neutrophil activation, adhesion, spreading, and migration and that the antiinflammatory effect was strongly associated with the abundance of lipid rafts. We established that these antiinflammatory effects could be observed in in vivo in mice and humans. Investigating the time course of the effects, we found that inhibition of neutrophil CD11b activation and expression by apoA-I was more rapid than that of HDL. Importantly, HDL not only inhibited potent activators of neutrophils (PMA & fMLP) but also attenuated priming of neutrophils by lipopolysaccharide. Cyclodextrin inhibited neutrophil CD11b activation similarly to apoA-I, whereas phospholipid liposomes had no effect.
As a main function of HDL and apoA-I is to facilitate cholesterol efflux, we examined the effect of cholesterol/lipid removal from neutrophils. We confirmed that granulocytes and monocytes bind HDL,27 and in addition we showed that neutrophils express the necessary receptors/transporters for cholesterol efflux. Efflux of radiolabeled cholesterol to HDL but not apoA-I was observed over a 60-minute period. Usually, the magnitude of cholesterol efflux to apoA-I is one third to one fifth that of the cholesterol efflux to HDL, thus it is possible that the short incubation period rendered the amount of labeled cholesterol effluxed to apoA-I below the detection threshold. Interestingly, lipid raft abundance was rapidly decreased in monocytes by both HDL and apoA-I,14 relating to an attenuation in CD11b expression. Although GM1 staining does not directly provide information on lipid order in cell membranes, increased abundance of rafts provides evidence of changes in the cell membrane in response to a stimulus. This indicated that lipid rafts may play a role in leukocyte activation, a hypothesis supported by a strong correlation between raft abundance and CD11b activation. As lipid rafts hold only a small proportion of cellular cholesterol, removal of specifically raft cholesterol may not have been detectable in the cholesterol efflux experiment. Interestingly, when neutrophils are loaded with cholesterol, adhesion is increased.7 The reasons for HDL triggering a slower response than apoA-I may be related to membrane structure or receptor abundance. It may also be a result of apoA-I, a lipid poor molecule, being able to extract more lipids than mature HDL, which not only extracts lipids from the cell membrane but also donates lipids.27 Furthermore, we demonstrated the dependence of the effects of apoA-I on the presence of ABCA1, which is a unidirectional transporter, whereas HDL required SR-BI, a bidirectional receptor.28 Whether ABCG1 is upregulated in neutrophils under conditions of oxysterol loading (inducing liver X receptor activation) to increase the effect of HDL is not known. It is also not known whether other reported antiinflammatory roles of apoA-I on neutrophils, including the inhibition of the oxidative burst,29,30

Figure 4. Functional inflammatory responses of neutrophils to cholesterol acceptors. A to F, Neutrophils were treated with PMA (0.1 μmol/L) (●) with or without HDL (50 μg/mL) (○) or apoA-I (40 μg/mL) (▼) for either 15 minutes (A to C) or 60 minutes (D to F) and perfused over a platelet monolayer. Total adhesion (A and D), stationary adhesion (B and E), and spreading (C and F) were quantified at 30, 60, 150, 300, and 600 seconds. ***P<0.001, **P<0.01, *P<0.05, apoA-I; ##P<0.05, #P<0.05, HDL; n=3. G and H, Neutrophil transmigration to fMLP was examined. Neutrophils were preincubated with PBS (control), HDL, or apoA-I for 15 minutes (G) or 60 minutes (H), seeded into the upper well, and allowed to migrate to fMLP for 30 minutes. **P<0.01; n=6.
involve ABCA1 and modulation of lipid rafts, but this is plausible given the timing and the role of apoA-I and not HDL.

The time-dependent inhibition of neutrophil activation by HDL and apoA-I was also investigated in functional in vitro assays mimicking the process required for leukocyte recruitment to sites of inflammation. Although apoA-I decreased neutrophil adhesion, spreading, and migration after 15 minutes, HDL required a longer incubation. As the majority of the neutrophils were either rolling or tethering, it is possible that apoA-I and HDL may also disrupt these processes. It must be recognized that in this model, leukocytes are activated before recruitment, whereas in vivo neutrophils are activated by immobilized chemokines in situ. This experiment provided functional evidence that apoA-I and HDL inhibit neutrophil adhesion in an environment free of potentially confounding factors.

Intravital microscopy was used to examine leukocyte-endothelial interactions in vivo. The entire leukocyte population, not just neutrophils, was examined in these studies; however, a recent report demonstrated that neutrophils were the dominant leukocyte to interact with the cremaster vessel during inflammation. We demonstrated that infusion of apoA-I in mice preactivated with TNF-α significantly attenuates neutrophil recruitment to the inflamed endothelium, thus inhibiting acute inflammation. This is consistent with previous in vivo studies demonstrating that infusion of rHDL or apoA-I up to 9 hours postsurgery can significantly inhibit neutrophil infiltration at sites of vascular inflammation in rabbits. In the current study, infusion of apoA-I inhibited the already present inflammation, whereas the extent of inflammation at the time of apoA-I injection in the rabbit study was unknown and is likely to have been only mild. Furthermore, Apoe−/− mice transgenic with apoA-I have fewer atherogenic lesions, with no decrease in vascular cell adhesion molecule-1 staining, suggesting that apoA-I acts on circulating cells. Moreover, as apoA-I cannot decrease endothelial activation and HDL can only inhibit activation, not reverse it, our findings largely describe the antiinflammatory role of apoA-I on leukocytes. Indeed, we observed

Figure 5. ApoA-I reduces leukocyte adhesion in an in vivo mouse model of inflammation. Mice were injected with either vehicle (PBS) or TNF-α before each experiment. At the beginning of each experiment, mice were injected intravenously with either vehicle (PBS) or apoA-I. The groups were as follows: control ( ), TNF-α ( ), apoA-I ( ), and apoA-I/TNF-α ( ). Stationary adhesion (A), rolling flux (B), and rolling velocity (C) were measured. **P < 0.001, *P < 0.01, ‘P < 0.05, TNF-α + apoA-I vs TNF-α; n = 3.

D. Western blot analysis of mouse plasma. Plasma was subjected to native gradient polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane and probed for apoA-I. Lipid-free apoA-I levels (saline vs apoA-I only vs TNF-α + apoA-I, P < 0.01) and HDL levels (saline vs apoA-I only vs TNF-α + apoA-I, P = 0.054; n = 3). Ratio of apoA-I:HDL is shown. S indicates saline; A, apoA-I; A + T, apoA-I + TNF-α. **P < 0.01. E. Agarose gel analysis of the lipid migratory profile in mouse plasma stained with Sudan black. cHDL indicates control purified HDL. Shown are pre-β-HDL (Beta) (saline vs apoA-I only vs TNF-α + apoA-I, P < 0.01) and α-HDL (Alpha) (saline vs apoA-I, P = 0.062; vs TNF-α + apoA-I, P = 0.05, n = 3).
that apoA-I infusion in TNF-α-stimulated mice led to a decrease in neutrophil activation. It was confirmed that following infusion, the majority of apoA-I remains poorly lipidated and the apoA-I that did acquire lipid became pre-β-HDL, suggesting that either lipid-free apoA-I or pre-β-HDL particles are responsible for the decrease in leukocyte recruitment. It remains unknown whether the pre-β-HDL particles are formed because of ABCA1-driven cholesterol efflux, HDL remodeling, or nonspecific binding of free lipid in circulation. However, the results do suggest that this has a direct effect on the circulating leukocytes but not the endothelium.

We have previously reported that patients with peripheral vascular disease, a chronic vascular pathology, have a significant reduction in monocyte activation as measured by CD11b expression 5 days after a bolus infusion of rHDL.25 In this study, we report assessment of neutrophil activation and show that there was also a significant reduction in neutrophil activation postinfusion. Given that average circulating half-life of a neutrophil is 10 to 12 hours, this finding suggests that rHDL infusion has long-lasting effects on the inflammatory environment. Although these findings are consistent with the findings from in vitro assays and the mouse model, there are many confounders that could make the correlation coincidental. It also remains to be determined how long the effect of a single infusion of rHDL on inflammation would last. This is an important issue as neutrophils can act as factories for cytokine production, aiding in fueling inflammation; thus, intervention with HDL or apoA-I could be an efficient way to combat inflammatory diseases.33

In summary, we have shown for the first time a potent and rapid antinflammatory effect of apoA-I on neutrophil recruitment in vivo. Infusion of rHDL in patients with peripheral vascular disease significantly reduces neutrophil activation. We have also demonstrated that apoA-I and HDL can inhibit neutrophil activation through a process that appears to be closely linked to lipid raft abundance and mediated through ABCA1 or, in the case of HDL, SR-BI. Accumulating evidence of multiple antinflammatory effects for HDL and apoA-I suggests that the cholesterol efflux pathway is an integral part of the innate immune system, regulating a number of cellular pathways.

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Disclosures
None.

References

Figure 6. rHDL infusion in patients with peripheral vascular disease attenuates leukocyte activation. CD11b expression was measured on neutrophils as a marker of activation before and 5 to 7 days after infusion with placebo (A) and rHDL (80 mg/kg) (B). CD11b was labeled on neutrophils in whole blood and measured via flow cytometry. Results were normalized to the FITC-isotype control antibody. rHDL, P<0.05, preinfusion vs postinfusion. C, Fold change in CD11b expression on blood neutrophils of placebo and rHDL. P<0.05, placebo vs rHDL.
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Supplemental Material

Methods

Blood collection
Blood was collected from healthy, consenting volunteers by venepuncture and drawn into syringes containing sodium citrate or acid-citrate-dextrose (ACD). This study was approved by the relevant Institutional Ethics Committee at either AMREP (animal studies) or the Alfred Hospital (human studies).

Neutrophil Isolation
Resting human neutrophils were isolated from whole blood (sodium citrate) by density centrifugation as previously described \(^1\). Briefly, blood was layered over the 1077 and the 1119 Histopaque solutions (Sigma, Australia) in a ratio of 2:1:1. The layered solution was centrifuged at 350g for 30 mins at room temperature. The neutrophils were collected and washed three times in PBS (without Ca\(^{2+}/Mg\(^{2+}\)) by centrifugation at 400g for 10 mins before being resuspended in PBS (with Ca\(^{2+}/Mg\(^{2+}\)). Cell number and purity was determined on an automated hematology analyzer (Sysmex, KX-21N, USA). Neutrophil preparations were only used if the neutrophils were greater than 95% pure.

Platelet isolation
Platelets were isolated from whole blood (ACD) by centrifugation as previously described \(^1\) and resuspended in tyrodes buffer (10 mmol/L hepes, 12 mmol/L NaHCO\(_3\), pH 7.4, 137 mmol/L NaCl, 2.7 mmol/L KCl, 5 mmol/L glucose) containing with Ca\(^{2+}/Mg\(^{2+}\) at 2.8x10\(^8\) platelets/mL.

Purification and production of the cholesterol acceptors
HDL was isolated from pooled normolipidemic plasma supplied by the Red Cross using sequential density ultracentrifugation using KBr (density 1.085-1.21g/mL). Human plasma apoA-I was isolated as previously described \(^2\) and purity determined using mass spectrometry. Dimyristoylphosphatidylcholine (DMPC) liposomes were prepared as previously described \(^2\) as was \(\beta\)-cyclodextrin (Sigma) \(^2\).

Neutrophil activation
CD11b expression was measured as previously described \(^2\). Isolated neutrophils were stimulated with either 0.1\(\mu\)M PMA or 1\(\mu\)g/mL LPS (Sigma, Australia) ± HDL (50\(\mu\)g/mL) or apoA-I (40\(\mu\)g/mL) and incubated with the FITC conjugated Ab to either the active epitope of CD11b (eBiosciences, USA, Clone CBRM1/5) or total CD11b (Serotec, USA, Clone ICRF44) at 37°C for a specified time. Cells were then
fixed with 4% paraformaldehyde. An isotype matched antibody was used as a negative control (FITC-anti-mouse IgG, Serotec, USA, Clone W3/25). CD11b was measured by flow cytometry using the FACS Calibur (Becton Dickinson). Analysis was conducted using the Cell Quest Pro software. Results were expressed as a percentage of the unstimulated control (100%).

Whole blood experiments were conducted using C57Bl/6 mouse blood. To measure CD11b blood was incubated with either PMA ± apoA-I or fMLP (10nM) ± apoA-I with anti-CD45-APC Cy7, anti-Ly6C/G-PerCP Cy5.5 (BD Biosciences), CD115-APC and anti-CD11b-FITC (eBiosciences) or for CD62L with anti-CD45-APC Cy7, anti-Ly6C/G-FTTC (Gr-1) CD115-APC and anti-CD62L-PerCP for 15mins before the red blood cells (RBCs) were lysed and the cells fixed in 2% PFA before running on the flow cytometer (LSRII, BD Biosciences). Neutrophils were gated from viable single cells and then selecting CD45\textsuperscript{hi}CD115\textsuperscript{lo}Ly6\textsuperscript{-}C/G\textsuperscript{hi} (Gr-1) and the expression of CD11b or CD62L was quantified using the flowJo analysis software. Expression was controlled using the appropriate fluorescently tagged Rat IgG2b isotype control antibodies (eBiosciences).

Receptor detection: flow cytometry
For ABCA1 detection neutrophils were incubated with an anti-ABCA1 antibody \(^3\) and a FITC conjugated secondary antibody, while SR-BI was detected with an anti-SR-BI antibody (Novus, USA) and a Texas Red conjugated secondary antibody. To assess non-specific binding of the secondary anti-bodies neutrophils were first incubated with isotype control antibodies before the addition of the respective secondary fluorescently tagged antibody. Primary incubations occurred for 1hr at 37°C followed by washing in PBS before a further 30 mins incubation with the respective secondary antibody. Cells were then fixed and expression was quantified using flow cytometry.

Immuno fluorescent microscopy
Isolated neutrophils were allowed to adhere on glass cover-slips before incubation as above with the respective anti-bodies. However DAPI (5μg/mL) was added in the final 30min incubation. The cells were then washed, fixed and mounted onto glass slides and viewed using an Olympus (Australia) microscope with the appropriate filters.

Quantitative RT-PCR
RNA was extracted from isolated human neutrophils and human monocyte derived macrophages using the RNeasy mini kit (Qiagen). RNA was quantified using a nano-drop (Thermo Scientific). Transcription to cDNA was performed using M-MLV reverse transcriptase (Invitrogen). RNA expression was measured using SYBR Green PCR master mix (Applied Biosystems) with primers to either ABCA1, ABCG1, SR-
BI and normalized to cyclophilin A using the ∆∆ CT method. Reactions were run on a stratagene Mx3000P (Agilent Technologies).

Receptor blockade
Receptors were blocked by incubating neutrophils with either an anti-ABCA1 antibody or anti-SR-BI antibody (Novus) for 4 hrs at 37°C.

ApoA-I binding
Isolated neutrophils were incubated at 37°C with or without apoA-I (40μg/mL) for 15mins. The cells were then washed in PBS and then probed with an anti-apoA-I Ab for 30mins, washed and then incubated with a FITC conjugated secondary Ab. Results are expressed as fold increase over control (isotype control + secondary Ab in apoA-I free neutrophils).

Cholesterol efflux
Neutrophils were loaded with ³H-cholesterol (0.4Ci/400μL treatments) for 4 hrs at 37°C. The neutrophils were then washed 3 times in PBS by centrifugation. Efflux to the respective cholesterol acceptors was measured in triplicate and allowed to occur for 15, 30 and 60 mins respectively in a total volume of 400 μL. After the efflux period cells were pelleted and the supernatant was collected and 100 μL aliquots in triplicate were used to quantify the cholesterol efflux. Results were expressed as percentage efflux of control (PBS).

Lipid raft abundance
For quantification of lipid raft abundance, isolated neutrophils were incubated at 37°C with the various lipid acceptors for 15, 30 or 60mins. The neutrophils were then washed in cold PBS by centrifugation and incubated with FITC-Cholera toxin B (CT-B) for 1hr at room temperature. The cells were fixed in 4% paraformaldehyde and lipid raft abundance measured by flow cytometry.

Shear-flow cell adhesion
Shear-flow experiments were conducted in glass capillaries (2x0.2x100mm) coated with a monolayer of spread platelets as previously described. Isolated human platelets were drawn into glass tubes by capillary action and allowed to adhere and spread to form a confluent monolayer for 15mins at 37°C, before blocking with 3% BSA in tryodes buffer followed by washing twice with tyrodes. Isolated neutrophils (1x10⁶/mL) were treated with PMA (1μmol/L) ± HDL (50μg/mL) or apoA-I (40μg/mL) 15 or 60mins at 37°C prior to perfusion. The neutrophils were perfused over the platelets under negative pressure (PHD 2000, Harvard Apparatus, USA), at a physiological shear-rate of 150s⁻¹ (1.1dyn/cm²) for 5
min with an additional washout period of 5min with tryodes. Neutrophil-platelet interactions were visualised using phase microscopy (X20 lens, Olympus, Australia) captured digitally (MDC-1004, Imperx, USA) at 30 frames/sec with XCAP™ software v2.2 (Epix, USA) and analysed off line using Image Pro-Plus v5.1 (Media Cybernetics, USA). Time 0 was defined by the first adhering neutrophil. Fields (274μm x 275μm) were recorded for 10sec at 0.5min, 1min (1 visual field each/time point were analysed), 2.5min, 5min and 10min (three visual fields each/time point were analysed) for off-line analysis.

Migration assay
Neutrophil migration was assessed using the previously described 2 Transwell (Costar, 8.0μM pores) migration assay. Neutrophils (1x10^5 cells/well) were pre-incubated in PBS ± HDL (50μg/mL) or apoA-I (40μg/mL) for either 15 or 60 mins before seeding into the upper chamber. Thus the upper chamber contained neutrophils ± HDL or apoA-I, while fMLP (0.1μM/L) used as the chemoattractant in the lower chamber for all conditions. Migration was allowed to occur for 30mins 37°C before the cells on the bottom surface of the membrane were fixed with 4% formaldehyde. Non-migrating cells were removed from the upper chamber and the migrated cells were stained with crystal violet. The cells were washed and the dye was extracted from the stained cells with 10% acetic acid. The O.D. was measured from each treatment at 595nm in triplicate and migration was expressed as a percentage of control (PBS).

Intravital microscopy
Leukocyte-endothelial interactions were studied in the cremaster vessels of male C57Bl/6 mice using intravital microscopy as previously described 5. Male mice (approximately 20g/6-10weeks of age) received an intra-peritoneal (i.p.) injection with either vehicle (PBS) or TNF-α (500ng) 4hrs prior to imaging. This concentration of TNF-α has previously been shown to induce systemic inflammation and significant increases in leukocyte recruitment to the inflamed endothelium 5. Mice were anaestetised using with ketamine hydrochloride (150mg/kg; Pfizer, Australia) and xylazine (10mg/kg; Troy Laboratories, Smithfield, NSW, Australia) i.p., followed by exposure of the jugular vein by surgery. A cannula was inserted into the right jugular vein for delivery of anesthetic or treatment. The mouse was placed on a customized stage and the cremaster surgically exposed, the connective tissue cleared and the muscle cauterized longitudinally and strung out flat by attaching silk sutures to the corner of the tissue. A cover-slip was placed over the exposed area and the tissue was kept at 37°C in Krebs buffer. The temperature of the mice was also regulated by placement on a thermo-plate. Imaging of the cremaster vessel was conducted on a stereo fluorescent microscope (Carl Zeiss Discovery V20, Germany) with 0.63X and 2.3X
objectives. Images were captured using a digital camera (Hamamatsu Orca-ER) and fluorescence illumination system (EXFO XCITE-120).

At time 0 of imaging either vehicle (PBS) or apoA-I (20mg/kg) was delivered via the jugular vein and 30sec recordings were captured at 0, 2.5, 5, 10, 15, 30, 45 and 60mins. The captured videos were analyzed off-line for stationary adhesion, rolling flux and rolling velocity as previously described. Stationary adherent cells were defined as the cells that remained stationary for 30secs throughout each respective recording time. Rolling flux was defined by averaging the number of cells that move past a fixed point on the venular wall and calculated to determine the number of cells rolling per minute. Leukocyte rolling/tethering velocity was determined by recording the time required for a leukocyte to roll along a 100 µm length of venule, and quantified for 20 leukocytes at each time interval. The analysis software used was AxioVision (Carl Zeiss, Germany).

CD11b activation in mice
C57Bl/6 mice were stimulated with TNF-α as above before been infused with apoA-I via tail vein injection. Blood was collected from the mice after TNF-α stimulation and then 15, 30, 45 and 60mins post apoA-I infusion. Blood was collected into EDTA lined tubes and incubated with an antibody cocktail (CD45-APC-Cy7, Ly6C-PerCP-Cy5.5 CD115-APC and CD11b-FITC (eBiosciences)) for 30mins on ice. The red blood cells were then lysed (BD Biosciences) and resuspended in PBS with 2% paraformaldehyde. The sample was then run on the flow cytometer and neutrophils gated as CD45hiCD115loLy6Cchi and CD11b expression was quantified. CD11b expression was controlled using a FITC-Rat IgG2b isotype control antibody (eBiosciences). Results were expressed as percentage of TNF-α stimulation.

Western blot
After each intravital experiment, blood from the mice was collected in sodium citrate lined syringes. Plasma was obtained by centrifugation at 1500g for 15mins. Equal amounts of plasma (10µL/lane) were run on a 4-20% gradient native gel (criterion, Bio-Rad) before western transfer to PVDF membrane. The membrane was blocked then probed with and anti-apoA-I monoclonal antibody (Abcam). The membrane was then washed and incubated with a sheep anti-mouse IgG-HRP antibody. After the final washing steps, enhanced chemiluminescence kit was used and the membrane was exposed for developing on Kodak film. Blots were scanned and quantified using ImageJ.

Agarose gel
Plasma samples were run on a 0.75% agarose gel in barbital buffer as previously described. Plasma (20μL) was pre-labeled with sudan black (10μL) for 1 hr on ice. The samples were then loaded and the gel was run for 2 hrs at 70V at 4°C. Images of the gel were acquired using a flat-bed colour scanner.

**Human rHDL clinical Study**

**Patient criteria**

This clinical study is an extension of the study previously reported by Shaw et al. All patients gave their informed consent to the study, which was approved by the Human Research Ethics Committee of the Alfred Hospital and conducted in accordance with the principles of the declaration of Helsinki 2000. PVD patients were recruited as those presenting with symptom limiting claudication and having a lesion in their superficial femoral artery as assessed by duplex ultrasonography which was deemed suitable for percutaneous revascularisation. Patients on current medical therapy including an exercise program were included (Supplementary Table 1). Excluding criteria included patients >40 years of age and those with other major co-morbidities including cancer with an expected survival of <12 months, a history of organ transplantation, HIV infection or serum creatinine >0.2 mmol/L. Enrolled patients continued all their existing medications except for statins which were discontinued from 1 week prior to the infusion and recommenced following the atherectomy/angioplasty.

**rHDL and infusion protocol**

The study was double blinded and the rHDL (CSL Behring AG, Bern, Switzerland; CSL-111; 80 mg/kg; comprised of human apoA-I and phosphatidylcholine from soy bean in a ratio of 1:150) or placebo (saline) was administered over a 4 hr period as previously described. Blood samples were taken for analysis pre-infusion and 5-7 days post-infusion.

**Plasma lipoproteins**

Plasma lipoproteins were measured as previously described. Lipid levels including total cholesterol, LDL, HDL and Triglycerides were analyzed using a Kodak Ektachem DT 60 analyzer.

**CD11b expression from PVD**

CD11b expression was measured from peripheral blood neutrophils from these patients before and 5-7 days post rHDL/placebo infusion. Blood, anti-coagulated with sodium citrate, was incubated with an anti-human CD11b antibody (Serotec, USA, Clone ICRF44) 1:40 dilution for 15 minutes at 37°C. Samples were then fixed and red blood cells lysed by a 10min incubation at room temperature with Optilyse B. Samples were controlled by using the isotype matched negative control. CD11b expression on neutrophils
was measured by flow cytometry, the neutrophil population was identified by size and granularity profiles. Analysis was conducted using Cell Quest Pro software.

Statistics

Values are presented as mean ± SEM or percentage of control ± SEM. All results were analysed for statistical significance using one-way ANOVA followed by Bonferroni post-hoc test, except the perfusion study which were analysed using a two-way ANOVA followed by Bonferroni post-hoc test, and CD11b expression data from the rHDL infusion study which was analysed using unpaired Student’s t-test. Statistical significance was set at $P<0.05$.

References

Supplementary Figures

Figure SI. Neutrophil activation is suppressed by direct interaction of apoA-I and not by PMA sequestration. A) Isolated neutrophils were stimulated with PMA (0.1µM) ± apoA-I (40µg/ml) or ± BSA (40µg/ml) for 15mins and then washed and fixed and CD11b expression was measured by flow cytometry. **P<0.01 c.f PMA, n=4.
Figure SII. Neutrophil activation is attenuated by apoA-I in whole blood.

A) Whole blood was stimulated with PMA (0.1µM) ± apoA-I or fMLP (10nM) ± apoA-I for 15mins before the RBCs were lysed, WBCs fixed and CD11b expression was determined via flow cytometry. ***P<0.01 c.f PMA, ^^P<0.05 c.f. fMLP, n=4.

B) Histogram of CD62L expression of neutrophils stimulated with PMA (Solid) apoA-I+PMA (dotted) and unstimulated (dashed). Isotype control (shaded).

C) Fold change in CD62L expression over control (isotype Ab). ***P<0.001 unstimulated vs PMA and ^P<0.05. Neutrophils were gated as CD45⁺, CD115⁻, Gr1⁺ cells.
**Figure SIII.** Receptor expression and apoA-I binding.

A) Isolated neutrophils were screened for SR-BI, ABCA1 and ABCG1 mRNA expression. Expression was normalized to the control gene cyclophilin A.

B) Expression of SR-BI, ABCA1 and ABCG1 in neutrophil as a percentage of the levels in macrophages.

C) Neutrophils were incubated with an anti-SR-BI Ab with a Texas Red conjugated secondary Ab, or D) an anti-ABCA1 Ab with a FITC conjugated secondary Ab and viewed via fluorescent microscopy, where nuclei were stained with DAPI (blue). Control= isotype control Ab + fluorescent secondary Ab.

E) Isolated neutrophils were incubated with or without apoA-I for 15mins, then probed with an anti-apoA-I Ab followed by a FITC conjugated secondary Ab. Results are expressed as fold increase over control (isotype control + secondary Ab), n=5, P<0.05.
Figure SIV. Unesterified cholesterol removal from neutrophils.

Neutrophils were incubated with apoA-I (40 µg/ml; ●) or HDL (50 µg/ml; ○) for the period indicated. Cells were washed, fix in 1% PFA and stained for 1hr with filipin (100 µg/ml). Cells were then washed before quantification via flow cytometry.
Figure SV. Cholesterol efflux and lipid raft abundance do not correlate. Regression analysis of CD11b activation vs cholesterol efflux, $R^2=0.002$ ($P=0.902$), apoA-I (●), HDL (○).
Figure SVI. ApoA-I attenuates leukocyte activation in TNF-α challenged mice. A) Mice were injected with TNF-α 4hrs prior to infusion with either saline or apoA-I. CD11b expression was measured 15, 30, 45 and 60mins post infusion to determine neutrophil activation. Results are expressed as percentage of pre-infusion. TNF-α+saline (●), TNF-α+apoA-I (○), n=4, *P<0.05. B) Quantification of the ratio of HDL and lipid poor apoA-I in apoA-I infused mice compared to HDL and lipid poor apoA-I in saline infused mice. n=3, **P<0.01, *P<0.05.