Anti-Inflammatory Functions of Apolipoprotein A-I and High-Density Lipoprotein Are Preserved in Trimeric Apolipoprotein A-I

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ABSTRACT
Raising high-density lipoprotein (HDL) levels is proposed as an attractive target to treat cardiovascular disease. However, a number of clinical studies examining the effect of HDL-raising therapies have been prematurely halted due to futility. Therefore there is a need for alternative therapies. Infusion of reconstituted HDL (rHDL) particles is still considered as a viable approach to increasing HDL levels. In this study we have profiled the anti-inflammatory effects of a trimeric-HDL particle. We show that trimeric apoA-I and rHDL particles promote cholesterol efflux to a similar rate as native apoA-I particles in both ABCA1-dependent and -independent pathways. Trimeric particles inhibited ICAM-1 and VCAM-1 expression and the ability of the endothelium to capture monocytes under shear flow. Monocyte activation, CD11b-dependent adhesion, and monocyte recruitment under shear flow conditions were perturbed by the trimeric particles. Our data suggest that trimeric rHDL particles can be constructed without any loss of function, preserving the anti-inflammatory effects of HDL that are key to its in vivo actions.

Introduction
Cardiovascular disease remains a leading cause of death worldwide. The cardioprotective properties of high-density lipoprotein (HDL) are well established (Murphy et al., 2009b), with plasma levels negatively correlating with cardiovascular events (Gordon et al., 1977). The ability of HDL to modulate a number of different cellular processes ranging from regulating hematopoiesis (Murphy et al., 2011a) to inhibiting activation of circulating cells (Murphy et al., 2008, 2011b) to having a major role in the reverse cholesterol transport system has made HDL an attractive potential therapy for cardiovascular disease (Murphy et al., 2010, 2012).

There are a number of therapeutic approaches to increase plasma HDL levels. Although the premature termination of the AIM-HIGH (Boden et al., 2011) and dal-OUTCOMES (Schwartz et al., 2009) studies for futility would appear to dispute the beneficial effect of HDL-raising therapies, it is possible that more dramatic and direct elevations of HDL levels with a high cholesterol efflux capacity are required to produce clinically meaningful results. One such method to achieve this would be to administer cholesterol-poor reconstituted HDL (rHDL) particles, which have been demonstrated in a number of short-term clinical studies to deliver beneficial outcomes (Murphy et al., 2009a). A study in patients with type II diabetes showed that a single infusion of native rHDL improves glucose metabolism and reduces a number of inflammatory parameters (Drew et al., 2009; Patel et al., 2009). We have also reported that a single infusion of rHDL into patients with peripheral vascular disease can decrease the inflammatory profile and remodel atherosclerotic plaques, attenuating vascular inflammation and decreasing plaque macrophage content, ultimately resulting in a more stable lesion (Shaw et al., 2008). In addition, a single infusion of rHDL can inhibit hematopoietic stem cell proliferation, mobilization, and monocytosis in ath-erosclerotic and leukemic mouse models (Westerterp et al., 2012; Murphy et al., 2011a), suggesting it could be used to treat myeloproliferative disorders. Finally, repeated infusions of rHDL have been shown not only to remodel but also regress atherosclerotic plaques in patients with acute coronary syndrome (Nicholls et al., 2006; Nissen et al., 2003; Tardif et al., 2007).

Recently, Ohnsorg et al. (2011) published on the reversed cholesterol transport capabilities of trimeric apoA-I, a recombinant high molecular mass variant of apoA-I, engineered by fusing three apoA-I molecules for the purpose of decreasing the rapid clearance properties of apoA-I, hence increasing plasma half-life (Graversen et al., 2008). In the current study, we explored the functional properties, including the anti-inflammatory capabilities, of the trimeric form of recombinant apoA-I (T-apoA-I) and trimeric-rHDL (T-rHDL) particles with native apoA-I and rHDL, respectively.

Materials and Methods
Blood Collection. Blood was collected from healthy consenting (approved by the Human Ethics Committee of the Alfred Hospital) volunteers by venipuncture and drawn into syringes containing sodium citrate.

Trimeric apoA-I, rHDL Particles, and Liposomes. Recombinant human apoA-I (apoA-Ig) and trimeric apoA-I and rHDL preparations were kindly provided by Hoffmann-La Roche, Basel, Switzerland. ApoA-I was constructed as previously described by Ohnsorg et al. (2011). Reconstituted HDL (rHDL) and dimyristoyl-phosphatidylcholine (DMPC) liposomes were prepared as previously described (Murphy et al., 2008). HDL was isolated from plasma using sequential ultracentrifugation (density 1.085 to 1.21 g/ml), and protein content was measured. All acceptors were used at the concentrations stated in each figure as calculated by protein for HDL particles or concentration of phospholipid for liposomes.

Particle Characterization. To measure the Stokes diameter, the respective apoA-I and rHDL particles were subjected to native 6% PAGE and Stokes diameter was measured as previously described (Hoang et al., 2012). To determine the number of apoA-I particles per rHDL particle, crosslinking studies were performed as previously described (Sviridov et al., 2000). Briefly, 10 μg of the respective particles was crosslinked for 30 min at room temperature in dithiobis (sucinimidylpro-pionate) (DSP; 200 μM); the reaction was then stopped by adding 50 mM of Tris-HCl for 15 min. Samples were then run on a 4–20% SDS-PAGE under denaturing conditions.

Cholesterol Efflux. Cholesterol efflux assays were performed as previously described (Ohnsorg et al., 2011). THP-1 was differentiated into macrophages using phorbol 12-myristate 13-acetate (PMA; 100 nM); the cells were then incubated with various concentrations of the apoA-I molecules for 4 h. Supernatant was collected and filtered centrifuged to remove cellular debris. and 100 μl was taken for counting on the scintillation counter (Perkin Elmer, MA). The remaining cells were washed, byzed by the addition of 5% Nonidet P-40, and an aliquot was taken for counting. Fractional efflux was calculated as the ratio of radioactivity measured in the supernatant to the sum of the radioactivity measured in both the cell lysate and supernatant.

Endothelial Cells. Human coronary aortic endothelial cells (HCAEC; Cell Applications, San Diego, CA) were cultured in 6-well or 12-well plates to form a confluent monolayer with HCAEC Media (Cell Applications, San Diego, CA). The cells were either used for perfusion studies (6 well) or for examining adhesion molecule expression (12 well). Freshly isolated human umbilical vein endothelia cells (HUVECs) were seeded onto cell culture coverslips in a 24-well plate and cultured to form a confluent monolayer.

Endothelial Adhesion Molecule Expression–Immunohistochemistry. HUVECs were grown on glass coverslips to form a confluent monolayer. Media were replaced with or without T-rHDL (1 mg/ml) or DMPC liposomes and incubated for 16 h, after which the cells were stimulated with tumor necrosis factor (TNF)-α (0.1 ng/ml). The cells were then fixed with 2% paraformaldehyde for 10 min and washed with phosphate-buffered saline (PBS). Endogenous peroxidase activity was blocked using peroxidase block (EnVision-kit, Dako Cytomation, Melbourne, VIC, Australia) for 5 min at room temperature (RT) followed by washing in Tris-buffered saline (TBS). The samples were then blocked with goat serum (1:10 in TBS) for 20 min at RT. The primary antibody to VCAM-1 (Dako Cytomation) (1:85 in TBS) was allowed to bind for 1 h at RT followed by washing with TBS. Detection of the primary antibody was achieved by adding a horseradish peroxidase-goat anti-mouse IgG for 30 min at RT followed by washing with PBS. The substrate-chromogen was then applied, and the sample was counterstained with Mayer’s hematoxilin nuclear staining solution (Bio-Optica, Milano, Italy). The samples were rinsed with dH2O before being dehydrated in ethanol and cleared in xylene. The coverslips were then mounted onto glass slides using Histomount (Invitrogen, Mulgrave, VIC, Australia). Images were captured using a 20 × magnification, and the percentage of positive stained cells was quantified using ImageJ.

Endothelial Adhesion Molecule Expression–Flow Cytometry. HUVECs or HCAECs were cultured as a confluent monolayer in 12-well plates and incubated in media with or without the various cholesterol acceptors (1 mg/ml) for 16 h prior to stimulation with TNF-α (0.1 ng/ml) for 5 h at 37°C. Cells were then washed with cold PBS and incubated with fluorescein isothiocyanate (FITC) conjugated antibodies to either ICAM-1 (Abs Serotec, clone 15.2) or VCAM-1 (AbD Serotec, Kidlington, United Kingdom, clone 1.G11B1) for 1 h at 4°C on a shaker. The cells were then washed twice with PBS (Ca/Mg free), and trypsin was added to remove the cells from the wells. After the cells began to lift, trypsin-neutralizing solution was added and the cells were transferred into flow cytometry tubes containing 4% paraformaldehyde.

Samples were compensated for by using the isotype matched negative control (FITC-anti-mouse IgG, AbD Serotec, clone W3/25). ICAM-1 and VCAM-1 expression was measured by flow cytometry using FACS Calibur (BD Biosciences; San Jose, CA). Analysis was conducted using the Cell Quest Pro software. Results were expressed as percentage of the unstained control (100%).

Monocyte Isolation. Resting human monocytes were isolated by density centrifugation with Lymphoprep (Axis Shield, Dundee, Scotland) followed by Dynal negative monocyte isolation kit (Invitrogen) as described previously (Murphy et al., 2008). Monocytes were resuspended in PBS and cell number was determined on an automated hematology analyzer (KX-21N; Sysmex, Mundelein, IL).

CD11b Expression–Flow Cytometry. Monocytes were stimulated with either 1 μmol/l PMA or 1 μg/ml LPS (Sigma, Sydney, NSW, Australia) in the presence or absence HDL (50 μg/ml) or apoA-I (40 μg/ml) and incubated with the FITC conjugated Ab the active epitope of CD11b (eBiosciences, San Diego, CA, Clone CBRM1/5) for 15 min at 37°C, unless otherwise stated. Cells were then fixed with 4% paraformaldehyde. The samples were run, data were analyzed, and results were expressed as in the HCAECs studies.

Perfusion Studies. Perfusion studies were conducted using the parallel plate flow-chamber (GlycotechGaithersburg, MD) as previously described (Murphy et al., 2008). HCAEC were cultured in 6-well plates to form a confluent monolayer with HCAEC Media (Cell Applications). Prior to perfusion, HCAECs were washed twice with PBS. Isolated monocytes (1 × 10⁶/ml) in PBS were perfused over HCAECs under negative pressure (PHD 2000, Harvard Apparatus, Holliston, MA), at a shear rate of 150 s⁻¹ (1.1 dyn/cm²) for 5 min, with an additional washout period of 5 min with PBS. To study the inhibitory effect of the rHDL molecules on monocye-dependent adhesion, monocytes were pretreated with 1 μmol/l of PMA ± 50 μg/ml rHDL for 15 min at 37°C prior to perfusion. To study the effect of the rHDL on endothelial-dependent adhesion, HCAECs were incubated for 16 h with rHDL (1 mg/ml) or PBS (control) prior to stimulation with TNF-α for 5 h. Monocyte–endothelial cell interactions were visualized using phase microscopy (20 × lens, Olympus, Mount Waverley, VIC, Australia) captured digitally (MDC-1004, Imperx, Boca Raton, FL) at 30 frames/s with XCAP software v2.2 (Epix, Buffalo Grove, IL) and analyzed offline using Image Pro-Plus v5.1 (Media Cybernetics, Bethesda, MD). Time 0 was defined by the first adhering monocyte. Fields (274 μm × 275 μm) were recorded for 10 s at 0.5 min, 1 min (1 visual field each/time point was analyzed), 2.5 min, 5 min, and 10 min (5 visual fields each/time point were analyzed) for offline analysis.

Static Adhesion Assay. Monocyte adhesion to immobilized fibronectin was performed as previously described (Murphy et al., 2008). Monocytes (1 × 10⁶/ml) were allowed to adhere for 15 min at 37°C with the appropriate treatment. Monocyte adhesion was quantified by phase microscopy (×40), counting five random fields for each slide. Treatments were carried out in triplicate. Results were expressed as the average amount of cells per field.

Statistical Analysis. Values are presented as the mean ± S.E.M. or percentage of control ± S.E.M. All results were analyzed for statistical significance using one-way analysis of variance followed by Bonferroni post hoc test, with the exception of perfusion studies, which were analyzed using a two-way analysis of variance followed by Bonferroni post hoc test. Significance was accepted at P < 0.05.
Results

Characterization of rHDL Particles. rHDL particles based on apoA-I_E and T-apoA-I were characterized using non-denaturing electrophoresis in native 6% gels and in cross-linking experiments (Fig. 1). The predominant population of rHDL particles assembled with apoA-I_E had a Stokes diameter of 9.2 nm (Fig. 1A). T-apoA-I formed a number of particles, with the predominant particle having a Stokes diameter of 9.5 nm. Analysis of crosslinking demonstrated that rHDL particles formed from apoA-I_E contained two molecules of apoA-I, whereas T-apoA-I formed particles that contained predominantly three molecules of T-apoA-I per rHDL particle (Fig. 1B). The level of heterogeneity of T-HDL was unexpectedly high for a particle with the same number of apolipoprotein molecules per particle. This may be a reflection of variability in lipid and protein packing in T-HDL resulting in a mixture of differently sized particles.

Trimeric apoA-I Facilitates Cholesterol Efflux Via ABC Transporters. The ability for T-apoA-I particles to promote cholesterol efflux was examined using THP-1-derived macrophages either unstimulated or treated with an LXR agonist (T01901317). Native apoA-I_E and T-apoA-I facilitated cholesterol efflux comparably over all concentrations tested in unstimulated cells (Fig. 2A). Moreover, the efficiency of these molecules to remove cholesterol was equal to purified native human apoA-I. As expected, T-rHDL removed cholesterol more efficiently than the nonlipidated acceptors. A significant difference in cholesterol efflux by T-rHDL was observed at 6.6 µg/ml. Similar results were observed in THP-1 macrophages activated with an LXR agonist. As expected, the overall level of cholesterol efflux was increased after LXR activation to all acceptors. Again the level of efflux was equal between native apoA-I and T-apoA-I over all concentrations examined, and the level of efflux was also equal to purified human apoA-I (Fig. 2B). Incubating macrophages with T-rHDL led to a significant increase in cholesterol efflux at 20 µg/ml.

Trimeric rHDL Inhibits Endothelial Adhesion Molecules. HDL has previously been shown to inhibit the expression of adhesion molecules on HUVECs. Immunohistochemistry and flow cytometry were employed to determine if T-rHDL particles could also inhibit the expression of endothelial adhesion molecules. Incubating endothelial cells with TNF-α promotes a dramatic increase in the number of VCAM-1-positive cells. Analysis of immunohistochemistry micrographs showed that T-rHDL particles could indeed inhibit VCAM-1 expression on endothelial cells stimulated with TNF-α (Fig. 3, A and E). T-rHDL particles significantly inhibited VCAM-1 expression to a greater extent than DMPC liposomes, and thus subsequent experiments focused on the rHDL particles (Fig. 3A). Flow cytometry was used to compare the anti-inflammatory ability of T-rHDL and rHDL particles. Both particles equally well reduced VCAM-1 expression (Fig. 3F). Similar results were obtained when comparing the ability of native and trimeric particles to inhibit ICAM-1 expression (Fig. 3G). These findings were confirmed in HCAECs. The rHDL molecules suppressed both VCAM-1 and ICAM-1 expression (Fig. 3, H and I). Incubating HCAECs with lipid-free apoA-I_E or DMPC liposomes failed to significantly inhibit adhesion molecule expression (Fig. 3, H and I).

Trimeric apoA-I Inhibits Monocyte Activation. Both HDL and apoA-I have recently been reported as having potent anti-inflammatory effects on monocytes as determined by measuring the conformational change of the adhesion molecule CD11b (Murphy et al., 2008). The efficacy of T-apoA-I or T-rHDL to inhibit monocyte activation was assessed. Stimulating monocytes with PMA caused a significant increase in CD11b activation. This activation of CD11b was significantly inhibited with only 5 µg/ml, with a nadir at 20 µg/ml for each of the respective apoA-I molecules (Fig. 4A). Additionally, there was no difference in the efficacy of each molecule, and the trimeric molecules were equally as efficient as native apoA-I_E. The ability of apoA-IE, T-apoA-I, or T-rHDL to inhibit monocyte activation stimulated with a receptor-dependent agonist LPS was also examined. Again, each of the apoA-I molecules inhibited CD11b activation to a similar extent (Fig. 4B).

Effect of rHDL Particles on Endothelial-Dependent Shear Flow Cell Adhesion. A shear flow cell adhesion model was employed to examine whether rHDL and T-rHDL were able to inhibit endothelial-dependent monocyte adhesion. Total adhesion, stationary adhesion, and rolling flux were assessed. Incubating endothelial cells with TNF-α prior to the perfusion of resting monocytes resulted in a significant increase in monocyte adhesion (Fig. 5, A and D–G, respectively). Preincubating endothelial cells with rHDL prior to stimulation with TNF-α resulted in a significant attenuation in total monocyte adhesion after 10 min of perfusion compared with endothelial cells stimulated with TNF-α alone (Fig. 5, A, H, and I). Preincubating endothelial cells with T-rHDL before stimulation resulted in a significant decrease in monocyte adhesion at the 5 min time point (Fig. 5, A, J, and K). T-rHDL was also able to reduce significantly stationary monocyte adhesion after 5 min of perfusion compared with TNF-α-treated cells. This was a more potent inhibition compared with rHDL, where a significant reduction in stationary adhesion was not observed until 10 min of perfusion (Fig. 5B). The number of monocytes rolling along the endothelium was significantly higher when the endothelial cells were pretreated with rHDL; there also was a tendency for a higher number of rolling monocytes in the T-rHDL group, suggesting that pretreatment with rHDL inhibited the ability of the endothelial cells to capture and arrest the monocytes (Fig. 5C).
apoA-I, various acceptors was assessed over a dose response. T-rHDL versus T-apoA-I and in (B) activated with an LXR agonist before cholesterol efflux to the ABC transporters. THP-1 macrophages were loaded with (A) Ac-LDL and in (B) activated with an LXR agonist before cholesterol efflux to the various acceptors was assessed over a dose response. T-rHDL versus T-apoA-I, *P < 0.05, ***P < 0.01, n = 3.

Effect of rHDL Particles on Monocyte-Dependent Shear Flow Cell Adhesion. To determine if the inhibition of CD11b activation by T-apoA-I particles translates into a decrease in monocyte inflammatory function, we aimed to examine the effect of these molecules on monocyte adhesion. Initially we examined CD11b-dependent monocyte adhesion using the extracellular matrix protein fibrinogen as the substrate (Gresham et al., 1989). Monocytes stimulated with PMA displayed a significant increase in adhesion to the fibrinogen matrix compared with control, correlating with the increase in CD11b activation. Incubation of PMA-stimulated monocytes with either of the apoA-I molecules significantly inhibited CD11b-dependent monocyte adhesion (Fig. 6A). Again there was no difference in the degree of reduction in monocyte adhesion between each of the respective apoA-I molecules. Next we examined the anti-inflammatory effects of the apoA-I molecules on monocyte adhesion to endothelial cells under shear flow conditions. Stimulating monocytes with PMA prior to perfusion over HCAECs lead to a significant increase in monocyte adhesion compared with control. Incubating PMA-stimulated monocytes with apoA-I prior to perfusion lead to a significant attenuation in adhesion after 5 min, continuing to the end of the perfusion period (10 min) (Fig. 6, B and E). Both T-apoA-I and T-rHDL also inhibited monocyte adhesion to HCAECs under shear flow after 5 min of perfusion, respectively (Fig. 6, B, F, and G). Furthermore, all apoA-I molecules returned adhesion levels to near basal conditions at 10 min of perfusion.

Discussion

Increasing HDL levels to reduce risk of cardiovascular disease is a strategy of choice for many research groups and industries. However, safely and effectively increasing functional cardioprotective HDL in disease has proven challenging. Previous studies suggest that oligomers of apoA-I have increased plasma half-life (Graversen et al., 2008; Pedersen et al., 2009). With this in mind, we explored the possibility that a stable trimer of apoA-I may be more effective than native apoA-I or rHDL. In the current study we describe a trimeric-apoA-I molecule that retains the anti-inflammatory properties of native apoA-I; this is the first report assessing the anti-inflammatory properties of apoA-I and/or rHDL containing three apoA-I molecules.

The ability of the trimeric particles to facilitate cholesterol efflux via the major reverse cholesterol transport pathways was first assessed. In acetylated-low-density lipoprotein-loaded THP-1 macrophages cholesterol efflux to the lipid-poor apoA-I molecules was similar and also comparable with native human apoA-I. T-rHDL facilitated significantly more efflux than lipid poor apoA-I, a finding that is likely due to the interaction with other cellular transporters and not just ABCA1 (Favari et al., 2009; Lorenzi et al., 2008). In LXR-activated THP-1 macrophages ABC transporter-mediated cholesterol efflux was again similar between the different apoA-I molecules, whereas the T-rHDL was only more effective at higher concentrations. Although lipid-poor apoA-I molecules are removing cholesterol via an ABCA1-dependent pathway, it has been shown previously that rHDL particles with three or more apoA-I molecules remove the majority of cholesterol via an ABCG1-dependent pathway in LXR-activated macrophages (Favari et al., 2009). Interestingly, although ABCA1 can efflux to rHDL particles, this appears to be only to small particles containing two apoA-I molecules with a low phospholipid concentration and at a significantly lower efficiency than lipid-free apoA-I (Favari et al., 2009). A previous study that employed the trimerization domain of human tetranectin fused to apoA-I to create a trimeric apoA-I molecule describe enhanced cholesterol efflux over native apoA-I at higher concentrations only (Graversen et al., 2008). However, both forms of apoA-I display equal binding efficiencies to ABCA1-expressing cells (Graversen et al., 2008).

The ability of the apoA-I molecules to inhibit endothelial inflammation was then examined. It has been previously shown that lipid-free apoA-I has a limited role in regulating endothelial adhesion molecules (Mcgrath et al., 2009), a finding we confirm in the current study using HCAECs. Consistent with the literature, we observed a significant inhibition of VCAM-1 expression with DMPC liposomes alone; however, this inhibition was dramatically enhanced by T-rHDL particles. We also observed via flow cytometry that both rHDL and T-rHDL were equal at inhibiting TNF-α–induced VCAM-1 and ICAM-1 expression. This inhibition of endothelial adhesion molecule
expression by the rHDL particles is likely to be via an SR-BI-dependent pathway as previously described (Kimura et al., 2006; McGrath et al., 2009; Murphy et al., 2006; Murphy and Woollard, 2010), showing the diversity of these rHDL particles to interact with a number of HDL receptors. The functional significance of the inhibition of endothelial inflammation was observed in shear flow cell adhesion studies. Preincubating endothelial cell monolayers with either form of rHDL prior to TNF-α stimulation and subsequent perfusion of monocytes resulted in a significant reduction in total and stationary adhesion. There was an increase in rolling adhesion with rHDL treatment, which is more than likely due to the reduction in ICAM-1 not allowing the monocyte to firmly adhere via receptors including CD11b (Diamond et al., 1991; Springer, 1994).

In a previous study we described a potent anti-inflammatory role for both apoA-I and HDL in attenuating monocyte activation (Murphy et al., 2008). All forms of apoA-I and T-rHDL appeared to have equal effects in inhibiting monocyte activation to both receptor and nonreceptor agonists. This finding translated into a decrease in CD11b-dependent

Fig. 3. T-rHDL inhibits endothelial cell adhesion molecules. (A–E) HUVECs were preincubated with either saline, 1 mg/ml of T-rHDL, or DMPC liposomes for 16 h prior to incubation with 0.1 ng/ml of TNF-α. VCAM-1 expression was detected via immunohistochemistry. **P < 0.01, ***P < 0.001 versus TNF-α, *P < 0.05 versus DMPC. Microscope images control (B), TNF-α (C), DMPC liposomes + TNF-α (D), T-rHDL + TNF-α (E). 20× objective. By using the above incubation conditions, VCAM-1 (F) and ICAM-1 (G) expression was measured in HUVECs by flow cytometry using FITC conjugated antibodies to the respective receptors. Results are expressed as percentage of control. *P < 0.05, **P < 0.01 versus TNF-α, n = 6. Flow cytometry was also employed to determine VCAM-1 (H) or CAM-1 (I) on HCAECs preincubated with the various cholesterol acceptors. *P < 0.05, **P < 0.01 versus TNF-α, n = 5.
adhesion as assessed by monocyte binding to fibrinogen (Gresham et al., 1989). By using shear flow cell adhesion experiments we were again able to demonstrate a functional effect of the apoA-I molecules. All molecules inhibited monocyte adhesion to the endothelial monolayer to a similar extent. Importantly, these trimeric particles did not appear to have any superphysiologic effects, suggesting they should be equally as safe as native particles in vivo.

In conclusion, we describe a trimeric apoA-I molecule that can form a rHDL particle comprised of three apoA-I molecules that retains the most important cardioprotective effects of native apoA-I and HDL. The trimeric complex can efficiently facilitate cholesterol efflux from lipid-loaded macrophages. T-apoA-I and T-rHDL are also able to modulate leukocyte recruitment to the endothelium by inhibiting endothelial and monocyte activation. Although further investigation of T-apoA-I in vivo is required, the data presented here suggest an alternative therapeutic approach to the sustained increase of apoA-I/HDL to treat cardiovascular-related diseases.

Fig. 4. T-apoA-I and T-rHDL inhibits monocyte activation. (A) isolated human monocytes were incubated with PMA (1 μmol/l) with or without the various apoA-I molecules in increasing doses (2.5–40 μg/ml). CD11b activation was assessed via flow cytometry and results were expressed as a percentage of control. *apoA-I compared with PMA, **T-apoA-I compared with PMA, and ***T-rHDL compared with PMA, n = 5. *P < 0.05 versus PMA (0 μg/ml), **P < 0.01 versus PMA (0 μg/ml), ***P < 0.001 versus PMA (0 μg/ml). (B) monocytes were stimulated with LPS (1 μg/ml) in the presence or absence of the cholesterol acceptors (40 μg/ml). Flow cytometry was used to determine the CD11b activation. *P < 0.05, **P < 0.01, n = 5.
Fig. 5. T-rHDL inhibits endothelial-mediated monocyte recruitment under shear flow. HCAECs were preincubated with rHDLs before stimulation with TNF-α and subsequent perfusion of purified monocytes. (A) total adhesion, (B) stationary adhesion and (C) rolling flux. T-rHDL inhibited total and stationary adhesion after 5 min, whereas rHDLε after 10 min compared with TNF-α. T-rHDL resulted in a significant increase in rolling flux after 5 min compared with TNF-α. Phase-contrast images at 5 and 10 min, respectively, control (D and E), TNF-α (F and G), rHDLε (H and I), and T-rHDL (J and K).

*P < 0.05 TNF-α + T-rHDL versus TNF-α, **P < 0.01 TNF-α + T-rHDL versus TNF-α, ***P < 0.001 TNF-α + T-rHDL versus TNF-α, ****P < 0.001 TNF-α + rHDLε versus TNF-α, ***P < 0.001 TNF-α + rHDLε versus TNF-α.
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Author Contributions

Participated in experiment designs: Murphy, Hoang, Aprico, Sviridov, Chin-Dusting.

Conducted experiments: Murphy, Hoang, Aprico.

Performed data analysis: Murphy, Hoang, Aprico.

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References
