Serum, but not monocyte macrophage foam cells derived from low HDL-C subjects, displays reduced cholesterol efflux capacity

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Abstract **The main antiatherogenic function of HDL is to promote the efflux of cholesterol from peripheral cells and transport it to the liver for excretion in a process termed reverse cholesterol transport. The aim of this study was to evaluate the cholesterol efflux capacity in low- and high-HDL subjects by utilizing monocytes and serum from 18 low-HDL and 15 high-HDL subjects. Low and high HDL levels were defined, respectively, as HDL ≤10th and HDL ≥90th Finnish age/sex-specific percentile. Cholesterol efflux from [^{3}H]cholesterol-oleate-acetyl-LDL-loaded monocyte-derived macrophages to standard apolipoprotein A-I (apoA-I), HDL$_2$, and serum was measured. In addition, cholesterol efflux from acetyl-LDL-loaded human THP-1 macrophages to individual sera (0.5%) derived from the study subjects was evaluated. Cholesterol efflux to apoA-I, HDL$_2$, and serum from macrophage foam cells derived from low- and high-HDL subjects was similar. The relative *ABCA1* and *ABCG1* mRNA expression levels in unloaded macrophages, as well as their protein levels in loaded macrophage foam cells, were similar in the two study groups. Cholesterol efflux from THP-1 foam cells to serum recovered from high-HDL subjects was slightly higher than that to serum from low-HDL subjects ($P = 0.046$). Cholesterol efflux from THP-1 macrophages to serum from study subjects correlated with serum apoB ($P = 0.033$), apoA-I ($P = 0.004$), apoA-II ($P < 0.0001$), and the percentage of apoA-I present in the form of preβ-HDL ($P = 0.0001$). Our data reveal that macrophages isolated from either low- or high-HDL subjects display similar cholesterol efflux capacity to exogenous acceptors. However, sera from low-HDL subjects have poorer cholesterol acceptor ability as compared with sera from high-HDL subjects.**—Nakanishi, S., R. Vikstedt, S. Söderlund, M. Lee-Rueckert, A. Hiukka, C. Ehnholm, M. Muilu, J. Metso, J. Naukkarinen, L. Palotie, P. T. Kovanen, M. Jauhiainen, and M-R. Taskinen. **Serum, but not monocyte macrophage foam cells derived from low HDL-C subjects, displays reduced cholesterol efflux capacity.** ***J. Lipid Res.*** **2009.** 50: **183–192.**

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Epidemiological and observational studies have clearly demonstrated an inverse relationship between the risk of premature coronary heart disease (CHD) and the level of HLD-cholesterol (HDL-C) (1, 2). The increased CHD risk associated with low HDL-C is apparent at all concentrations of LDL-cholesterol (LDL-C) (3). The mechanism(s) underlying the protective role of HDL are still far from resolved. The best-established mechanism relates to the ability of HDL to promote efflux of cholesterol from macrophage foam cells that represent an early hallmark of atherosclerotic lesions. The effluxed cholesterol is then transported to the liver for excretion into bile and feces, a process known as reverse cholesterol transport (RCT) (4). Several processes promote cholesterol efflux from cells. One is cholesterol efflux to lipid-poor apolipoprotein A-I (apoA-I), a process mediated by ABCA1 (5, 6). Another involves the ABCG1 transporter with large spherical HDL particles as lipid acceptors (7, 8). ABCA1 and -G1 may

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work in concert and together provide an efficient defense mechanism against atherosclerosis (9, 10). A third efflux mechanism is provided by the scavenger receptor class B type I (SR-BI) (11). This receptor facilitates bidirectional flux of free cholesterol between cells and lipoproteins but may not contribute to macrophage RCT in vivo (12). The important role of the ABCA1 transporter in the regulation of plasma HDL levels was confirmed when a homozygous defect in ABCA1 was discovered to cause a virtual absence of HDL (13–15). Subsequently, several studies have aimed at clarifying the role of ABCA1 mutants in HDL metabolism and risk of CHD (16–20) but, overall, the results have not been conclusive. We recently reported (21) that in subjects with familial low HDL, a defective ABCA1 function in macrophages could be a potential contributor to impaired RCT.

Another study found efflux defects in subjects with low HDL, but *ABCA1* mutations were present in only a minority of the subjects, suggesting a contribution of additional pathways to HDL deficiency (22). These observations led us to investigate whether monocyte-derived macrophages from low- and high-HDL subjects would display different cholesterol efflux capacity to apoA-I, HDL_2, or serum. In addition, we assessed whether sera from low- and high-HDL subjects differ as cholesterol acceptors from human THP-1 macrophages. Relative mRNA expression levels of *ABCA1* and *ABCG1* were recorded to assess the possible contributions of the two efflux pathways. Because a previous study (23) reported a proinflammatory state in monocytes and monocyte-derived macrophages from low HDL-C subjects, we also measured mRNA expression of selected inflammatory genes in monocytes isolated from the study subjects.

SUBJECTS AND METHODS

Study subjects

We recruited 18 low-HDL-C subjects (10 men, 8 women) and 15 high-HDL-C subjects (6 men, 9 women) for this study. Low HDL-C was defined as ≤10th Finnish age/sex-specific percentile (24) (HDL-C ≤0.9 mmol/l in men and ≤1.1 mmol/l in women). High HDL-C was defined as ≥90th Finnish age/sex-specific percentile (24) (for men: HDL-C ≥1.7 mmol/l under 55 years, ≥1.6 mmol/l over 55 years; and for women HDL-C ≥2.0 mmol/l under 35 years and 50–55 years, and ≥1.9 mmol/l 35–50 years and over 60 years). To recruit the study subjects, the EUFAM (European Multicentre Study of Familial Dyslipidemias) database was used (25) to identify subjects with low HDL-C. The high-HDL-C group consisted of healthy spouses from families with familial dyslipidemia, and two healthy siblings of subjects with familial dyslipidemia. Study subjects came from different families, and were not related to each other. Both low-HDL-C and high-HDL-C subjects were required to have corresponding HDL-C levels measured in our research center on at least three occasions. The 18 low-HDL subjects included 10 subjects who had CHD, whereas all 15 high-HDL subjects were free of CHD. Exclusion criteria were estrogen therapy, diabetes mellitus, renal or hepatic disease, malignancy, alcohol abuse, thyroid disease, and age ≤20 years. Each participant filled in a standard questionnaire on medication, drinking, and smoking habits. Each study subject gave written informed consent before participating in the study. All samples were collected in accordance with the Helsinki Declaration, and the Ethics Committee of the Helsinki University Central Hospital approved the study design.

Biochemical analyses

Venous blood samples from the study subjects were drawn after an overnight fast. Serum and EDTA plasma were separated by centrifugation and stored at −80°C until analysis. Serum total cholesterol (TC) and triglycerides (TGs) were determined using an automated Cobas Mira analyzer (Hoffman-La Roche, Basel, Switzerland) by fully enzymatic methods (Hoffman-La Roche kits #0722138 and #0715166, respectively). Serum HDL-C was quantified by phosphotungstic acid-magnesium chloride precipitation procedures (Hoffman-La Roche kit #0720674). Serum LDL-C was calculated from the Friedewald formula [LDL cholesterol = TC−(HDL-C)−TG/2.2] (26). Concentrations of apoA-I, apoA-II, and apoB were measured by immunoturbidometric methods (for apoA-I, Wako Chemicals GmbH, Neuss, Germany; for apoA-II, Wako Chemicals GmbH and our own polyclonal antibody produced in rabbits against human apoA-II; and for apoB, Orion Diagnostica, Espoo, Finland). Plasma glucose concentration was analyzed by the glucose dehydrogenase method (Precision-G Blood Glucose Testing System; Medisense, Abbott, IL). The level of high-sensitivity C-reactive protein was determined using a commercial kit (Konelab kit #981798; Thermo Electron Corporation, Vantaa, Finland). PLTP activity was measured using a radiometric assay (27) with minor modifications (28). PLTP concentration was measured with ELISA (29). CETP activity was measured with radioisotope assay (30). Serum apoE concentration was quantitated by ELISA (31).

Analytical methods

HDL_2 and HDL_3 were separated and isolated by ultracentrifugation, and their composition was analyzed as described (32). HDL 2b, 2a, 3a, 3b, and 3c subspecies distribution and HDL mean particle size were determined by native gradient gel electrophoresis (33) with minor modifications. Briefly, electrophoresis was performed on the d ≤1.210 kg/l lipoprotein fraction isolated ultracentrifugally from plasma. We used the Hoefer miniVE vertical electrophoresis system (Amersham Biosciences, San Fransisco, CA) with native 4–22% polyacrylamide gradient gels (10 × 10.5 cm, PAA:BIS 19:1) prepared in the laboratory. Samples were applied in a volume of 5 μl containing four parts lipoprotein fraction and one part 40% sucrose. Samples were electrophoresed at 125 V for 24 h at 4°C in a running buffer (90 mM TRIS, 80 mM boric acid, and 3 mM EDTA, pH 8.53). Gels were stained for 1 h with 0.04% Coomassie blue G-250 and destained overnight with 5% acetic acid. Gels were densitometrically scanned with Kodak digital science 1D system (Eastman Kodak Co., Rochester, NY) and analyzed with ImageMasterTM 1D software (version 4.00, Amersham Pharmacia Biotech, Newcastle, UK).

We used a high-molecular-weight calibration kit (Pharmacia) for standardization. The molecular size intervals for HDL subspecies 2b, 2a, 3a, 3b, and 3c were used (33), and for each subspecies, the relative area under the densitometric scan is reported. Mean HDL particle size was calculated by multiplying the mean size of each HDL subclass by its relative area under the densitometric scan (34).

Quantification of preβ-HDL was performed by crossed immunoelectrophoresis (35, 36). For the analysis of plasma preβ-HDL formation capacity, plasma samples from each subject were preincubated at 37°C or 4°C for 16 h with the LCAT inhibitor iodoacetamide (2 mmol/l). The preβ-HDL area is expressed as a percentage of the sum of α-HDL and preβ-HDL areas. Preβ-HDL concentration is given as the absolute amount of apoA-I present in preβ-HDL particles (mg/dl serum).

Measurement of cholesterol efflux from cultured macrophage foam cells derived from the study subjects to lipid-free apoA-I, HDL_2, and serum

Human monocyte-derived macrophages were obtained from human whole blood by cell culturing. Fasting blood (70 ml) was drawn into tubes containing citrate as anticoagulant. Buffy coat was promptly separated by low-speed centrifugation (1,500 *g*) at room temperature. The recovered buffy coat was diluted up to 40 ml with PBS, layered over Ficoll-Paque, centrifuged (800 *g*, 30 min), and the mononuclear cells were recovered as a cell layer. To eliminate platelets from the mononuclear cells, the cells were washed three times with PBS. Finally, the cell pellet was suspended in DMEM. The mononuclear cells were plated onto 24-well plates (1.5 million cells per well), and the cells were allowed to attach for 1 h. After attachment, cells were washed three times with PBS and serum-free macrophage medium (GIBCO) with added granulocyte macrophage colony-stimulating factor (GM-CSF) (Biosite).

The medium was then changed every 2–3 days. After 7 days, when the monocytes had been converted to macrophages, they were loaded with radiolabeled cholesterol by incubation for 48 h with [^{3}H]cholesteryl oleate-acetyl-LDL (25 μg of protein/well). The method used for labeling the acetylated LDL (37) yielded preparations of [^{3}H]cholesteryl oleate bound to acetyl-LDL with specific activities ranging from 50–90 dpm/ng protein. Loading macrophages with labeled [^{3}H]cholesteryl oleate-acetyl-LDL typically increased the cellular content of [^{3}H]cholesteryl esters (38). We followed a standard procedure for cholesterol loading of macrophages with acetyl-LDL in which a dose-dependent response is found up to at least 50 μg/ml acetyl-LDL. Importantly, a time-dependent linear increase in macrophage cholesteryl esters was observed by incubating the cells with 25 μg/ml acetyl-LDL for up to 48 h. The majority of cholesterol was in the esterified form, and the cholesteryl ester mass in the foam cells after loading represented, on average, 76 ± 5.2% of the total cholesterol mass. Regarding reproducibility of the loading, the determination of cholesteryl ester contents in the lipid extracts from the generated foam cells yielded 116 ± 11.8 μg/mg cellular protein (mean ± SD; range 101–126 μg/mg cellular protein). Regarding the extent of cholesterol increase, we observed that compared with the nonloaded macrophages, cholesteryl ester mass in the foam cells had increased 30- to 34-fold. The specific activity of the cholesteryl esters at the start of the efflux phase was 710 ± 42 dpm/μg cholesteryl ester. The variation of the specific activity in the cholesteryl esters (calculated as SD, percentage of the mean value) was within the range of 4% to 8% among the experiments. Corresponding results derived from experiments using human monocytic THP-1 cells typically have lower variability, owing to the homogeneous responses of established cell lines (see below). The criteria we use for defining a macrophage is based on the morphology of the differentiated cells, as reported previously (39). Adherent monocytes differentiated in the presence of GM-CSF maintained the rounded shape typical of the monocyte precursors. We have also demonstrated that during differentiation, macrophages display high relative mRNA expression levels of CD 68.

To measure cholesterol efflux from the radiolabeled macrophage foam cells, three cholesterol acceptors were used: *i*) human lipid-free apoA-I (10 μg/ml medium; kindly provided by Dr. Peter Lerch of the Swiss Red Cross), *ii*) HDL_2 (25 μg/ml medium), and *iii*) serum (1%, v/v) from normolipidemic controls. The control serum we used was a serum pool from two normolipidemic donors, and the blood was withdrawn after an overnight fasting period. After incubation for 16 h at 37°C, the medium was collected and centrifuged at 2,500 rpm for 5 min to remove detached cells. Radioactivity in the medium was determined by liquid scintillation counting (Wallac WinSpectral 1414; Wallac, Turku, Finland). The cells were washed twice with PBS and lysed with 0.2 M NaOH. The cell lysates were analyzed for radioactivity and for total cell protein. The cholesterol efflux was calculated as disintegrations per minute in medium normalized to macrophage protein content (dpm in medium/μg cell protein). Efflux values to incubation medium in the absence of acceptors were subtracted from those in the presence of acceptors.

Expression of ABCA1 and ABCG1 protein

Expression of ABCA1 and ABCG1 protein in macrophages from low- and high-HDL subjects was determined after loading of macrophages with acetyl-LDL. After 7 days of culturing, the macrophages were loaded with acetyl-LDL (25 μg of protein/well) in DMEM supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin for 48 h. Macrophages were washed twice with cold PBS on ice. After washing, the cells were collected in PBS and centrifuged at 2,600 rpm for 15 min at 4°C. The supernatant was removed, and cells were lysed with buffer containing 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1% Triton X-100, 1% SDS, and complete EDTA-free protease inhibitor cocktail (Roche Diagnostics Gmbh, Mannheim, Germany). Before analysis, samples were sonicated twice for 15 s on ice to shear DNA and reduce sample viscosity, and centrifuged at 13,000 rpm for 5 min. The protein concentration of the samples was determined by the method of Lowry et al. (40) using BSA as a standard. For SDS-PAGE, 20 μg of protein from each sample was loaded onto 5% SDS-PAGE gels for analysis of ABCA1 and onto 12.5% SDS-PAGE gels for analysis of ABCG1. After electrophoresis, proteins were transferred to Hybond-C extra nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ), and polyclonal rabbit anti-ABCA1 (1/500 dilution; Novus Biologicals, Littleton, CO) and polyclonal rabbit anti-ABCG1 affinity-purified antibodies (1/1,000 dilution; Novus Biologicals) were used for detection of ABCA1 and ABCG1. HRP-conjugated goat anti-rabbit IgG (1/2,000 dilution; BioRad, Hercules, CA) and ECL (GE Healthcare, Buckinghamshire, UK) were used for visualization.

Expression analyses of *ABCA1*, *ABCG1*, *apoE*, *TNF-α*, *IL-6*, and *MCP-1* mRNA

Monocyte-derived macrophages from low- and high-HDL subjects were subjected to mRNA extraction before cholesterol loading. Macrophage mRNA was extracted and quantified as described (41). Quantitative PCR, using the SYBR-Green assay (Applied Biosystems), was used to measure the relative abundance of transcripts. Two-step RT-PCR was carried out using the Taq-Man Gold RT-PCR kit. To compare the relative *ABCA1* and *ABCG1* expression levels between monocyte-derived macrophages isolated from low- and high-HDL subjects, the relative mRNA expression in each sample was normalized against the expression of the housekeeping gene *GAPDH*, previously shown to be stably expressed in these cells (21).

In addition, *apoE* mRNA expression, as well as expression levels of selected inflammation markers *TNF-α*, *IL-6*, and *MCP-1*, were determined in unloaded monocyte-derived macrophages using a protocol similar to that given above.

Cholesterol efflux from THP-1 macrophage foam cells to sera from the study subjects

Human THP-1 monocytes were purchased from the American Type Culture Collection (Manassas, VA; catalog no. TIB-202). The monocytes were grown and maintained in complete RPMI 1640 medium containing 10% (v/v) FBS, 10 mM HEPES, pH 7.4, 100 U/ml penicillin, and 100 μg/ml streptomycin at

37°C under 5% CO_2 and 95% air, until the experimental treatments. To differentiate the monocytes into macrophages, the cells were plated onto 24-well plates and treated with 100 nM phorbol 12-myristate 13-acetate (Sigma-Aldrich, St. Louis, MO) in the growth medium for 72 h prior to the experiment. The macrophages were washed twice with PBS and loaded by incubation in the presence of [^{3}H]cholesteryl oleate-acetyl-LDL (25 μg of protein/well) in RPMI 1640 supplemented with 5% (v/v) lipoprotein deficient serum, 10 mM HEPES, pH 7.4, and penicillin/streptomycin for 48 h. After loading, the cells were washed twice with PBS, and to measure cholesterol efflux, the cholesterol-loaded THP-1 macrophages were incubated in serum-free RPMI 1640 supplemented with 10 mM HEPES, pH 7.4, and antibiotics, with the serum (0.5%) from the study subjects as a cholesterol acceptor. After incubation for 16 h at 37°C, the medium was collected and centrifuged at 2,500 rpm for 5 min to remove detached cells. Radioactivity in the medium and cells and the protein concentration of the cell lysates were analyzed, and cholesterol efflux was expressed as disintegrations per minute in medium/μg cell protein.

Statistical analysis

Statistical comparisons of clinical and biochemical parameters were performed with SAS v.8.02 (SAS Institute, Inc.). Results are expressed as means ± SD for continuous variables and as frequencies or percentages for categorical variables. Continuous variables with skewed distribution were $\log_{10}$-transformed before the analyses and were compared between groups by general linear model ANCOVA, whereas the values in text, tables, and figures are presented as nontransformed. $P < 0.05$ was considered significant (two-tailed). The frequency distribution of the categorical variables was compared between groups with the χ^2 test. The relationships of biochemical and clinical characteristics were examined by Pearson's correlation and Spearman correlation analysis, as appropriate.

RESULTS

Characteristics of the study subjects

Clinical and biochemical characteristics of the study subjects are presented in **Table 1**. The age distribution was similar in the two groups. As expected, HDL-C, TC, apoA-I, and apoA-II were significantly higher in the high-HDL subjects, whereas the low-HDL subjects had significantly higher levels of TG and a bigger waist circumference and body mass index than high-HDL subjects. LDL-C and apoB-100 were similar in the two groups.

The distributions of HDL_2 (d = 1.063–1.125 g/ml) and HDL_3 (1.125–1.21 g/ml) recovered by ultracentrifugation are presented in **Table 2**. In the low-HDL subjects, 32% of their HDL-associated cholesterol was recovered in the HDL_2 fraction, whereas in the high-HDL subjects, 56% of the total HDL cholesterol represented HDL_2-C. Basal level of preβ-HDL particles in the two study groups was similar (Table 2). However, upon incubation in the presence of the LCAT inhibitor iodoacetamide, sera from low-HDL subjects generated higher levels of preβ-HDL (as percentage of total HDL) as compared with the high-HDL group (low-HDL, 36.3% vs. high-HDL, 27.5%, $P = 0.028$).

To characterize the HDL subspecies distribution in low- and high-HDL subjects, we performed native gradient gel electrophoresis (Table 2). Evidently, in low-HDL subjects, the large-sized particles were significantly decreased, whereas in high-HDL subjects, the large HDL_{2b} particles represented the majority of HDL particles. Together, HDL_{2b} and HDL_{2a} represented approximately 70% of all HDL particles in high-HDL subjects. Further, low-HDL subjects had smaller HDL mean particle size than high-HDL subjects.

TABLE 1. Clinical and biochemical characteristics of the study subjects

	Low-HDL Subjects	High-HDL Subjects	*P*
N (men/women)	18 (10/8)	15 (6/9)	NS
Current smoking (%)	7 (38.9)	4 (26.7)	NS
Coronary heart disease (%)	10 (55.6)	0 (0)	0.0005
Age (years)	52.3 ± 13.2	56.6 ± 8.9	NS
Systolic blood pressure (mmHg)	138 ± 22	133 ± 20	NS
Diastolic blood pressure (mmHg)	79 ± 11	81 ± 9	NS
Waist (cm)	94.6 ± 11.4	83.2 ± 11.0	0.007
Body mass index (kg/m^2)	27.4 ± 4.5	23.7 ± 2.4	0.008
Total cholesterol (mmol/l)	4.35 ± 1.17	5.83 ± 0.94	0.0004
Triglycerides (mmol/l)	1.66 ± 1.04	1.01 ± 0.33	0.008
LDL cholesterol (mmol/l)	2.62 ± 0.97	3.09 ± 0.88	NS
HDL cholesterol (mmol/l)	0.97 ± 0.19	2.29 ± 0.36	<0.0001
ApoA-I (g/l)	1.21 ± 0.14	1.82 ± 0.16	<0.0001
ApoA-II (g/l)	0.32 ± 0.05	0.45 ± 0.10	<0.0001
ApoB (g/l)	0.97 ± 0.29	0.92 ± 0.24	NS
ApoE (μg/ml)	15.4 ± 11.4	12.6 ± 6.3	NS
Glucose (mmol/l)	5.63 ± 0.50	5.32 ± 0.73	NS
hsCRP (mg/l)	1.42 ± 1.20	1.57 ± 2.47	NS
PLTP activity (nmol/ml/h)	5,860 ± 1,335	6,313 ± 1,536	NS
PLTP mass (μg/ml)	5.82 ± 1.24	8.14 ± 1.70	<0.0001
LA-PLTP (μg/ml)	3.22 ± 1.16	5.20 ± 1.57	0.0002
HA-PLTP (μg/ml)	2.60 ± 0.42	2.94 ± 0.46	0.032
CETP activity (nmol/ml/h)	24.34 ± 6.89	25.19 ± 5.26	NS
LCAT activity (nmol/ml/h)	9.40 ± 2.18	7.62 ± 2.13	0.024

LA-PLTP, low-activity PLTP; HA-PLTP, high-activity PLTP: hsCRP, high-sensitivity C-reactive protein; NS, nonsignificant. Data are expressed as means ± SD or median. The two values from the top are evaluated using χ2 tests.

TABLE 2. Protein/lipid composition of HDL particles of the study subjects

	Low-HDL Subjects	High-HDL Subjects	*P*
Preβ-HDL before incubation (%)	5.0 ± 1.7	5.0 ± 2.3	0.951
Preβ-HDL after incubation (%)	34.1 ± 6.3	28.8 ± 6.6	0.028
Preβ-HDL mass before incubation (mg/dl)	6.1 ± 2.6	9.3 ± 4.4	0.017
Preβ-HDL mass after incubation (mg/dl)	40.8 ± 8.6	52.4 ± 12.4	0.004
HDL_2-C (mmol/l)	0.35 ± 0.14	1.23 ± 0.44	<0.0001
HDL_2 particle mass (mg/dl)	82 ± 22	251 ± 72	<0.0001
HDL_3-C (mmol/l)	0.62 ± 0.11	0.93 ± 0.18	<0.0001
HDL_3 particle mass (mg/dl)	212 ± 32	276 ± 41	<0.0001
HDL_2-C/HDL_3-C	0.57 ± 0.05	1.34 ± 0.15	<0.0001
HDL_{2b} (%)	17.2 ± 5.1	38.8 ± 12.7	<0.0001
HDL_{2a} (%)	32.6 ± 6.7	31.9 ± 5.9	0.757
HDL_{3a} (%)	31.7 ± 4.6	19.9 ± 6.0	<0.0001
HDL_{3b} (%)	15.0 ± 5.4	7.0 ± 2.7	<0.0001
HDL_{3c} (%)	3.5 ± 1.4	2.4 ± 1.4	0.037
HDL particle size (nm)	9.11 ± 0.21	9.76 ± 0.34	<0.0001

Data are expressed as means ± SD.

The analysis of percent mass composition revealed both HDL_2 and HDL_3 displaying significant enrichment of TG in the low-HDL subjects (**Fig. 1**).

Cholesterol efflux from monocyte-derived macrophage foam cells isolated from low- and high-HDL subjects

To study whether macrophage foam cells derived from low- or high-HDL subjects display differences in cholesterol efflux to apoA-I (10 μg/well), HDL_2 (25 μg/well), and 1% (v/v) control serum, we incubated macrophage foam cells in the presence of these acceptors (**Fig. 2**). Cholesterol efflux was similar from macrophages obtained from low- (n = 16) or high- (n = 13) HDL subjects to apoA-I (64 ± 24 vs. 57 ± 27 dpm/μg cell protein, *P* = 0.422), to HDL_2 (88 ± 23 vs. 101 ± 51 dpm/μg cell protein, *P* = 0.755), and serum (140 ± 37 vs. 143 ± 65 dpm/μg cell protein, *P* = 0.767).

Expression of *ABCA1* and *ABCG1* mRNA and protein

The transcripts of *ABCA1* and *ABCG1* were analyzed in cultured unloaded monocyte-derived macrophages. Sufficient amounts of mRNA for the analysis of *ABCA1* were available from 14 low- and 13 high-HDL subjects and for the analysis of *ABCG1* from 13 low- and 13 high-HDL subjects. The relative *ABCA1* and *ABCG1* mRNA expression levels in monocyte-macrophages did not differ between low- and high-HDL subjects (the relative expression of *ABCA1* ± SD, low-HDL subjects, 1.93 ± 1.86, n = 14 vs. high-HDL subjects, 1.25 ± 1.03, n = 13, *P* = 0.253; the relative expression of *ABCG1* ± SD, low-HDL subjects, 1.91 ± 1.39, n = 13 vs. high-HDL subjects, 1.30 ± 0.98, n = 13, *P* = 0.213). In addition, expression of ABCA1 and ABCG1 protein in macrophage foam cells did not differ between low- and high-HDL subjects as measured by Western blot analysis (ABCA1, low-HDL subjects, 76,924 ±

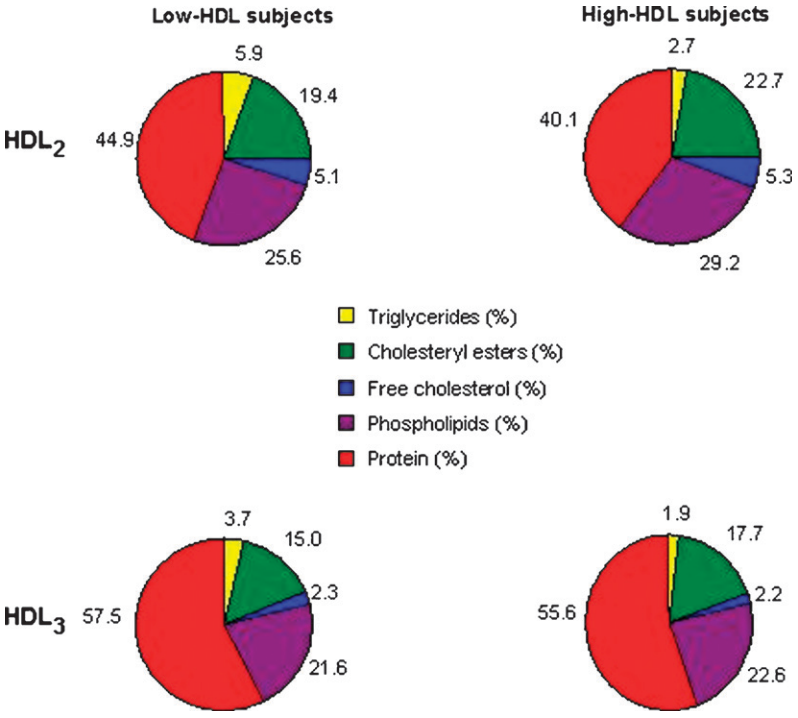

Fig. 1. The mass composition of the HDL_2 and HDL_3 particles of low- and high-HDL subjects.

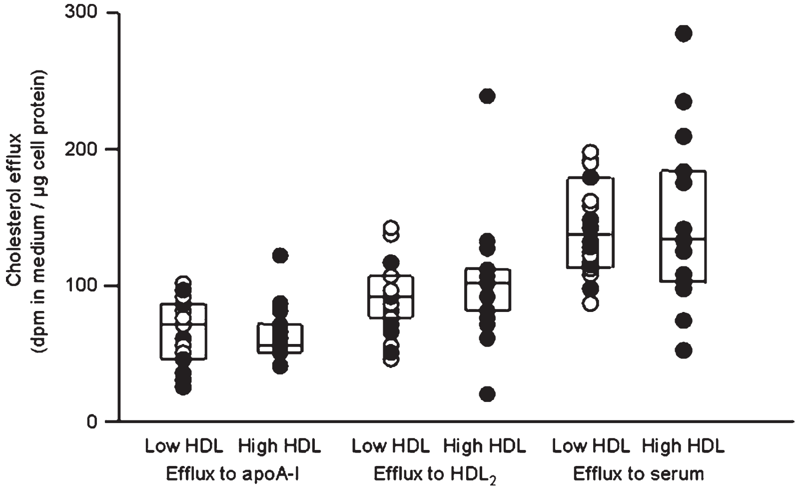

Fig. 2. Cholesterol efflux from monocyte-macrophages derived from low- and high-HDL subjects. Cellular cholesterol efflux to apolipoprotein A-I (apoA-I), HDL$_2$, and normolipidemic serum (1%) from macrophage foam cells normalized to macrophage protein content was analyzed. Box plots demonstrate the median and the lower and upper quartiles. The open and closed circles indicate the data from low-HDL subjects with and without coronary heart disease (CHD), respectively. Low-HDL subjects, n = 16; high-HDL subjects, n = 13.

46,993 pixels, n = 11 vs. high-HDL subjects, 84,340 ± 50,839 pixels, n = 4, low HDL/high HDL ratio 0.91, P = 0.795; ABCG1, low-HDL subjects, 82,087 ± 49,197 pixels, n = 14 vs. high-HDL subjects, 72,107 ± 22,835, n = 7, low HDL/high HDL ratio 1.14, P = 0.619). Representative ABCA1 and ABCG1 Western blots are shown in the inset in **Fig. 3**.

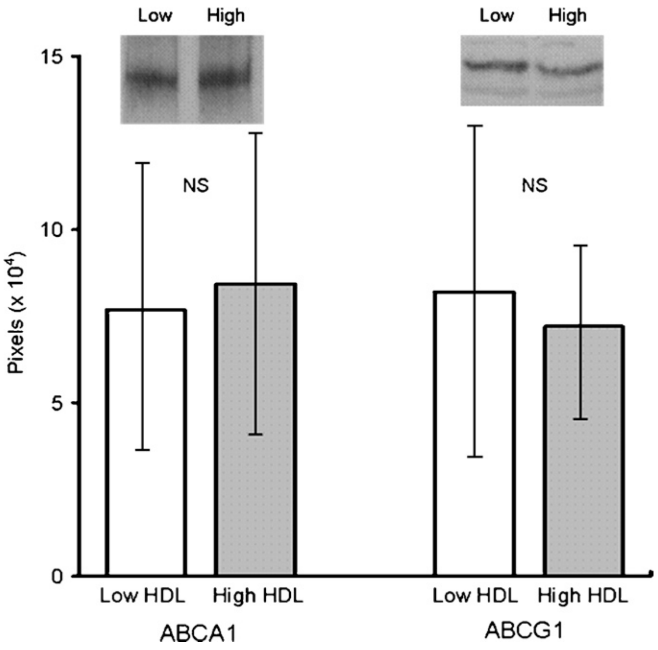

Fig. 3. Expression of ABCA1 and ABCG1 mRNA and protein. For mRNA expression analysis of ABCA1 and ABCG1, unloaded monocyte-derived macrophages from low- and high-HDL subjects were subjected to mRNA extraction. Quantitative RT-PCR was done to measure the relative abundance of transcripts, and the relative mRNA expression in each sample was normalized against the expression of the housekeeping gene GAPDH. Data are expressed as means ± SD. ABCA1, low-HDL subjects, n = 14 versus high-HDL subjects, n = 13; ABCG1, low-HDL subjects, n = 13 versus high-HDL subjects, n = 13. For expression analysis of ABCA1 and ABCG1 protein, macrophages from low- and high-HDL subjects were loaded with acetyl-LDL and lysed after loading, and expression of ABCA1 and ABCG1 protein was analyzed with Western blotting. ABCA1, low-HDL subjects, n = 11 versus high-HDL subjects, n = 4; ABCG1, low-HDL subjects, n = 14 versus high-HDL subjects, n = 7.

In addition, we evaluated inflammation status of the isolated macrophages by analyzing expression levels of the major inflammatory genes *TNF-α*, *IL-6*, and *MCP-1*. The expression levels of these inflammation markers were similar in low-HDL and high-HDL subjects. Further, we observed no significant difference in inflammation markers between low-HDL subjects with or without CHD (data not shown).

Cholesterol efflux from THP-1 macrophage foam cells to sera from low- and high-HDL subjects

To investigate the ability of sera from low- and high-HDL subjects to function as cholesterol acceptor, we used

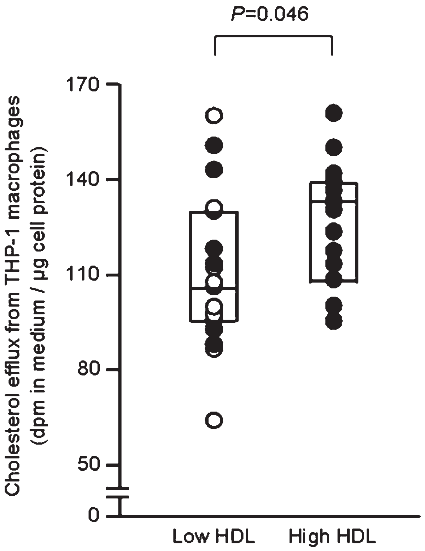

Fig. 4. Cholesterol efflux from human THP-1 macrophages to serum from low- and high-HDL subjects. THP-1 macrophages were first loaded with radiolabeled acetyl-LDL. Cholesterol efflux to serum (0.5%) was measured and normalized to THP-1 macrophage foam cell protein. Box plots display the median and the lower and upper quartiles. The open and closed circles indicate the data from low-HDL subjects with and without CHD, respectively. Low-HDL subjects, n = 18; high-HDL subjects, n = 15.

cholesterol-loaded human THP-1 macrophage foam cells (**Fig. 4**). Cholesterol efflux from THP-1 foam cells to 0.5% (v/v) serum from high-HDL subjects (n = 15) was slightly higher than that to 0.5% (v/v) sera from low-HDL subjects (n = 18) (127 ± 19 vs. 111 ± 24 dpm/µg cell protein, $P = 0.046$). In addition, cholesterol efflux from THP-1 foam cells to individual sera showed a positive association with serum apoB ($r = 0.373$, $P = 0.033$), apoA-I ($r = 0.485$, $P = 0.004$), and apoA-II ($r = 0.654$, $P < 0.0001$) (**Fig. 5**), as well as HDL_3 phospholipids ($r = 0.564$, $P = 0.002$), HDL_3 cholesteryl ester ($r = 0.412$, $P = 0.030$), HDL_3 free cholesterol ($r = 0.517$, $P = 0.005$), HDL_3 particle mass ($r = 0.451$, $P = 0.016$), percentage of preβ-HDL ($r = 0.626$, $P = 0.0001$), preβ-HDL concentration ($r = 0.754$, $P < 0.0001$), preβ-HDL concentration after incubation at 37°C ($r = 0.486$, $P = 0.005$), and HDL-C ($r = 0.359$, $P = 0.040$).

We also analyzed serum CETP, LCAT, and PLTP activities as well as PLTP mass to gain insight into whether these important regulators of HDL metabolism differ between the low- and high-HDL subjects. CETP and PLTP activities were similar between the two groups. Interestingly, low-HDL subjects displayed significantly higher LCAT activity and lower PLTP mass (Table 1). Because PLTP in serum exists in low-activity (LA) and high-activity (HA) forms, we also analyzed their distribution. The HA-PLTP mass slightly differed between the two groups, whereas the high-HDL subjects had significantly higher levels of LA-PLTP. *ApoE* mRNA expression levels in unloaded monocyte-derived macrophages, as well as serum apoE concentration, were similar between low-HDL and high-HDL subjects.

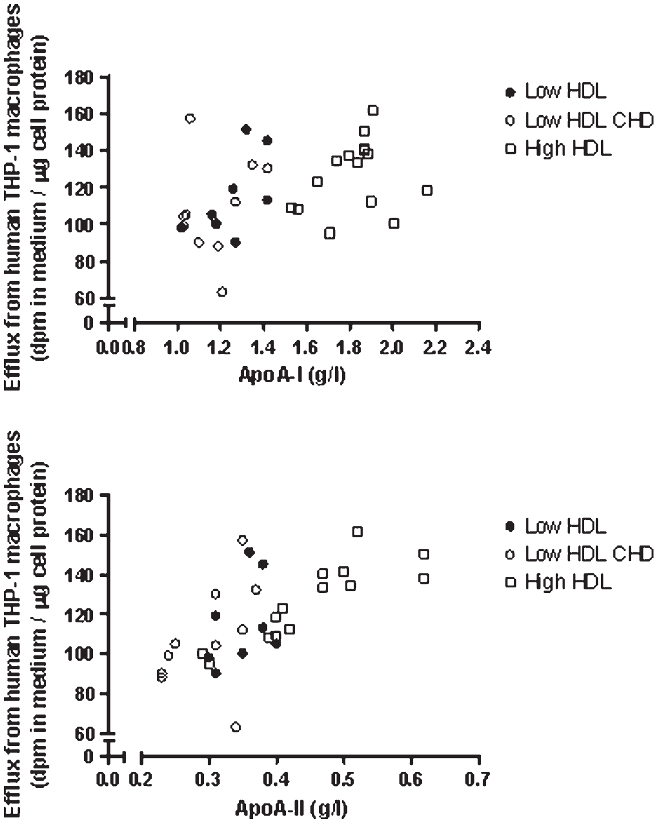

Fig. 5. Relationships between plasma concentrations of apoA-I and apoA-II and cholesterol efflux from THP-1 macrophage foam cells. The closed circle, open circle, and square indicate the data from low-HDL subjects without CHD, low-HDL subjects with CHD, and high-HDL subjects, respectively. In the low-HDL group, correlation coefficients between apoA-I and apoA-II and cholesterol efflux from human THP-1 macrophages were 0.448 ($P = 0.06$) and 0.420 ($P = 0.08$), respectively. In the high-HDL group, correlation coefficients between apoA-I and apoA-II and cholesterol efflux from human THP-1 macrophages were 0.290 ($P = 0.29$) and 0.883 ($P < 0.0001$), respectively. The slopes for apoA-I and apoA-II were not different between the low- and high-HDL groups ($P = 0.47$ and 0.69, respectively).

DISCUSSION

In this study, we investigated whether monocyte-macrophages from low- and high-HDL-C subjects display different potential to facilitate cholesterol efflux or whether the sera derived from these subjects differ as cholesterol acceptors. As major findings in the present study, we observed that cholesterol efflux to lipid-free apoA-I, HDL_2, and standard serum was similar from macrophage foam cells derived from low- and high-HDL subjects. However, cholesterol efflux from THP-1 macrophage foam cells to serum recovered from high-HDL subjects was higher than that to serum from low-HDL subjects.

The present data extend our previous findings that macrophages derived from low-HDL subjects expressed cholesterol efflux to lipid-free apoA-I similar to those isolated from the control subjects (21). In addition, we observed no signs of defective cholesterol efflux to HDL_2 or serum. These data imply that neither the ABCA1 pathway nor the ABCG1 pathway is significantly impaired. Importantly, there were no differences in cholesterol efflux levels in the low-HDL subjects with CHD (n = 10) compared with those without CHD (n = 8). Neither *ABCA1* or *ABCG1* mRNA expression nor their protein levels in macrophages displayed differences. These comprehensive data suggest only marginal, if any, differences in cholesterol efflux between the macrophages derived from low-HDL and high-HDL subjects. Regarding the differences in plasma HDL-C levels, this result is not unexpected, because several other mechanisms modify HDL levels and the contribution of macrophage-derived HDL-C is quantitatively only marginal (42). In accordance with our data, Kiss et al. (22), who studied the genetic etiology of 124 low-HDL subjects, found that a large number of low-HDL subjects had normal cholesterol efflux from isolated monocyte-macrophages to apoA-I and HDL. Although about 30% of low-HDL subjects exhibited cellular cholesterol efflux defects, the majority of these subjects did not harbor functional mutations in *ABCA1*. A recent observation that the activated proinflammatory state of the monocytes and macrophages in low-HDL subjects may contribute to the pathophysiological consequences of low HDL prompted us to test for this in our sample (23). In the present study, quantitative RT-PCR analysis for relative expression of selected inflammatory genes in unloaded macrophages revealed no difference in inflammatory status between low-HDL and high-HDL subjects or between low-HDL subjects with or without CHD. Although we did not observe any differences in the inflammatory status of unloaded macrophages derived from low-HDL and high-HDL subjects, we have to remember that these monocytes are differentiated into macrophages under cell culture conditions and may thus differ from those present in the vessel wall in vivo. The function of endothelial cells, as well as cells in the arterial intima, e.g., macrophages, is essential for the development of atherosclerotic lesions. Recently, Waldo et al. (39) reported that the monocytes differentiated in the presence of alternative macrophage development cytokines, GM-CSF, to produce granulocyte macrophage CSF macrophages (GM-Mac), or macrophage colony-stimulating factor (M-CSF) to produce M-Mac, differ in their gene expression patterns and thus, in their potential for promoting atherosclerosis. They also demonstrated that M-CSF upregulates in addition to CD 68 also CD 14 mRNA expression, whereas GM-CSF does not affect the expression of CD 14. Also, immunostained human coronary arteries showed that macrophages with antigen expression similar to that of M-Mac [CD68(+)/CD14(+)] were predominant within atherosclerotic lesions, whereas macrophages with antigen expression similar to that of GM-Mac [CD68(+)/CD14(−)] were predominant in areas devoid of disease. In our present study, we have used GM-CSF for the differentiation of monocytes, whereas the method used by Kiss et al. (22) was not reported, and this could be one factor in the discrepancy in cholesterol efflux between low-HDL subjects. Further studies with a higher number of study subjects are needed to further characterize the effects of differentiation on the function of macrophages in cholesterol efflux. Another important aspect that could possibly explain these differences is the fact that the study population of our previous investigation consisted of affected family members from carefully characterized Finnish low-HDL pedigrees, whereas the target population of the present study consisted of subjects from the EUFAM database (low-HDL subjects) and their healthy spouses or healthy siblings (high-HDL subjects).

In addition to macrophage function, several studies have evaluated the cholesterol efflux capacity to individual serum samples (4). Cholesterol efflux from THP-1 macrophages to the individual sera showed a positive association with serum apoB, apoA-I, apoA-II, relative and absolute amounts of preβ-HDL, and HDL-C. Notably, ABCG1 mediates cholesterol efflux to mature HDL and, to a lesser extent, to apoB-containing lipoproteins (8). Our data are also in accordance with recent findings that ABCA1-dependent efflux is highly dependent on the availability of preβ-HDL (43–45). These results demonstrate that preβ-HDL may be the preferred acceptor for the concerted action of the ABCA1 and ABCG1 pathways, leading to high efflux capacity from THP-1 macrophage to serum with high preβ-HDL content. The amount of preβ-HDL was similar in serum samples from low-HDL and high-HDL subjects when expressed as a percentage from total, i.e., α-HDL and preβ-HDL together. However, the amount of serum preβ-HDL was higher for high-HDL subjects when expressed as milligrams per deciliter, and therefore this could promote higher cholesterol efflux to sera of high-HDL subjects. In addition, cholesterol efflux from THP-1 macrophages correlated with phospholipids and particle mass of HDL, consistent with earlier efflux studies demonstrating that the phospholipid content of HDL is an important determinant of cholesterol efflux (8, 9, 46). In accordance with previous studies (35, 47), our low-HDL subjects had smaller HDL mean particle size, a decreased proportion of large HDL particles, and reduced preβ-HDL concentration. The effect of HDL particle size on efflux capacity, and the impact of large HDL particles on cholesterol removal were recently demonstrated (45, 48, 49). As expected, high-HDL subjects demonstrated a higher proportion of large HDL_{2b} particles. The higher efflux capacity to the sera of these subjects suggests a central role of the ABCG1 pathway in cholesterol removal from macrophage foam cells. Notably, HDL_{2b} particles have been found to be the most important determinant of carotid atherosclerosis evaluated as increased intima-media thickness (47). Our results clearly demonstrate the importance of the distribution and composition of HDL subpopulations when evaluating serum cholesterol efflux capacity. In addition to ABC transporters, SR-BI and aqueous diffusion are connected to cholesterol efflux. However, based on recent results, the role of SR-BI in cholesterol removal from macrophages is rather small and ABCA1 and ABCG1 are the two major players in the efflux process from cholesterol-enriched macrophages (50). Another, nonspecific pathway for cholesterol egress, aqueous diffusion, also plays a role. However, a recent report by Adorni et al. (50) demonstrated that this pathway is functional in nonloaded macrophages and therefore quantitatively is not relevant in our system with cholesterol-loaded cells.

LCAT, CETP, and PLTP are all involved in the remodeling of HDL particles (51) and affect RCT. Also, apoE is an important factor participating in HDL formation and maturation, in hepatic uptake of HDL (52), and in RCT (49). In addition, macrophage apoE promotes cholesterol efflux and reduces atherosclerosis (53). The difference in cholesterol efflux between sera from the two extreme groups could not be explained by alterations in the serum levels of PLTP and CETP. Neither did the apoE level differ between the low- and high-HDL subjects. PLTP mass was significantly increased in the high-HDL group because of the increase of the LA-PLTP form. Phospholipid transfer activity of PLTP has been associated with various pathophysiological conditions; however, little information is available concerning the relationship between PLTP mass and disease. Interestingly, an inverse relationship between serum total PLTP concentration and CHD risk was recently reported (54). However, the physiological role of LA-PLTP remains unknown.

There are certain limitations to the present study. First, the limited number of low-HDL and high-HDL subjects available for the cholesterol efflux experiments attenuates the statistical power. Second, macrophages differentiated from isolated circulating monocytes may not accurately represent those cells located in the subendothelial space, the actual site of cholesterol efflux. Third, although our data suggest higher cholesterol efflux to serum from high-HDL subjects, it would have been informative to analyze cholesterol efflux to distinct HDL subclasses isolated from the study subjects.

In conclusion, monocyte-macrophages isolated from either low- or high-HDL subjects did not differ in their ability to facilitate cholesterol efflux to exogenous acceptors, apoA-I, HDL_2, and whole serum. However, serum from high-HDL subjects promoted higher cholesterol efflux from THP-1 macrophage foam cells than serum from low-HDL subjects, most probably due to the higher proportion of both HDL_{2b} and preβ-HDL particles. The low levels of HDL_{2b} and preβ-HDL particles may critically limit the efflux capacity and thereby promote atherosclerosis in low-HDL subjects. Currently, it is very important to realize that HDL level measured as total HDL-C level is not a valid parameter to measure the antiatherogenicity of serum, because lower HDL-C values need not impart excess coronary disease and, vice versa, higher HDL-C levels may not always confer a protective benefit. Our study demonstrates that the determination of the distribution of HDL subclasses is essential when evaluating antiatherogenic properties of serum and its ability to function as a cholesterol acceptor. Physiologically, this is an important issue and has to be considered when HDL levels are modified pharmaceutically.

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