



Published in final edited form as:

*Circ Res.* 2013 June 21; 113(1): e1–e9. doi:10.1161/CIRCRESAHA.113.301112.

## Pegylation of HDL Decreases Plasma Clearance and Enhances Anti-Atherogenic Activity

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### Abstract

**Rationale**—Infusions of apoA-I, mimetic peptides or HDL remain a promising approach to treatment of atherosclerotic coronary disease. However, rapid clearance leads to a requirement for repeated administration of large amounts of material and limits effective plasma concentrations.

**Objective**—Since pegylation of purified proteins is commonly used as a method to increase their half-life in the circulation, we determined whether pegylation of apoA-I or HDL would increase its plasma half-life and in turn its anti-atherogenic potential.

**Methods and Results**—Initial pegylation attempts using lipid-poor apoA-I showed a marked tendency to form multi-pegylated (PEG) species with reduced ability to promote cholesterol efflux from macrophage foam cells. However, pegylation of human *holo*-HDL or reconstituted phospholipid/apoA-I particles (rHDL) led to selective N-terminal mono-pegylation of apoA-I with full preservation of cholesterol efflux activity. The plasma clearance of PEG-rHDL was estimated following injection into hypercholesterolemic *ApoE*<sup>-/-</sup> mice; the half-life of pegylated apoA-I following injection of PEG-rHDL was increased about 7-fold compared to apoA-I in non-pegylated rHDL. Compared to non-pegylated rHDL, infusion of PEG-rHDL (40 mg/kg) into hypercholesterolemic *ApoE*<sup>-/-</sup> mice led to more pronounced suppression of bone marrow myeloid progenitor cell proliferation and monocytosis, as well as reduced atherosclerosis and a stable plaque phenotype.

**Conclusions**—We describe a novel method for effective mono-pegylation of apoA-I in HDL particles, in which lipid binding appears to protect against pegylation of key functional residues. Pegylation of apoA-I in rHDL markedly increases its plasma half-life and enhances anti-atherogenic properties *in vivo*.

### Keywords

HDL; apoA-I; atherosclerosis; hematopoiesis; cholesterol

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#### DISCLOSURES

A.R.T is a consultant for Merck, Pfizer, Roche, Amgen, CSL, and Arisaph. The other authors have no conflicts to report.

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## INTRODUCTION

Extensive epidemiological data has shown an inverse relationship between HDL levels and coronary heart disease<sup>1,2</sup>. There is also convincing evidence for a protective role of HDL against atherogenesis in animal models<sup>3,4</sup> notably following infusion of HDL or rHDL in hypercholesterolemic animal models<sup>5-7</sup>. However, the development of new therapies that increase plasma HDL levels has proven challenging. This was highlighted by the failure of torcetrapib and dalcetrapib, two different inhibitors of cholesteryl ester transfer protein, and of ER-niacin in recent clinical trials (AIM-HIGH and HPS2-THRIVE)<sup>8-12</sup>. In addition, carriers of the *LIPG* 396Ser allele in endothelial lipase had higher HDL cholesterol concentrations but this allele was not associated with an altered risk of myocardial infarction<sup>13</sup>, suggesting that raising HDL cholesterol by inhibition of endothelial lipase will not reduce atherosclerosis risk<sup>14</sup>. Ongoing clinical studies with more potent, apparently non-toxic CETP inhibitors<sup>9</sup> will provide further evaluation of CETP inhibition but will not test the “HDL hypothesis” because these agents substantially lower LDL levels as well as increasing HDL.

A promising approach to reducing coronary atherosclerosis is the infusion of cholesterol-poor reconstituted HDL (rHDL)<sup>15-17</sup>. In two small-scale randomized clinical trials, infusions of wild type or the Milano variant of apoA-I in complexes with phospholipids into patients who had recently experienced acute coronary syndromes reduced atheroma volume in coronary arteries<sup>15-17</sup>. Infusion of rHDL in patients with peripheral vascular disease (PVD) also resulted in significant remodeling of the atheroma and suppression of inflammation<sup>16</sup>. These results most likely relate to the ability of cholesterol-poor rHDL particles to act as highly efficient acceptors of cholesterol from macrophage foam cells as well as other vascular cells involved in atherogenesis<sup>18,19</sup> and to promote reverse cholesterol transport<sup>20</sup>. Animal models have revealed that raising HDL levels by genetic methods or infusion of rHDL inhibits vascular inflammation<sup>21</sup>, significantly remodels atherosclerotic lesions<sup>7,19,22-24</sup> and improves endothelial function<sup>25</sup>.

Recent studies also indicate that dysregulated cholesterol homeostasis in hematopoietic stem and multipotent progenitor cells (HSPC) increases the risk of atherosclerosis as cholesterol accumulation in these cells promotes cell proliferation and causes monocytosis and neutrophilia, which contribute to accelerated atherosclerosis<sup>26,27</sup>. Accordingly, increasing HDL in hypercholesterolemic *ApoE*<sup>-/-</sup> mice reduced HSPC proliferation and monocytosis<sup>26</sup>, and decreased mobilization of HPSCs and extramedullary hematopoiesis<sup>28</sup>, suggesting a potential further application of rHDL infusions in the treatment of myeloproliferative neoplasms.

A major drawback of rHDL infusions in clinical practice is the need for repeated administration of a relatively large amount of material, due to the rapid clearance of HDL from the plasma<sup>17</sup>. The mechanisms responsible for turnover of plasma HDL are still poorly defined, though liver and kidney are the major organs taking up HDL<sup>29</sup>. One approach to reducing clearance has been to generate multimers of recombinant apoA-I. ApoA-I multimers had increased molecular size, decreased clearance relative to apoA-I monomers<sup>30</sup> and caused reduced atherosclerosis in hypercholesterolemic mice<sup>30</sup>. Current methods to produce rHDL rely on the use of bile salts<sup>17</sup>. While the amphipathic nature of bile salt used in the preparation of rHDL facilitates rHDL particle formation, the residual bile salt in the rHDL preparation could cause adverse effects and therefore limit the quantity of the rHDL that can be administered<sup>17</sup>. These considerations suggest the need for novel strategies to improve the therapeutic efficacy of rHDL preparations.

Protein pegylation, a process of covalent attachment of polyethylene glycol polymer chain to the target protein, has been used extensively to increase the therapeutic efficacy of protein drugs<sup>31</sup>. However, protein pegylation has been used primarily for modification of purified proteins. It is not clear whether pegylation can also increase the plasma half-life of proteins complexed with other biological molecules such as lipids in lipoprotein particles and whether this modification affects their biological activities. The goal of this study was to determine if pegylation of lipid-poor apoA-I or rHDL particles could increase the biological half-life of the preparation while preserving anti-atherogenic functions.

## METHODS

### Animals

C57BL6/J or *ApoE*<sup>-/-</sup> mice were from Jackson Laboratory. For atherosclerosis studies, *ApoE*<sup>-/-</sup> mice were fed a Western type diet (WTD) (TD88137; Harlan Teklad) for the indicated period of time. Where indicated, vehicle (saline), rHDL, or PEG-rHDL was injected at the indicated dose into the mice via the tail vein. Purified human ApoA-I or rHDL (CSL-111) was provided by CSL Behring AG, Bern, Switzerland; CSL-111 is comprised of human apoA-I and phosphatidylcholine from soybean in a ratio of 1:150. The Columbia University animal ethics committee approved this study.

### Pegylation of apoA-I or HDL

Holo-HDL was purified from human plasma as previously described using KBr density gradients<sup>32</sup>. Human apoA-I, HDL or rHDL were pegylated with M-PEG-ALD of MW 20000 or 40000. M-PEG-ALD was purchased from JenKem Technology USA (Allen, TX). After fully equilibrated to room temperature from -20°C storage and dissolved in an aliquot of 50 mM sodium acetate, pH 5.5, 10 mM sodium cyanoborohydride solution, M-PEG-ALD was immediately mixed at an indicated molar ratio with apoA-I, HDL or rHDL reconstituted in the same solution with gentle agitation. The final concentration of apoA-I, HDL or rHDL was ranging from 3 to 6 mg/ml. The mixture was incubated at 4°C for 16 to 72 hours. At the end of incubation, the reaction was quenched by addition of an aliquot of 1M Tris solution to the mixture to make the final concentration 100 mM Tris. The pegylated apoA-I, HDL or rHDL were subjected to SDS-PAGE and Coomassie Brilliant Blue staining for evaluation of the pegylation efficiency. The unmodified control was processed similarly without M-PEG-ALD. For cholesterol efflux assays or infusion of the pegylated or non-pegylated apoA-I, HDL or rHDL preparations into mice, same amounts of quenched, inactivated M-PEG-ALD were added to the non-pegylated apoA-I, HDL or rHDL preparations and the pegylated or non-pegylated preparations were dialyzed against phosphate buffered saline for final formulation. To determine the molecular mass of pegylated human apoA-I, we isolated pegylated apoA-I with a modified protocol of a method previously reported<sup>33</sup>. Briefly, pegylated apoA-I preparation was subjected to SDS-PAGE and the unfixed, unstained PEG-apoA-I band was excised from the gel after its location in the gel was estimated using a sample run in the same gel in parallel. After passive elution from the gel strip, the pegylated apoA-I sample was repeatedly diluted with phosphate buffered saline and concentrated with Amicon Centrifugal filters. The molecular mass of PEG-apoA-I was determined by MALDI-TOF.

### Plasma clearance

An aliquot of unmodified or pegylated apoA-I, HDL or rHDL at the indicated dose was injected into the mice via tail vein. At the indicated time point, an aliquot of blood was collected from the mice. The blood samples were subjected to SDS-PAGE and Western analysis with anti-human apoA-I antibodies. Native or PEG-apoA-I was quantified by densitometry analysis with ImageJ.

### Cholesterol efflux

Cholesterol efflux from mouse peritoneal macrophages or THP-1 cell derived macrophage like cells was performed as described previously<sup>32</sup>. Briefly, the cells were cholesterol loaded by incubation with the indicated amount of acetyl-LDL containing [<sup>3</sup>H]cholesterol for 16 hours in the presence of 1  $\mu$ M TO901317. The cells were then washed and cholesterol efflux was initiated by addition of indicated amount of cholesterol acceptors before the media and cells were collected for analysis. Cholesterol efflux was expressed as the percentage of the radioactivity released from the cells into the medium relative to the total radioactivity in cells plus medium.

### Analysis of blood leukocytes

Total white blood cell count in freshly collected mouse blood was performed using hematology cell counter (Oxford Science Inc.). Monocytes and neutrophils were identified from whole blood as previously described<sup>26</sup>. Monocytes were identified as CD45<sup>hi</sup>CD115<sup>hi</sup> and neutrophils were identified as CD45<sup>hi</sup>CD115<sup>lo</sup>Ly6-C/G<sup>hi</sup> (Gr-1).

### Analysis of hematopoietic stem cells

Hematopoietic stem and progenitor cells from the BM were analyzed by flow cytometry as previously described<sup>26</sup>. Isolated bone marrow was incubated with a cocktail of antibodies against lineage-committed cells (B220, CD19, CD11b, CD3e, TER-119, CD2, CD8, CD4, Ly6-C/G: All FITC, eBioscience), Sca1-Pacific Blue and ckit-APC Cy7. HSPCs were identified as lin<sup>-</sup>, Sca1<sup>+</sup> and ckit<sup>+</sup> (LSK) while the hematopoietic progenitor subsets were separated by using antibodies to CD16/CD32 (Fc $\gamma$ RII/III) and CD34. CMPs were identified as lin<sup>-</sup>, Sca1<sup>-</sup>, ckit<sup>+</sup>, CD34<sup>int</sup>, Fc $\gamma$ RII/III<sup>int</sup>, GMPs as lin<sup>-</sup>, Sca1<sup>-</sup>, ckit<sup>+</sup>, CD34<sup>int</sup>, Fc $\gamma$ RII/III<sup>hi</sup>. Cell cycle analysis was performed using DAPI (Sigma) in cells that had been stained with the above markers and then incubated in cytofix/cytoperm buffer (BD Biosciences).

Flow cytometry was performed using an LSRII running FACS DiVa software. Flow cytometry data was analyzed using FlowJo software (Tree Star Inc.).

### Quantification of aortic atherosclerosis lesions

The lesions located in the aorta and aortic sinuses were analyzed using Oil-Red O staining. Quantification of Oil-Red O staining was performed off-line using Adobe Photoshop CS5 and presented as the percentage of the total surface area of the aorta. Lesion area in the aortic root was quantified by morphometric analysis of H&E stained sections as described<sup>34</sup>. Collagen staining was performed using picrosirius red as per the manufactures instructions (PolySciences, Inc). Macrophage staining was performed by staining with antibody against F4/80 (Abcam).

### Statistical analysis

One-way ANOVA with post-hoc analysis and *t* tests were performed using GraphPad Prism version 5.00 for Mac OS X (GraphPad Software, San Diego California USA, [www.graphpad.com](http://www.graphpad.com)) or STATVIEW 5.0 (Abacus Concepts, Inc). The post-test was Fisher's PLSD and the threshold for significance was *p* = 0.05. Data shown are mean  $\pm$  SEM.

## RESULTS

### Characterization of N-terminally pegylated ApoA-I

Ideally, pegylation of apoA-I would not adversely affect apoA-I lipidation or its capacity to promote cholesterol efflux. ApoA-I structure and lipidation have been extensively studied. The carboxyl terminal amphipathic alpha-helix structure of apoA-I appears to be critical for

lipid binding and its lipidation by ABCA1 activity<sup>35</sup>. In contrast, ABCA1-mediated cholesterol efflux is relatively insensitive to mutagenesis-induced changes in the N-terminal helix-bundle domain<sup>35</sup>. Thus, we reasoned that amino terminal pegylation of apoA-I might not disrupt the part of the structure of apoA-I necessary for mediating cholesterol efflux. In order to test this idea, we took advantage of an acidic reaction condition which favors selective pegylation of the amino terminal residue of the target protein<sup>36</sup>. When a reactive linear PEG molecule with average molecular size of 20 kDa was used for pegylation at 4°C, this strategy yielded a predominant single species of pegylated apoA-I with retarded migration in SDS polyacrylamide electrophoresis (SDS-PAGE) (Fig. 1A). The apparent Mr (~70 kDa) was higher than expected (i.e. human apoA-I: 28 kDa + PEG: 20 kDa = 48 kDa), likely reflecting aberrant migration, a common observation that has been attributed to PEG-SDS interactions when running pegylated proteins by SDS-PAGE<sup>37</sup>. We also used mass spectrometry to determine the molecular mass of the gel purified PEG-apoA-I band. This showed a molecular mass of approximately 48 kDa, as expected for mono-pegylated human apoA-I. Importantly, cholesterol efflux from macrophage foam cells was preserved using this method of pegylation (Fig. 1B). This indicated that mono-pegylation of apoA-I does not alter its function.

However, the targeted pegylation of lipid-poor apoA-I at low temperature was suboptimal as the majority of the apoA-I was not pegylated. We attempted to increase the yield of pegylated apoA-I by adjusting variables such as the temperature and time of pegylation; however, this resulted in multi-pegylated species, suggesting pegylation of amino acid residues of apoA-I in addition to the N-terminus (Fig. 1C). Further, the multi-pegylated apoA-I had reduced capacity to promote cholesterol efflux from macrophage foam cells (Fig. 1D), indicating that unmodified amino acids of apoA-I other than the N-terminus are required to preserve functionality.

### Characterization of pegylated HDL

In an attempt to reduce the pegylation of residues other than the N-terminus of apoA-I, we assessed the use of human *holo*-HDL particles as the substrate for protein pegylation. We reasoned that use of native HDL rather than lipid-poor apoA-I might increase the specificity and efficiency of targeted pegylation, as functionally important amino acid residues might be shielded from pegylation by lipid molecules in HDL particles. Under conditions where lipid-poor apoA-I gave rise to multi-pegylated species, pegylation of HDL gave rise mainly to mono-pegylated apoA-I species, as shown by SDS-PAGE and Coomassie Blue staining (Fig. 2A). After pegylation, >90% of human apoA-I in HDL particles migrated at the Mr of the mono-pegylated apoA-I species. The band that appears in the native HDL preparation of a similar size to PEG-HDL in Fig 2A is likely albumin, a common contaminant of HDL purified from plasma. We also performed a western analysis using an anti-apoA-I antibody and no band of that size was detected in the native HDL preparation confirming that it was not an apoA-I product (Fig 2B).

We next carried out studies to determine if PEG-HDL promoted cholesterol efflux from macrophage foam cells. In order to rule out the potential effect of free PEG molecules in the efflux assay, the same amount of quenched PEG preparation was added to the unmodified HDL preparation during the efflux assay. The targeted pegylation of HDL particles did not affect the ability of HDL to promote cholesterol efflux. Cholesterol efflux from macrophages to the native HDL or PEG-HDL showed a similar dose response (Fig. 2C).

In order to evaluate the pharmacokinetics of PEG-HDL particles we infused PEG-HDL into chow-fed wild type mice. This preparation of PEG-HDL also contains a portion of non-pegylated human apoA-I which served as an internal control. The half-life of HDL protein (apoA-I) estimated by Western analysis of an aliquot of plasma was approximately 9.2 hours

(lower Mr band in Fig. 2C), comparable to the estimated half life of human apoA-I infused into chow-fed wild type mice as reported by others<sup>30</sup>. Infusion of native human HDL at similar dose gave rise to a similar half-life of human apoA-I as determined from the internal standard (not shown). The turnover time of PEG-HDL, estimated by the clearance of the distinct PEG-apoA-I, was increased to ~48 hours as compared with that of unpegylated human HDL (Fig. 2D higher Mr band = PEG-apoA-I vs lower Mr band = non-PEG-apoA-I), indicating ~5-fold increase in circulation time. We also noted a non-specific band that was detected by western blot with anti-apoA-I antibody (Fig. 2D). However, we have confirmed this was not a modified form of human apoA-I as this band was also detected in plasma from mice that received a saline infusion (Fig. SI – lane 1).

### Characterization of pegylated reconstituted HDL

For further characterization of properties of pegylated HDL, we used a preparation of rHDL that has been employed in clinical studies<sup>16,17</sup>. Targeted N-terminal pegylation of rHDL also yielded a largely homogeneous species of mono-pegylated apoA-I (Fig. 3A). In order to verify that apoA-I was pegylated at the N-terminus, the single species of pegylated apoA-I was isolated and subjected to Edman N-terminal protein sequencing. Only ~20% of the pegylated apoA-I could be sequenced by Edman degradation while, as a control, ~98% of apoA-I that was subjected to similar treatment but without pegylation could be sequenced (not shown). This suggests that the majority (~80%) of the mono-pegylated apoA-I was N-terminally pegylated. This sequencing result confirmed the method of preferential N-terminal protein pegylation, and suggests it can be applied to proteins complexed with other biological components so that amino acid residues that are essential for function are protected. In line with this idea cholesterol efflux assays showed that rHDL and PEG-rHDL promoted similar levels of cholesterol efflux from LXR activated human THP-1 macrophages (Fig. 3B). We next carried out studies to examine rHDL or PEG-rHDL clearance from plasma in a hypercholesterolemic mouse model. rHDL or PEG-rHDL at a dose of 60 mg/kg (based on rHDL protein content) was injected intravenously in *ApoE*<sup>-/-</sup> mice fed on a WTD. The clearance of human apoA-I or PEG-apoA-I from plasma was assessed by SDS-PAGE and Western blotting. Again as in Figure 2, some non-PEG-rHDL was present in the PEG-rHDL infused mice which served as an internal control. This along with the comparison to mice infused with rHDL clearly demonstrated the increased half-life of PEG-rHDL (Fig. 3C higher Mr band = PEG-HDL vs lower Mr band = non-PEG-HDL). Using densitometry we found that PEG-rHDL had a markedly reduced clearance rate and an approximately 7-fold prolonged half-life ( $t_{1/2}$ =48.7 h for PEG-rHDL vs  $t_{1/2}$ =7.7 h for rHDL and  $t_{1/2}$ =7.5 h for non-PEG-rHDL internal control,  $n=5$ /group,  $p<0.001$ ) (Fig. 3C,D). We also noted some intermediate bands in that reacted with the anti-apoA-I antibody in the mice infused with PEG-rHDL (Fig. 3C – right panel). It is possible that these could be partially degraded pegylated apoA-I molecules as it has previously been reported that apoA-I within HDL particles can be cleaved *in vivo*<sup>38,39</sup>. Again the non-specific band detected was not a by-product of PEG-rHDL preparation or a modification as this band was also detected in plasma from mice that received a saline infusion (Fig. SI).

### PEG-rHDL is more efficient at reducing hematopoietic stem and progenitor cell proliferation and monocytosis

In order to test the idea that pegylation of rHDL would extend its half-life and thus promote a more prominent anti-atherogenic effect, we infused rHDL or PEG-rHDL into WTD-fed *ApoE*<sup>-/-</sup> mice. We and others have shown that hypercholesterolemic *ApoE*<sup>-/-</sup> mice develop monocytosis which appears to contribute to accelerated atherosclerosis<sup>26,40</sup>, and we showed that rHDL infusion could reduce monocyte levels in this mouse model<sup>26</sup>. Thus, monocyte counts could potentially act as a biomarker for the *in vivo* efficacy of rHDL infusions. In order to detect a potential difference in effectiveness of rHDL vs PEG-rHDL, we infused

mice with two doses of 40 mg rHDL or PEG-rHDL protein/kg body weight separated by one week. This dosing schedule was chosen, as we had previously shown that 40mg/kg of rHDL was a submaximal dose, having a modest effect on inhibiting monocytois in WTD-fed *ApoE*<sup>-/-</sup><sup>26</sup>. At the 40mg/kg dose in the present study, rHDL infusion had no effect on the count of total white blood cells, monocytes or neutrophils 7 days after the second infusion (Fig. 4A, B). In contrast, PEG-rHDL significantly reduced these parameters relative to rHDL or the vehicle control. Our previous studies showed that effects of rHDL on monocyte counts reflected decreased proliferation of HSPCs and myeloid progenitors in the bone marrow<sup>26</sup>. Consistent with its effect of blood counts, PEG-rHDL infusion also more markedly decreased the count and proliferation of hematopoietic stem and progenitor cells (HSPC), common myeloid progenitors (CMPs) and granulocyte-macrophage progenitors (GMPs) (Fig. 4C, D).

### PEG-rHDL is more effective at inhibiting atherosclerosis and stabilizing lesions

We initially conducted a dose response study using rHDL infusions in *ApoE*<sup>-/-</sup> mice fed a WTD for 4 wks to ascertain the amount of rHDL to use in our comparison to PEG-rHDL. Our goal was to find a dose of rHDL that failed to significantly improve atherosclerotic lesions. We administered two infusions of rHDL (40 mg, 80 mg or 120 mg protein/kg body weight) separated by one week. Doses of rHDL at 80 and 120, but not 40 mg/kg, significantly reduced the area of Oil-Red O staining of the aortic arch compare to mice treated with vehicle (Fig. SII). Therefore we decided to compare rHDL with PEG-rHDL at a dose of 40 mg/kg.

We examined the impact of rHDL or PEG-rHDL at a dose of 40 mg/Kg on the development of atherosclerosis in the hypercholesterolemic *ApoE*<sup>-/-</sup> mice. We observed no changes in total plasma or HDL cholesterol levels measure 7 days post the second infusion (Fig. SIII). Infusion of PEG-rHDL but not rHDL at equal doses (40mg/kg) significantly reduced the area of Oil-Red O staining of the aorta, indicating a reduction in aortic lipid content (Fig. 5A). Compared to our pilot experiment (Fig. SII) these results may suggest that PEG-rHDL administered at 40mg/kg has an efficacy similar to rHDL at 80mg/kg. Assessment of lesion formation in the proximal aorta showed no change in overall lesion area (Fig. 5B), which is consistent with a number of previous reports<sup>30</sup>. However, we observed a significant reduction in the macrophage content and cellularity of proximal aortic lesions of PEG-rHDL treated mice which was accompanied by a reduction in necrotic core size (Fig. 5 C,D,E). Lesions of PEG-rHDL treated mice also showed increased collagen (Fig. 5F). Taken together these results suggest that PEG-rHDL effectively remodeled lesions to what is considered a more stable phenotype, similar to that reported with rHDL infusions in humans with PVD<sup>16</sup> and animal models where HDL levels were increased<sup>7,19,22-24</sup>.

## DISCUSSION

We have developed a novel formulation of HDL prepared by targeted pegylation of HDL particles. While our initial studies focused on pegylation of lipid-poor apoA-I, attempts to scale up preparations by increasing temperature or PEG concentration were unsuccessful due to formation of multi-pegylated apoA-I species that were inefficient in cholesterol efflux assays. However, pegylation of plasma HDL or rHDL led to formation of functional, N-terminally, mono-pegylated apoA-I, likely because key amino acid residues were protected by binding to lipids. Following injection of this preparation, the half-life of apoA-I was increased about 7-fold. Most importantly, PEG-rHDL was significantly more effective than control rHDL at reducing myelopoiesis, monocyte counts, and atherosclerosis.

The apparent increase in efficiency of PEG-rHDL in reducing myelopoiesis and atherosclerosis was likely related to the increased half-life of apoA-I in the preparation.

Since the half-life of apoA-I in HDL particles is several-fold more than that of HDL lipids, it is likely that apoA-I is recycled through several generations of HDL particles, probably being released by lipases that act on HDL, followed by lipidation of apoA-I via ABCA1 in various tissues<sup>41,42</sup>. However, it is important to note that we do not expect pegylation of rHDL to alter or enhance its cholesterol efflux ability on a molar basis compared to native rHDL. We speculate that compared to native apoA-I, mono-pegylated apoA-I may be less susceptible to glomerular filtration and degradation in the kidney or to catabolic uptake in the liver, allowing continued interaction with ABCA1 and ABCG1 in bone marrow progenitors, circulating leukocytes and macrophage foam cells.

The pegylation approach has several advantages. PEGs have been used to modify multiple protein drugs and their safety in humans has been rigorously examined<sup>43</sup>. The long-term, repeated use of some pegylated protein drugs, e.g. pegylated erythropoietin, is common in clinical practice<sup>44</sup>. A major use of protein pegylation is to increase hydrodynamic size of the target protein and reduce its glomerular filtration and degradation in kidney in order to increase its therapeutic concentration in the circulation<sup>43</sup>, desirable properties for apoA-I and HDL. While pegylation does not lead to conformational changes of the polypeptide chains in most cases<sup>43</sup>, it has the potential to reduce the immunogenicity of the target protein<sup>43</sup>.

Various formulations of cholesterol acceptors intended to promote cholesterol efflux from macrophage foam cells and RCT *in vivo* have been studied. In addition to rHDLs, infusions of synthetic amphipathic peptides complexed with phospholipids have been investigated<sup>45,46</sup>. However, on a molar basis the ability of synthetic peptides to promote cholesterol efflux from macrophage foam cells is much less than rHDL<sup>45,46</sup>. Infusion of a large amount of the peptide-phospholipid complexes is needed in order to achieve effective concentrations, which may increase the potential toxicity, for example in the kidney following glomerular filtration. In addition, the synthetic amphipathic peptides contain an artificial amino acid sequence, which has the potential to induce adverse immune responses.

Various strategies have been studied as a means of reducing the renal clearance of apoA-I. This included recombinant trimeric apoA-I, which showed an increased circulation time when injected into animals either as lipid-free or lipidated forms<sup>30</sup>. However, this approach has some intrinsic limitations. Cost-effective large scale manufacturing of recombinant proteins free of adverse foreign components remains a challenge, particularly for apoA-I with expected therapeutic doses higher than most recombinant protein drugs. Also, trimeric apoA-I, as a complex of recombinant fusion proteins of human apoA-I and the trimerization domain of human tetranectin, has the potential to be recognized as non-self protein and to induce adverse immune responses. The lipidated trimeric apoA-I also appears to be less efficient in promoting cholesterol efflux from macrophage foam cells relative to lipidated recombinant apoA-I monomers, at equal molecular mass<sup>30,47</sup>, however trimeric apoA-I preparations still retain many of the known anti-inflammatory functions of native apoA-I<sup>48</sup>.

Our study has several limitations. We compared the efficacy of rHDL and PEG-rHDL at a submaximal dose, which was chosen to assess a potential benefit of the pegylated preparation. For synthetic HDL preparations, the ability to use a lower dose represents a major advantage, both in terms of cost and convenience of preparations and also in terms of potential toxicities. We showed a beneficial effect of PEG-rHDL on aortic atherosclerosis as revealed by Oil-Red O staining, consistent with reduced macrophage foam cells content. However, lesion area in the proximal aorta was not reduced, similar to some previous studies using rHDL infusion strategies<sup>19</sup>. Nonetheless, macrophage content, lesion cellularity and necrotic core size were decreased and collagen content was increased, consistent with lesion stabilization<sup>7,19,22-24</sup>. PEG-rHDL, relative to rHDL, showed increased



ability to suppress HSPC proliferation and monocytosis. This indicates that monocyte and leukocyte counts may be useful biomarkers to assess effectiveness of different HDL based therapies *in vivo*. Together, the improved pharmacokinetics and enhanced anti-atherogenic activity of PEG-rHDL suggest that it may be a useful preparation, with certain advantages such as lower dosing and decreased potential for toxicity.

Several recent clinical and genetic studies have led the generic “HDL hypothesis” to be called into question<sup>8-13</sup>. However, we believe this hypothesis should be refined to emphasize HDL functionality especially its ability to promote macrophage cholesterol efflux and reverse cholesterol transport<sup>49,50</sup>. While recent studies of HDL raising with niacin have not shown clinical benefit, the macrophage cholesterol efflux potential of HDL from subjects treated with niacin is at best marginally improved<sup>51</sup>. HDL raising with dalcetrapib also did not produce clinical benefit<sup>52</sup> but lipoprotein changes may have been suboptimal due to incomplete CETP inhibition. Variants in some genes associated with increased HDL levels were not associated with expected effects on CAD<sup>13</sup>, but none of these variants are known to influence the production of apoA-I or HDL. Finally, a large body of preclinical evidence strongly supports the concept that interventions to increase macrophage cholesterol efflux and reverse cholesterol transport, as is likely to occur with rHDL infusions, are associated with reduced atherosclerosis<sup>49,50</sup>. Whether rHDL infusions succeed in improving clinical outcomes remains to be proven. If such therapies do prove successful, targeted N-terminal pegylation of various forms of apoA-I or related peptides, which may be accomplished by initial introduction into a lipid containing HDL particle prior to pegylation, may represent a novel method for extending the effectiveness, reducing the dose and decreasing toxicity of the therapeutic HDL preparation.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## Acknowledgments

### SOURCES OF FUNDING

A.J.M. was supported by a post-doctoral fellowship from the American Heart Association (AHA; 12POST11890019). This project was funded by the NIH grants HL87123 and HL107653.

## Nonstandard Abbreviations

<b>PEG</b>	Pegylation
<b>HSPC</b>	hematopoietic stem and multipotent progenitor cells

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## Novelty and Significance

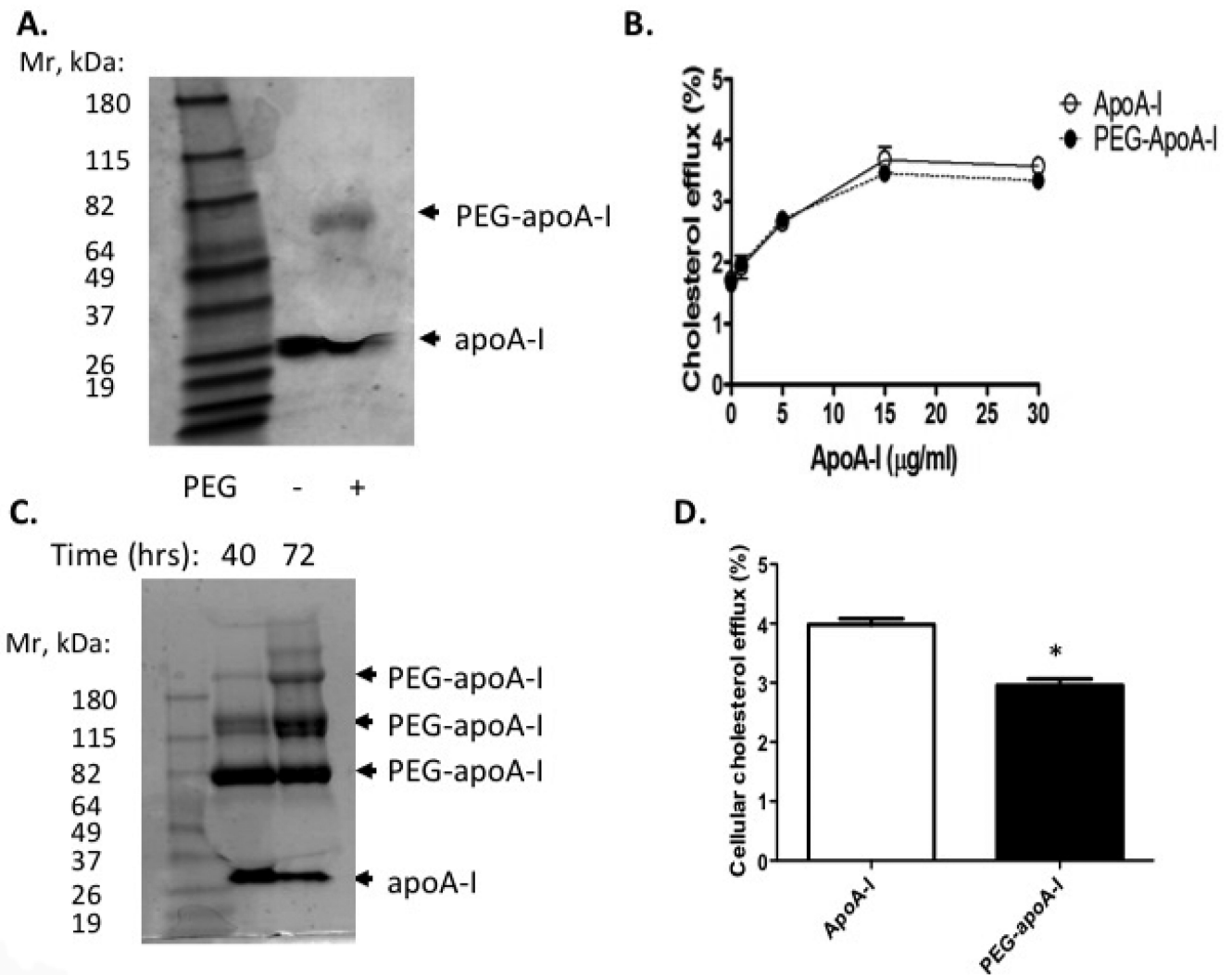
### *What Is Known?*

- Raising HDL by direct infusion of reconstituted HDL (rHDL) appears to be a promising therapeutic approach to treating cardiovascular disease (CVD).
- However, the relatively short half-life of the current preparations may require frequent dosing.
- Reconstituted HDL for clinical use has been difficult to manufacture, expensive, and toxicity has limited dosing and usefulness.

### *What New Information Does This Article Contribute?*

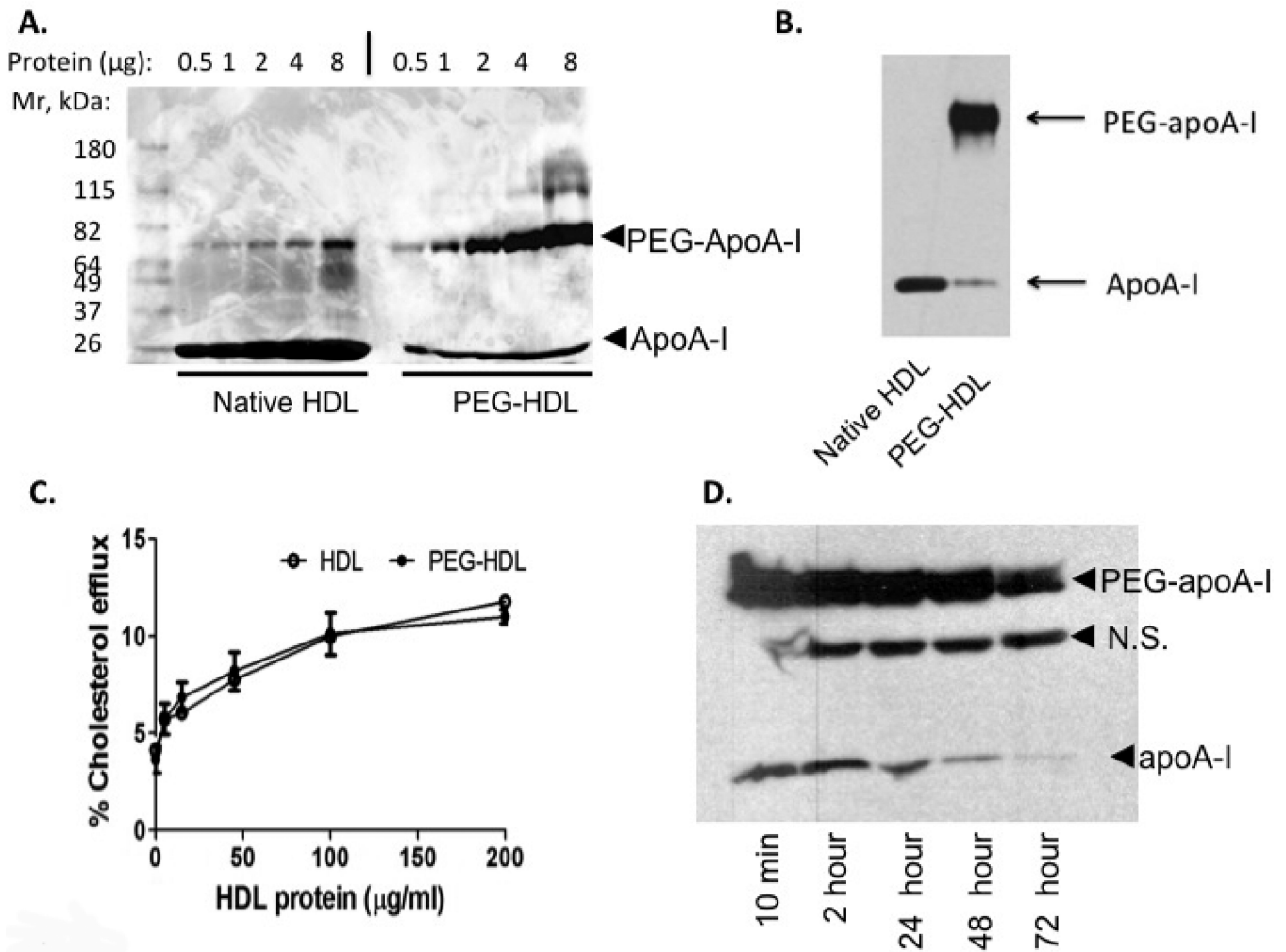
- We developed a method for efficient, N-terminal monopegylation of HDL, which greatly extends the in vivo half-life of HDL and increases its anti-atherogenic properties.
- This extends the concept of protein pegylation to pegylation of a particle, with beneficial results.
- The use of pegylated HDL may reduce the dosing requirement for reconstituted HDL preparations, with greater efficacy and reduced potential for side-effects.

Directly raising HDL levels by rHDL is thought to be a promising approach in the treatment of cardiovascular disease. However, rHDL is cleared fairly quickly from the circulation, limiting its bioavailability. We describe a technique to effectively mono-pegylate the N-terminus of apoA-I in HDL particles which extends its in vivo half-life. Pegylation of HDL particles can be applied to *holo*-HDL, rHDL and possibly peptide/lipid-complexes, increasing efficacy, decreasing dosage and potentially reducing side-effects. This approach could be applied to the treatment of atherosclerosis and myeloproliferative neoplasms.



**Figure 1. Characterization of pegylated human apoA-I**

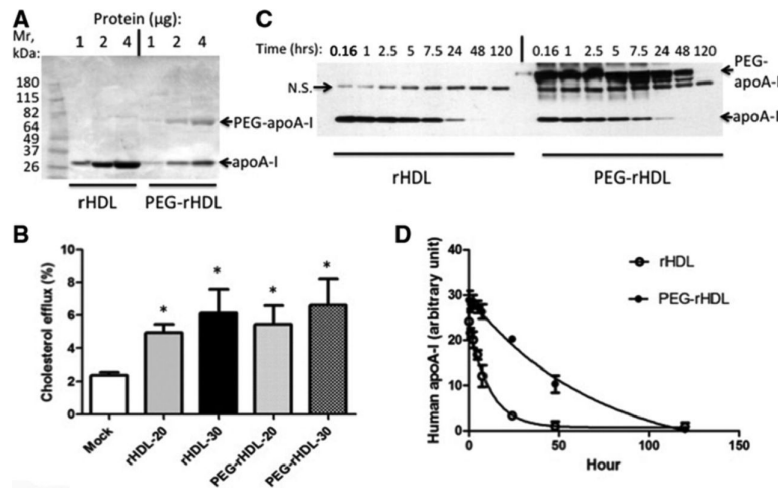
(A) ApoA-I pegylated with M-PEG-ALD 20K at 4°C for 16 hours and analyzed by SDS-PAGE and Coomassie Blue staining. (B) Cholesterol efflux for 3 hours from cholesterol-loaded mouse peritoneal macrophages to apoA-I or PEG-apoA-I prepared as in (A). (C) ApoA-I pegylated with M-PEG-ALD 20K at 4°C for 40 and 72 hours and subjected to SDS-PAGE and Coomassie Blue staining. (D) Cholesterol efflux for 3 hours from cholesterol-loaded mouse peritoneal macrophages to apoA-I or PEG-apoA-I (10  $\mu\text{g}$  protein/ml) prepared as in (C, 72 hour incubation).



### Figure 2. Characterization of pegylated human HDL

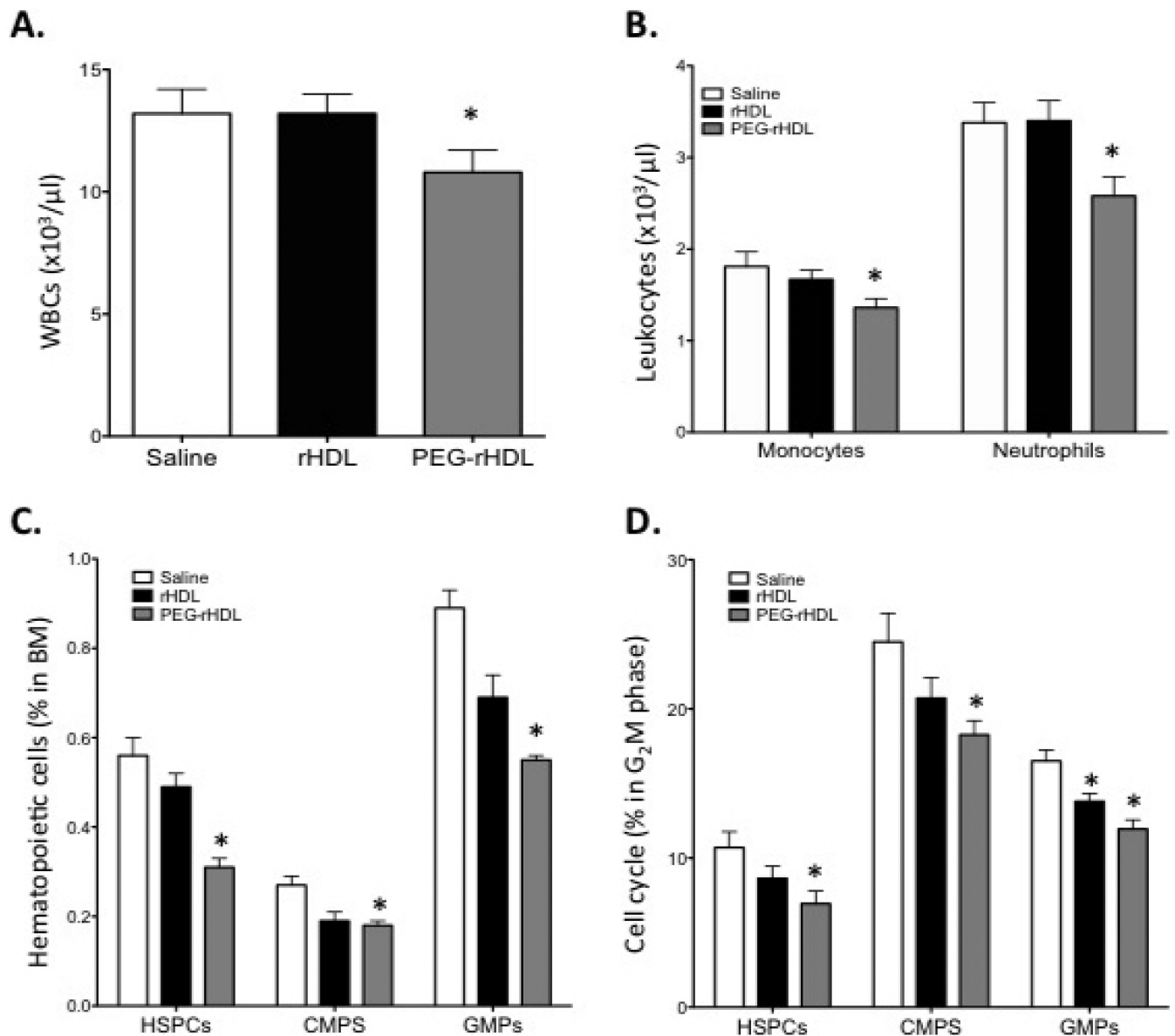
(A) HDL with or without pegylation using M-PEG-ALD 20K at 4°C for 24 hours and analyzed by SDS-PAGE and Coomassie Blue staining. The minor higher Mr band in native HDL samples is likely albumin in the HDL preparations. (B) Western analysis using an anti-apoA-I antibody to HDL and PEG-HDL. (C) Cholesterol efflux for 3 hours from cholesterol-loaded macrophage cells derived from THP-1 cells to HDL or PEG-HDL prepared as in (A). (D) Plasma clearance of PEG-HDL following injection of 40mg/kg PEG-HDL prepared as in (A) and analyzed by Western analysis. N.S.= non-specific band.





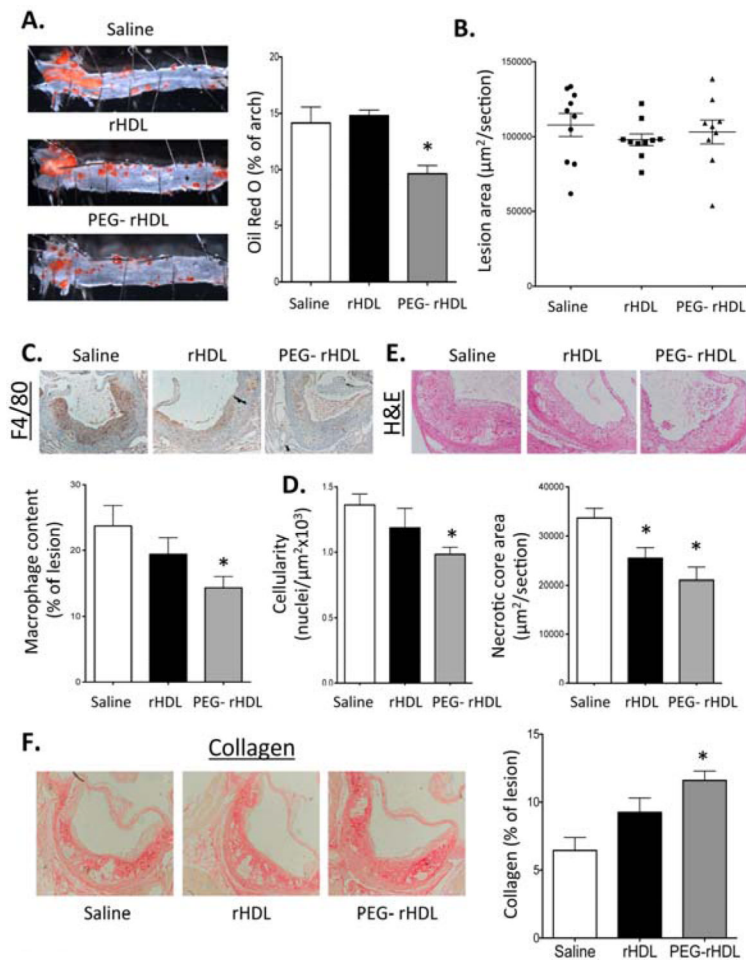
### Figure 3. Characterization of pegylated human rHDL

(A) rHDL pegylated with M-PEG-ALD 20K at 4°C for 16 hours and analyzed by SDS-PAGE and Coomassie Blue staining. (B) Cholesterol efflux for 3 hours from cholesterol-loaded LXR activated THP-1 macrophages to 20 or 30 μg rHDL or PEG-rHDL protein/ml. (C) Plasma clearance of rHDL or PEG-rHDL following injection of 60mg/kg rHDL or PEG-rHDL protein into circulation and analyzed by Western analysis. N.S.= non-specific band. (D) Densitometry quantification and analysis of (C), n=4.



**Figure 4. Effects of rHDL or PEG-rHDL on leukocytosis and HSPC proliferation associated with ApoE deficiency**

*ApoE*<sup>-/-</sup> mice fed a WTD for 8 weeks (n=10/group) were given a single infusion of rHDL or PEG-rHDL (40 mg/kg) weekly for two weeks. 7 days post the second infusion, (A) peripheral blood WBC, (B) neutrophil and monocyte counts as well as (C) bone marrow progenitor cell abundance and (D) proliferation were determined by flow cytometry. Data are presented as mean  $\pm$  SEM, n=10/group. \**P*<0.05 vs Saline.



**Figure 5. Quantification of aortic atherosclerosis lesions**

*ApoE*<sup>-/-</sup> mice fed a WTD for 8 weeks were infused with rHDL or PEG-rHDL 40mg/kg weekly for two weeks. 7 days post the second infusion, the mice were euthanized lesion characteristics were analyzed. (A) The percentage of the aortic arch stained with Oil-Red O. (B) Lesion area in the proximal aorta. (C) Macrophage content as assessed by F4/80 staining in the proximal aorta. (D) Cellularity of the lesions in the proximal aorta determined by nuclei/area. (E) Necrotic core size from H&E stained sections of the proximal aorta. (F) Collagen staining in the proximal aorta by picosirius red. Data are presented as mean  $\pm$  SEM, n=10/group. \**P*<0.05 vs Saline.