Online supplement for:

Pegylation of HDL Decreases Plasma Clearance and Enhances Anti-atherogenic Activity

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Materials and Methods

Animals

C57BL6/J or *Apoe*$^{-/-}$ mice were from Jackson Laboratory. For atherosclerosis studies, *Apoe*$^{-/-}$ 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 indicted 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[1]. 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 cyanoborahydride 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[2]. 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[1]. Briefly, the cells were cholesterol loaded by incubation with the indicated amount of acetyl-LDL containing [^{3}H]cholesterol for 16 hours in the presence of 1 μM 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 while 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[3]. Blood was collected from the tail into EDTA lined tubes and immediately placed on ice. All following steps were performed on ice. RBCs were lysed and WBCs were centrifuged, washed and resuspended in flow-buffer (Hanks balanced salt solution + 0.1% BSA w/v, 5mM EDTA). Cells were stained with a cocktail of antibodies against CD45-Alexa Fluor

450 (Invitrogen), Ly6-C/G-PerCP-Cy5.5 (BD Pharmigen), CD115-APC (eBioscience). Monocytes were identified as $CD45^{hi}CD115^{hi}$ and neutrophils were identified as $CD45^{hi}CD115^{lo}Ly6\text{-}C/G^{hi}$ (Gr-1).

Hematopoietic Stem Cells. Hematopoietic stem and progenitor cells from the BM were analyzed by flow cytometry as previously described[3]. BM was harvested from femurs and tibias by flushing with ice cold PBS. Spleen was minced into small pieces, gently rubbed against the mesh in cell strainer and flushed with PBS. The cell suspension was centrifuged at 500 g for 5 mins, supernatant aspirated and the cells were resuspended in RBC lysis buffer (for 5 min). Lysis was stopped by adding excess amount of flow buffer and the cell suspension was once again centrifuged, aspirated and single cell suspension was prepared by gently pipetting up and down with 200 μl of flow buffer. This was followed by incubation 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^-, $Sac1^+$ and $ckit^+$ (LSK) while the hematopoietic progenitor subsets were separated by using antibodies to CD16/CD32 (FcγRII/III) and CD34. CMPs were identified as lin^-, $Sca1^-$, $ckit^+$, $CD34^{int}$, $Fc\gamma RII/III^{int}$, GMPs as lin^-, $Sca1^-$, $ckit^+$, $CD34^{int}$, $Fc\gamma RII/III^{hi}$. 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 mice were euthanized and perfusion fixed with 10% buffered formalin via the left ventricle for 5 minutes. The lesions located in the aorta and aortic sinuses were analyzed using Oil Red O staining. To measure lesions in the aorta, the whole aorta, including the ascending arch, thoracic, and abdominal segments, was dissected, gently cleaned of adventitial tissue, cut longitudinally, stained with Oil Red O (Sigma, St. Louis, MO) followed by washing and mounting on a silicone coated dish. Aortas were viewed on an Olympus SZX16 dissecting microscope with a 0.8X objective and images were captured using a spot Insight Mosaic camera. Quantification of ORO staining was performed off-line using Adobe Photoshop CS5 and presented as the percentage of the total surface are of the aorta. Lesion area also was quantified by morphometric analysis

of H&E stained sections of aortic root as described[4]. Paraffin-embedded serial sections were prepared from the aortic root and average lesion size was determined from six sections per mouse. 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) 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 ± SEM.

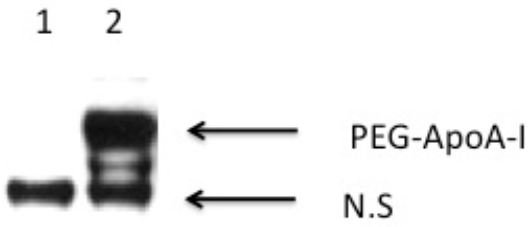

Figure I. Chow-fed C57BL6/J mice were given a single infusion of saline or PEG-rHDL (60 mg/kg). 24 hours post infusion western blot analysis with an anti-apoA-I antibody was performed on total plasma. Lane 1= saline, lane 2 = PEG-rHDL. N.S = non-specific binding of the anti-body to an unknown plasma protein.

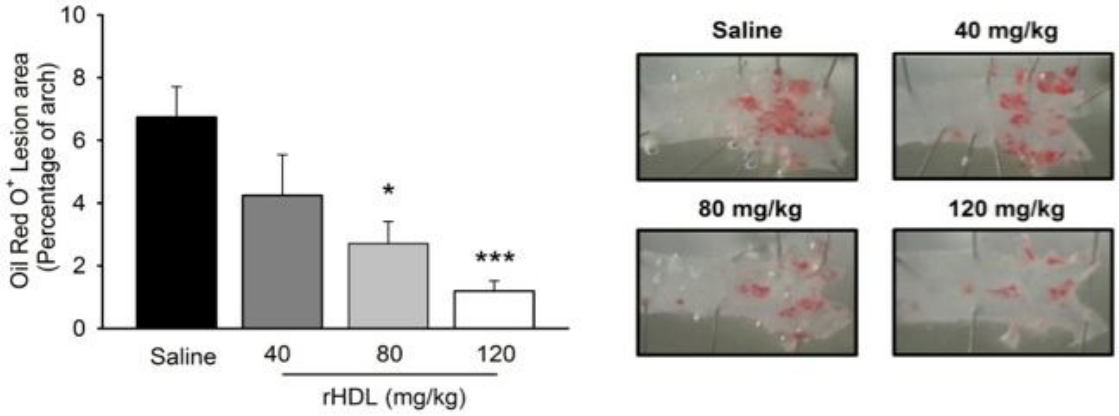

Figure II. *Apoe*$^{-/-}$ mice fed a WTD for 4 weeks were infused with rHDL at 40, 80 or 120 mg/kg weekly for two weeks. 7 days post the second infusion the percentage of the aortic arch stained with Oil-Red was quantified. *P<0.05, ***P<0.001 vs Saline, n=8, data presented as means ± SEM.

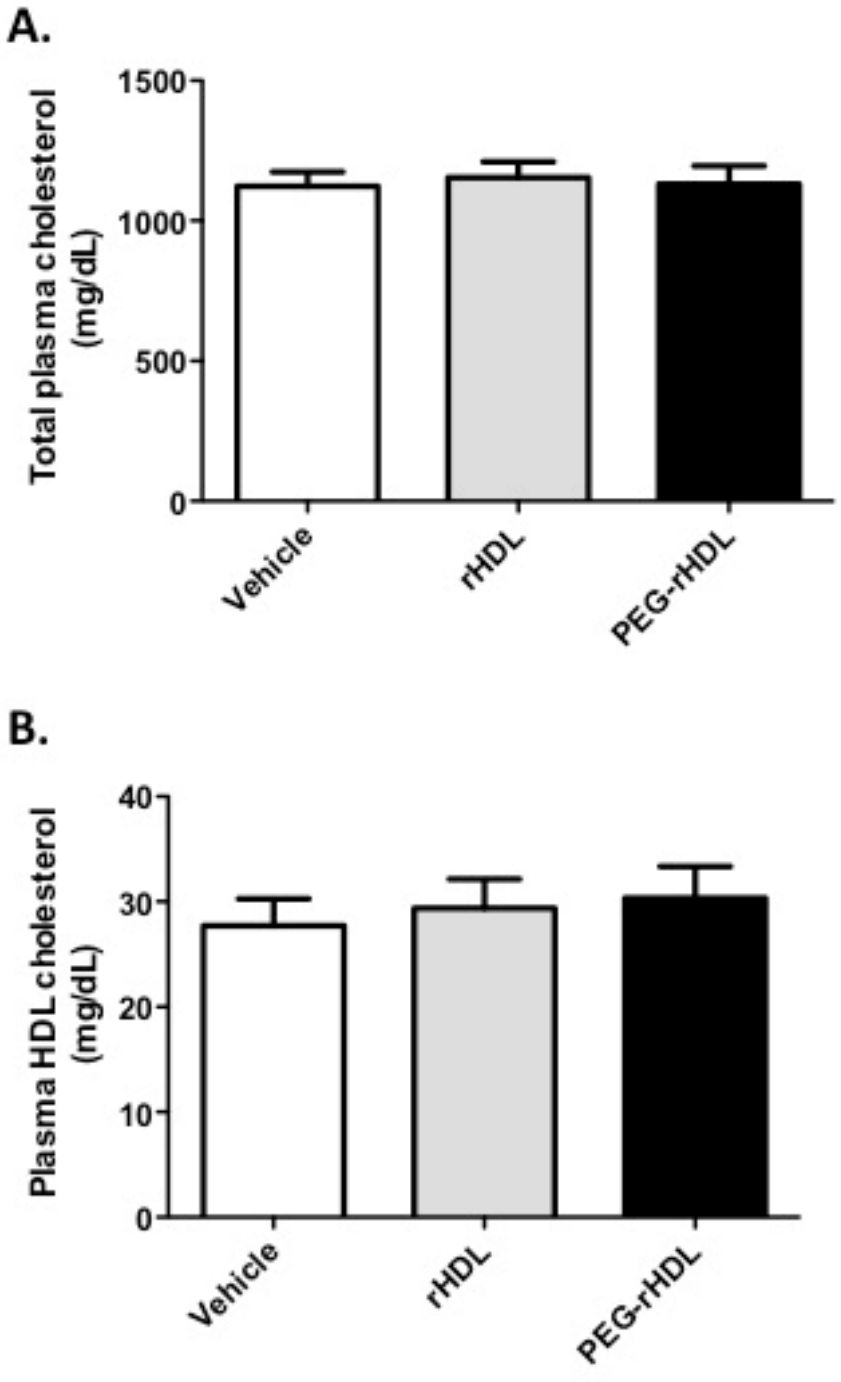

Figure III. *Apoe*$^{-/-}$ mice fed a WTD for 8 weeks were infused with rHDL or PEG-rHDL 40mg/kg weekly for two weeks. 7 days post the second infusion (A) Total plasma cholesterol and (B) HDL plasma cholesterol levels were analyzed. Data are presented as mean ± SEM, n=10/group.

References:

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