

# Endogenous apolipoprotein A-I stabilizes ATP-binding cassette transporter A1 and modulates Toll-like receptor 4 signaling in human macrophages

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**ABSTRACT** Apolipoprotein A-I (ApoA-I) is the main functional protein component of human high-density lipoproteins. ApoA-I shows various anti-inflammatory and atheroprotective properties toward macrophages; however, endogenous apoA-I expression has not been investigated in macrophages. We have shown that endogenous apoA-I gene is expressed in human macrophages at both mRNA and protein levels. Endogenous ApoA-I is localized in intracellular vesicles and at the external side of the plasma membrane in association with ATP-binding cassette transporter A1 (ABCA1) and lipid rafts in macrophages. We have shown that endogenous ApoA-I stabilizes ABCA1, moreover, down-regulation of ApoA-I by siRNA results in an increase of Toll-like receptor 4 (TLR4) mRNA and membrane surface protein expression, as well as an enhancement of bacterial lipopolysaccharide (LPS)-induced expression of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), interleukin 1 $\beta$  (IL-1 $\beta$ ), and inducible nitric oxide synthase (NOS2) genes in human macrophages. TNF- $\alpha$  stimulates ApoA-I expression and secretion ( $1.2 \pm 0.2$  vs.  $4.3 \pm 0.9$  ng/mg total protein) in macrophages. Obtained results suggest that endogenous ApoA-I has anti-inflammatory properties, presumably due to ABCA1 stabilization in macrophages; these results elucidate the cell type-specific mechanism of the TNF- $\alpha$ -mediated regulation of apoA-I gene expression in monocytes and macrophages.—Mogilenko, D. A., Orlov, S. V., Trulioff, A. S., Ivanov, A. V., Nagumanov, V. K., Kudriavtsev, I. V., Shavva, V. S., Tanyanskiy, D. A., Perevozchikov,

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APOLIPOPROTEIN A-I (ApoA-I) is the main structural and functional component of human high-density lipoproteins (HDLs; ref. 1). A high level of HDL-associated ApoA-I in plasma protects blood vessels against atherosclerotic lesion formation (2). However, the mechanism of antiatherogenic action of ApoA-I is not yet well understood. The dominant hypothesis suggests that ApoA-I plays a key role in HDL-mediated reverse cholesterol (CS) transport, including CS efflux from macrophages to HDLs with subsequent transport to liver (3). Other data suggest that ApoA-I can realize antiatherogenic properties through its anti-inflammatory (4, 5), antioxidant (6), and antithrombotic (7) activities. Synthesis of ApoA-I protein generally occurs in the liver (hepatocytes) and small intestine (enterocytes), which secrete ApoA-I as a part of HDL or very low-density lipoproteins (VLDLs; by liver) and chylomicrons (by intestine) (8).

To date, there are controversial data about synthesis of Apo A-I protein in human myeloid cells. Early studies did not find any apoA-I mRNA in human macrophages by Northern assay (9). Other groups of researchers have shown the apoA-I mRNA in human peripheral blood mononuclear (PBM) cells by using RT-PCR (10, 11). Moreover, there are the apoA-I-specific clones in expressed sequence tag (EST) libraries derived from human monocytes and macrophages.

Abbreviations: ABCA1, ATP-binding cassette transporter A1; ApoA-I, apolipoprotein A-I; CS, cholesterol; DAPI, 4,6-diamidino-2-phenylindole; ELISA, enzyme-linked immunosorbent assay; EST, expressed sequence tag; HDL, high-density lipoprotein; LPS, lipopolysaccharide; LSCM, laser scanning confocal microscopy; M $\beta$ CD, methyl- $\beta$ -cyclodextrin; PBM, peripheral blood mononuclear; PMA, phorbol 12-myristate 13-acetate; TLR4, Toll-like receptor 4; TNF- $\alpha$ , tumor necrosis factor- $\alpha$ ; VLDL, very low density lipoprotein.

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Using mouse models of atherosclerosis, Fazio and colleagues (12–14) showed that the expression of exogenous human apoA-I in mouse macrophages decreases the level of atherosclerotic lesions of aorta and increases CS efflux from macrophages but has no influence on the levels of ApoA-I and HDL-associated CS in serum (13). These observations suggest that the macrophage-specific expression of ApoA-I may be important for preventing of atherosclerotic lesions.

In the current work, we have shown endogenous apoA-I gene transcription and ApoA-I protein synthesis and secretion by human monocytes and macrophages. Our results suggest that macrophage-synthesized ApoA-I protein appears to stabilize ATP-binding cassette transporter A1 (ABCA1) in macrophages and show possible involvement of macrophage-synthesized ApoA-I in control of inflammation and atherosclerotic lesion formation. Endogenous ApoA-I seems to down-regulate Toll-like receptor 4 (TLR4) signaling pathway, thereby diminishing lipopolysaccharide (LPS)-induced activation of expression of several proinflammatory genes. In contrast to hepatic cells, proinflammatory cytokine tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) activates apoA-I gene transcription and ApoA-I protein secretion by human macrophages.

## MATERIALS AND METHODS

### Antibodies and recombinant proteins

Mouse monoclonal antibodies against human  $\beta$ -actin (cat. no. ab3280), against human TLR4 (ab30667), rabbit polyclonal antibodies against human ABCA1 (ab7360), against human PDI (ab3672), goat FITC-labeled secondary antibodies against mouse IgG (ab6669-1) and goat rhodamine-labeled secondary antibodies against rabbit IgG (ab7051-1) were purchased from Abcam (Cambridge, UK). Mouse monoclonal antibodies against human ApoA-I (0650-0050) were purchased from AbD Serotec (Oxford, UK). Goat polyclonal antibodies to human ApoA-I were described previously (15). Rabbit polyclonal antibodies against human apolipoprotein E were a kind gift from Dr. I. A. Oleinik (Institute of Experimental Medicine, Russian Academy of Medical Science, St. Petersburg, Russia). Antibodies against human CD14 (Pc5-labeled, IM0643), CD4 (PE-labeled, 6607101), CD45 (PE-labeled, IM1833), and CD11b (FITC-labeled, IM0530U) were purchased from Beckman Coulter (Brea, CA, USA). Antibodies against human CD68 (AF647 labeled, sc-20060) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Human recombinant TNF- $\alpha$  (T0157) was purchased from Sigma (St. Louis, MO, USA). Cholera toxin subunit B (recombinant), Alexa Fluor 647 conjugate was purchased from Invitrogen (Carlsbad, CA, USA).

### Cell cultures

Human acute monocytic leukemia cell line THP-1, human hepatoma cell line HepG2, and human normal skin fibroblast VH-10 cells were obtained from the Cell Culture Bank of the Institute of Cytology (Russian Academy of Sciences, St. Petersburg, Russia). THP-1 cells were cultivated in RPMI containing 10% FCS with 5% CO<sub>2</sub> at 37°C. VH-10 fibroblasts were

cultivated in DMEM containing 5% FCS with 5% CO<sub>2</sub> at 37°C. The differentiation of THP-1 cells into macrophages was performed by treatment of the cells with phorbol 12-myristate 13-acetate (PMA; 50 ng/ml, 24 h; Sigma). THP-1 cells were washed 24 h after PMA treatment and incubated for 48 or 144 h (cultivating medium was changed every 2 d).

### Human peripheral blood monocyte-derived macrophages

Human PBM cells were isolated by Percoll-Hypaque gradient centrifugation from healthy donor blood. Mature monocyte-derived macrophages were obtained after 3, 5, or 7 d of cultivation in RPMI containing 10% FCS with 5% CO<sub>2</sub> at 37°C. In some experiments, PBM cells have been treated by trypsin to remove residual plasma ApoA-I adherent on cell membranes. Cholesterol depletion from macrophage membranes was performed using methyl- $\beta$ -cyclodextrin (M $\beta$ CD; 10 mM, 1 h; Sigma). For TNF- $\alpha$  administration experiments, THP-1 cells or peripheral blood monocytes were seeded on 24-well plates at the density of  $5 \times 10^5$  cells/well in 1 ml of RPMI with 10% FCS. PBM-derived macrophages were cultivated for 3, 5, or 7 d. After 24 or 48 h of incubation with TNF- $\alpha$ , cells were washed 3 times with phosphate-buffered saline (PBS; pH 7.5), harvested, and used for RNA and protein isolation. The cultivation medium was used for Western blot analysis or ELISA experiments.

### Animals

Male C57BL/6 mice were purchased from Rapolovo (Russian Medicine Academy Nursery, St. Petersburg, Russia). Mice used in experiments reported in the present work were maintained and handled in accordance with institutional ethical committee guidelines, and these experiments were performed in concordance with the U.S. Institute of Laboratory Animal Research *Guide for the Care and Use of Laboratory Animals* (16). Mice were sacrificed by cervical dislocation, and resident peritoneal macrophages were harvested from male mice (6–8 wk old, weighing 18–22 g) without thioglycolate stimulation. The cells harvested by peritoneal lavage were plated in 24-well plates at the cell density of  $1 \times 10^6$  cells/well and incubated 3 h in RPMI medium supplemented with 10% FCS at 37°C, with 5% CO<sub>2</sub>. Cells were then washed 3 times with PBS and incubated overnight under the conditions stated above. The livers of sacrificed mice were used for RNA isolation.

### Reverse transcription

Total cellular RNA was isolated from cultivated cells by RNA STAT-60 reagent (Tel-Test, Gainesville, FL, USA) in accordance with manufacturer's guidelines. After digestion with RNase-free DNase I (Roche Applied Science, Minneapolis, MN, USA) the concentration of total RNA and RNA purity was determined using an Avaspec-2048 spectrophotometer (Avantes, Apeldoorn, The Netherlands). Ribosomal RNA band integrity was confirmed by electrophoresis in 1% agarose gel. RNA (2  $\mu$ g) was subjected to reverse transcription, using a dT-16 primer (Invitrogen) and M-MuLV reverse transcriptase (Promega, Madison, WI, USA) to generate first-strand cDNA.

### Real-time PCR

The primers and TaqMan probes for human GAPDH and apoA-I real-time PCR reactions were described previously

(17). The primers and TaqMan probes for ABCA1 were described by Mogilenko *et al.* (18).

The following sets of primers and TaqMan probes were designed using Primer3 software (<http://fokker.wi.mit.edu/primer3>): human  $\beta$ -actin (5-hACB: AGCCTTCCTTCCTGGGC; 3-hACB: CGGATGTCCACGTCACACT; h-hACB: C $\gamma$ 5-TGGAGTC-CTGTGGCATCCACGA-RTQ2); human apoE (hApoE-5: GGTCA-CATTCTCCTGGCAGGA; hApoE-3: GCGCAGGTAATCCCAAAAG; h-hApoE: R6G-GCCAGGCCAAGGTGGAGCAA-RTQ1); mouse  $\beta$ -actin (5-m- $\beta$ -actin: CTGGCACCACACCTTCTACA; 3-m- $\beta$ -actin: CTTTTACGGTTGGCCTTAG; h-m- $\beta$ -actin: ROX-GCACCTGT-GCTGCTCACCG); and mouse apoA-I (mApo1rt: TATGTGGA TGCGGTCAAAGA; mApo2rt: ACGGTTGAACCCAGAGTGTC; mApo-h: FAM-CCTCCTCCTTGGGCCAACAGCT-RTQ1).

The following sets of SYBR Green I primers were used: human TLR4 (5-TLR4: AGAAGTGCAGGTGCTGGATT; 3-TLR4: AA ACTCTGGATGGGGTTTCC); human TLR2 (5-TLR2: TCG-GAGTTCTCCCAGTGTTF; 3-TLR2: GAGCAATGGGCACAAT-GAG); human TLR3 (5-TLR3: AGCGGGTGTTTTTGAACATA; 5-TLR3: TCTTCGCAAACAGAGTGCAT), human TLR8 (5-TLR8: TGCTGCAAGTTACGGAATGA; 3-TLR8: CGCATAACTCACAG-GAACCA); human TNF- $\alpha$  (5-TNF- $\alpha$ : ATGAGCACTGAAAGCAT-GATCC; 3-TNF- $\alpha$ : GAGGGCTGATTAGAGAGAGGTC); human MCP-1 (5-MCP-1: CCCAGTCACCTGCTGTTAT; 3-MCP-1: AG-ATCTCCTTGGCCACAATG); human interleukin (IL)-6 (5-IL-6: AGGAGACTTGCTGGTAAA; 3-IL-6: CAGGGTGGTTATTG-CATCT); human CD14 (5-CD14: GGAAGACTTATCGACCA TGGA; 3-CD14: GCTGAGGTTCCGAGAAGTTG); human IL-15 (5-IL-15: CCCCAGGAAATCAAAGAT; 3-IL15: TGGCTCAA-CAAATCAACAG); human inducible nitric oxide synthase (NOS2; 5-NOS2: CTCTATGTTTGGGGGATGT; 3-NOS2: TTCTTCGC-CTCGTAAGGAAA); human CXCL10 (5-CXCL10: CCACGTGTT-GAGATCATTGC; 3-CXCL10: TTCTTGATGGCCTTCGATTG); human IFN- $\beta$ 1 (5-IFN- $\beta$ 1: CATTACCTGAAGGCCAAGGA; 3-IFN- $\beta$ 1: CAGCATCTGCTGGTTGAAGA); and human IL-1 $\beta$  (5-IL-1 $\beta$ : GGACAAGCTGAGGAAGATGC; 3-IL-1 $\beta$ : TCGTTATCCCATGT-GTCGAA).

Real-time PCR reactions were performed by using the CFX96 real-time PCR system (Bio-Rad, Hercules, CA, USA). All sets of TaqMan primers and probes were designed in a way so they are not able to amplify the genomic DNA templates (one of primers locates at the junction of two exons in the case of each pair of primers). The negative (no-reverse transcriptase), as well as the no-template control reactions were carried out to verify the absence of DNA template contamination and probe hybridization with genomic DNA for each real-time PCR reaction. For SYBR Green I PCR, the melting curves were run to prove the specificity of the amplicons. The relative abundance of mRNAs of tested genes was assessed by GAPDH and  $\beta$ -actin detection in the same reaction. The levels of mRNA of genes are presented as the results of GAPDH and  $\beta$ -actin normalization, as described previously (19). The number of cycles ( $C_t$  value) required to reach a threshold level of fluorescence that is  $\sim 10$  SD (of fluctuations in background fluorescence) above the mean background fluorescence was determined for each PCR reaction and primer set by using of the CFX96 real-time PCR system and automated software (Bio-Rad). The relative amount of mRNA (in percentage for the control sample) was calculated by the relation  $(2^{C_{tc} - C_{ts}}) \times 100$ , where  $C_{tc}$  is cycle threshold for the control, and  $C_{ts}$  is cycle threshold for the sample.

### ApoA-I/ABCA1 coimmunoprecipitation

For ApoA-I/ABCA1 immunoprecipitation experiments, membrane proteins were prepared as described previously (20). Coimmunoprecipitation was performed using rabbit polyclonal antibodies against human ABCA1 (ab7360; Ab-

cam) and mouse monoclonal antibodies against human ApoA-I (0650-0050; AbD Serotec), as described by Mogilenko *et al.* (18).

### ApoA-I knockdown in macrophages by siRNA

Small interfering RNA (siRNA) oligonucleotides targeted to human ApoA-I (sc-63361; Santa Cruz Biotechnology) and scrambled control RNA oligonucleotides (sc-37007; Santa Cruz Biotechnology) were used. PBM-derived macrophages were cultivated in DMEM containing 10% lipoprotein-deficient FCS (HyClone, Logan UT, USA) for 48 h and then were transfected by siRNAs using ExGen 500 reagent (Fermentas, Vilnius, Lithuania) for 72 h in accordance with manufacturer's guidelines.

### Immunostaining, FACS, and cell sorting

THP-1 cells or PBM-derived macrophages were fixed with 4% formalin for 10 min at 22°C, washed 3 times with PBS containing 0.1 M glycine, and incubated 40 min at 22°C with blocking buffer [PBS containing 1% BSA, 3% FCS, unspecific human IgG (1  $\mu$ g/ml), and 0.02% Tween-20]. In some experiments, cells were incubated with the addition of 0.1% Triton X-100 for cell membrane permeabilization. THP-1 cells or PBM-derived macrophages were treated with mouse monoclonal antibodies against human ApoA-I (1:250 dilution) or/and rabbit polyclonal antibodies against human ABCA1 (1:300 dilution) in PBS containing 1% BSA and 0.02% Tween-20 for 2 h at 22°C, washed 3 times with PBS, and incubated with secondary FITC-labeled goat polyclonal antibodies against mouse IgG (1:500 dilution) and/or Rhodamin-labeled goat polyclonal antibodies against rabbit IgG (1:500 dilution) in PBS containing 1% BSA and 0.02% Tween-20 for 1 h at 22°C. After that, cells were washed 3 times with PBS and fixed in PBS containing 1% formalin for FACS, or stained in Vectashield 4,6-diamidino-2-phenylindole (DAPI)-containing medium (Vector Laboratories, Burlingame, CA, USA) for laser scanning confocal microscopy (LSCM). THP-1 cells or PBM-derived macrophages were treated with secondary antibodies but not incubated with anti-ApoA-I and/or anti-ABCA1 antibodies were used as a control of immunostaining specificity. THP-1-derived monocytes and macrophages (not permeabilized) were stained with anti-ApoA-I or anti-Actin (specificity control) antibodies with further staining with secondary FITC-labeled antibodies and propidium iodide to prove that ApoA-I is localized at the outer surface of cell membrane. LSCM analysis was performed using Leica TCS SPE LCSM (Leica Microsystems, Wetzlar, Germany) and Carl Zeiss LSM710 (Carl Zeiss, Oberkochen, Germany). FACS analysis and cell sorting were carried out using Epics Altra flow cytometer (Beckman Coulter) and Expo32 software (Beckman Coulter).

### Western blot analysis

Cells were lysed in RIPA-50 buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA, 0.1% SDS, 0.01% Na $_3$ S, and 1 mM PMSF, pH 7.4). For concentration of ApoA-I in the culture medium, acetone precipitation of proteins was used. Proteins were separated by 10% SDS-PAGE, transferred to nitrocellulose membrane (Hybond-C, 0.45- $\mu$ m pore size; Amersham Biosciences, Piscataway, NJ, USA) and immunoblotted with antibodies. Immunoreactive bands were quantified using enhanced chemiluminescence system of detection.

## Enzyme-linked immunosorbent assay (ELISA)

Human ApoA-I in the culture medium and cell lysate were detected by the sandwich ELISA with mouse monoclonal antibodies against human ApoA-I and goat polyclonal antibodies conjugated with horseradish peroxidase. Diaminobenzidine was used as a chromogenic substrate.

## CS efflux

The PBM-derived macrophages (d 5 of differentiation) were loaded by  $^{14}\text{C}$  CS (2  $\mu\text{Ci}/\text{ml}$ ) dissolved in FCS for 24 h. The medium was then replaced by fresh RPMI 1640 with 10% FCS (CS efflux to serum lipids), or RPMI 1640 with delipidated FCS and human HDLs (50  $\mu\text{g}/\text{ml}$ ) (CS efflux to HDLs). After 24 h the medium was collected, and cells were washed 4 times with PBS. The medium was centrifuged at 3000 g for 5 min to remove cell debris. Then, 20  $\mu\text{l}$  of the supernatant (1/5 of total volume) was mixed with scintillation liquid and counted for radioactivity. The cells were lysed in 0.1 ml of 0.5 N NaOH overnight with constant shaking. Further, 40  $\mu\text{l}$  of cell lysates were mixed with scintillation liquid and counted for radioactivity. The CS efflux was expressed as a percentage of radioactivity in the medium relative to the total radioactivity (cells plus medium).

## Statistical analysis

Results are presented as means  $\pm$  SE. Statistical analyses of differences between groups were performed using an unpaired *t* test or Dunnett's criterion for multiple comparisons. Differences were considered statistically significant at values of  $P < 0.05$ . All statistical analyses were performed using Statistica 5.0 (StatSoft, Tulsa, OK, USA).

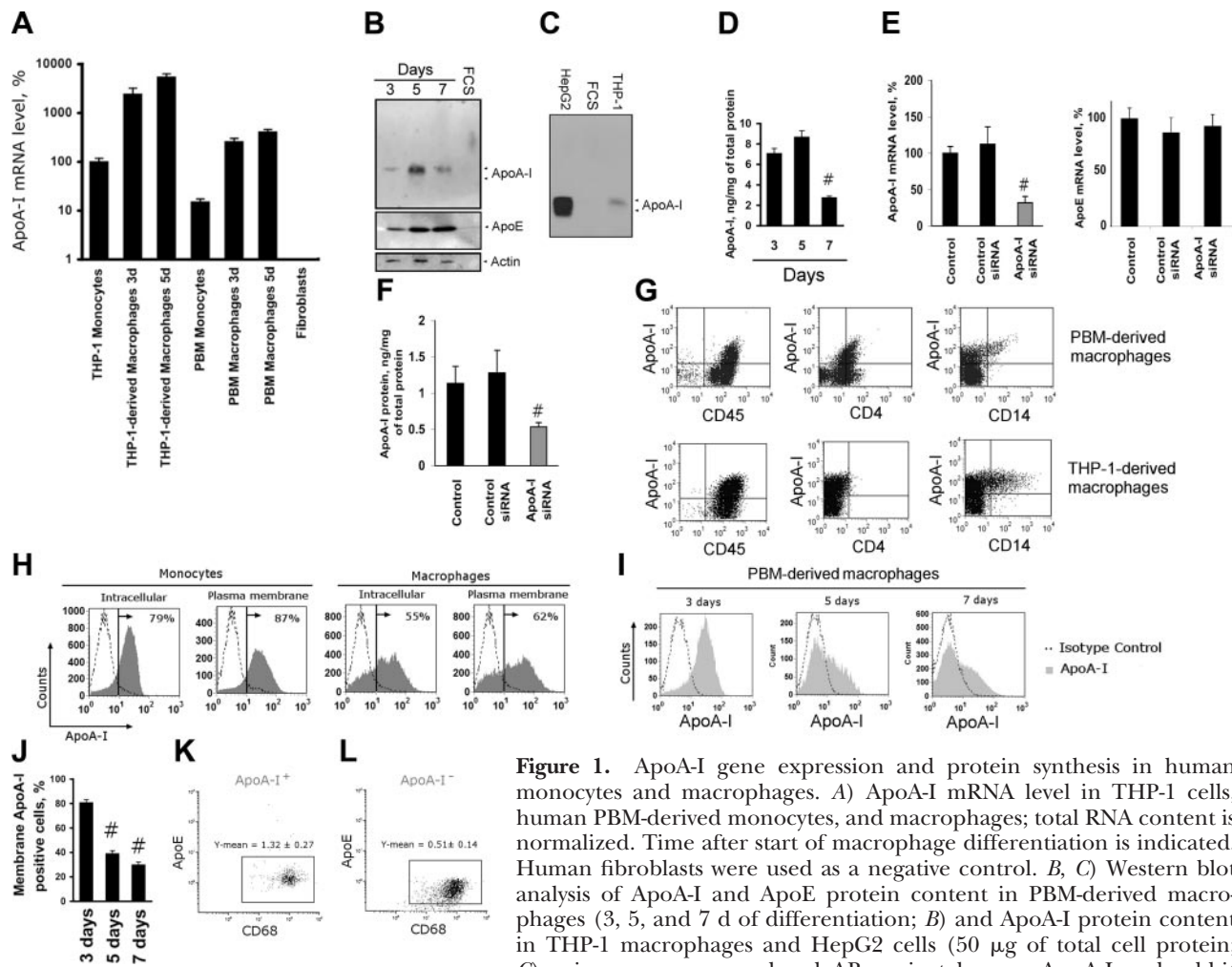
## RESULTS

### Human PBM-derived monocytes/macrophages and THP-1 cells express endogenous apoA-I gene

Computer analysis of apoA-I ESTs indicates that apoA-I gene expression may be realized not only in hepatocytes and enterocytes, but also in some other cell types, including monocyte-macrophage cells. For identification of endogenous apoA-I gene expression in human monocyte-macrophage cells, we have used real-time RT-PCR. The low levels of ApoA-I mRNA are detected in THP-1 monocytes and macrophages and human PBM-derived monocytes and macrophages, but no apoA-I mRNA has been found in normal human skin VH-10 fibroblasts (negative control; Fig. 1A). The specificity of the primers used for spliced apoA-I RNA was confirmed by cloning and sequencing the products of quantitative PCR. ApoA-I gene expression was up-regulated during monocyte-macrophage differentiation (Fig. 1A). The relative levels of apoA-I mRNA in human monocyte-macrophage cells were appreciably less than in human hepatoma cell line HepG2 (data not shown). We have found that mouse peritoneal macrophages also express endogenous apoA-I gene, and the ratio of mouse apoA-I mRNA content in macrophages *vs.* liver is comparable with that for human apoA-I mRNA in macrophages *vs.* HepG2 cells (data not shown). Culti-

vation of THP-1 cells or PBM-derived macrophages without human serum proteins allowed us to examine endogenous ApoA-I protein synthesis in the cells by using Western blot assay. To exclude a possible contamination of PBM-derived macrophages with plasma ApoA-I protein, PBM cells were thoroughly washed from plasma proteins after isolation. In some experiments, PBM cells were treated by trypsin to remove residual plasma ApoA-I adherent on cell membranes (data not shown). Newly synthesized ApoA-I was found in PBM- and THP-1-derived macrophages in experiments using mouse monoclonal AB against human ApoA-I (Fig. 1B, C), and those results have been confirmed by using goat polyclonal AB against human ApoA-I (data not shown). Figure 1B shows the dynamics of ApoA-I protein level during the differentiation of PBM monocytes to macrophages. The elevation of ApoA-I at d 5 of differentiation suggests that the signal corresponds to the newly synthesized ApoA-I protein, but not to residual plasma ApoA-I. Interestingly, the level of apolipoprotein E (ApoE), the apolipoprotein whose synthesis is known as positively regulated during macrophage differentiation, has maximum value at d 7, whereas the level of ApoA-I is decreased at that time point (Fig. 1B). To exclude the possible cross-reactivity of the AB against human ApoA-I with bovine ApoA-I from FCS used for cell cultivation, the Western assay was performed with FCS. There were no human ApoA-I specific signals in FCS (Fig. 1B, C). ELISA was used to quantitatively determine ApoA-I concentration in THP-1 macrophages (Fig. 1D). ApoA-I protein concentration in THP-1 macrophages was 6–8 ng/mg total cell proteins at d 3 to 5 of differentiation and  $\sim$ 3 ng/mg total proteins at d 7 of differentiation (Fig. 1D), while concentration of ApoA-I protein in human HepG2 hepatoma cells was  $420 \pm 50$  ng/mg total proteins (data not shown). These data suggest that the level of expression of apoA-I in THP-1-derived macrophages is relatively low (1–2% *vs.* HepG2 cells). For further corroboration of endogenous ApoA-I synthesis, we have used siRNA against human apoA-I. Treatment of PBM-derived macrophages with siRNA against apoA-I results in a decrease of both apoA-I mRNA and ApoA-I protein (Fig. 1E, F) but does not change the level of ApoE mRNA in transfected cells (Fig. 1E, negative control). Taken together, these results confirm the endogenous ApoA-I synthesis in human macrophages.

Differentiation of THP-1 monocytes to macrophages also leads to the development of two populations of macrophages: ApoA-I-rich and ApoA-I-poor cells, according to the levels of membrane-associated and intracellular ApoA-I protein as determined by FACS, whereas most THP-1 monocytes have a high level of membrane-associated and intracellular ApoA-I (Fig. 1G, H). FACS analysis was used to determine the macrophage phenotype of studied PBM- and THP-1-derived macrophages. The ApoA-I-rich cells have a  $\text{CD45}^+$ ,  $\text{CD4}^+$ ,  $\text{CD14}^+$  phenotype, which is typical for macrophages (Fig. 1G). As shown in Fig. 1I, the level of



**Figure 1.** ApoA-I gene expression and protein synthesis in human monocytes and macrophages. *A*) ApoA-I mRNA level in THP-1 cells, human PBM-derived monocytes, and macrophages; total RNA content is normalized. Time after start of macrophage differentiation is indicated. Human fibroblasts were used as a negative control. *B*, *C*) Western blot analysis of ApoA-I and ApoE protein content in PBM-derived macrophages (3, 5, and 7 d of differentiation; *B*) and ApoA-I protein content in THP-1 macrophages and HepG2 cells (50  $\mu$ g of total cell protein; *C*) using mouse monoclonal AB against human ApoA-I and rabbit

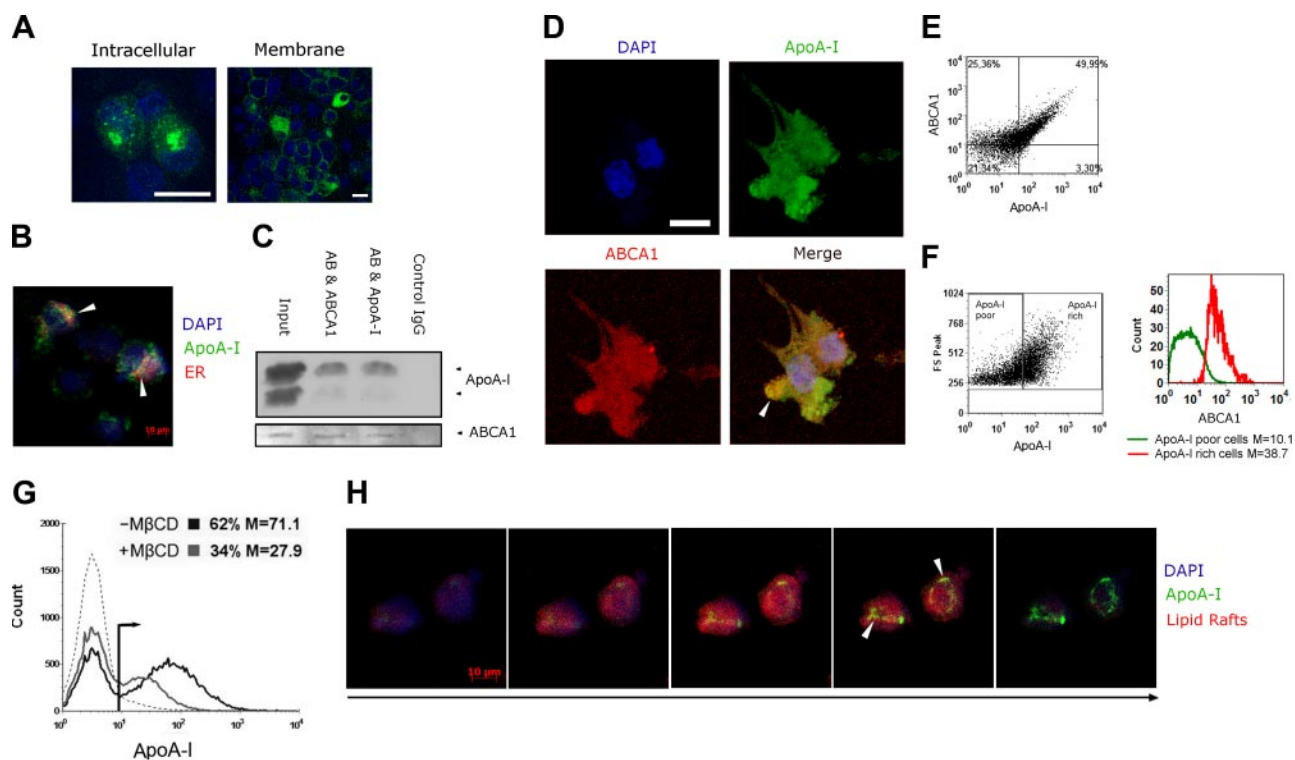
polyclonal AB against human ApoE. *D*) ELISA analysis of ApoA-I protein content in THP-1 macrophages (3, 5, and 7 d of differentiation). *E*, *F*) apoA-I and apoE mRNA levels (normalized; *E*) and intracellular ApoA-I protein level (*F*) in PBM-derived macrophages treated with ApoA-I siRNA or control siRNA at d 5 of differentiation. Values are presented as means  $\pm$  the SE of 4 independent experiments.  $^{\#}P < 0.05$ ; Dunnett's criterion. *G*) FACS analysis of membrane-associated endogenous ApoA-I in CD4<sup>+</sup>, CD45<sup>+</sup>, and CD14<sup>+</sup> PBM- and THP-1-derived macrophages. Cells were cultivated in the absence of human plasma proteins. *H*) Level of membrane-associated and intracellular ApoA-I protein in THP-1 monocytes and THP-1-derived macrophages (FACS; results show percentage of ApoA-I<sup>+</sup> cells). *I*, *J*) Level of membrane-associated ApoA-I protein and membrane-associated ApoA-I<sup>+</sup> cells during differentiation of PBM-derived macrophages (FACS). Values are presented as means  $\pm$  the SE of 3 independent experiments.  $^{\#}P < 0.05$ ; Dunnett's criterion. *K*, *L*) FACS analysis of membrane-associated ApoE in CD68<sup>+</sup> ApoA-I rich (ApoA-I<sup>+</sup>) and ApoA-I poor (ApoA-I<sup>-</sup>) PBM-derived macrophages.

membrane-associated ApoA-I is decreased during differentiation of PBM-derived macrophages, and the number of membrane-associated ApoA-I expressing macrophages (membrane ApoA-I rich cells) is also reduced, from 80% of the cells at d 3 to 1/3 of the total macrophage population at d 7 of differentiation (Fig. 1I, J). Interestingly, similar data have been published earlier for ApoE (21). FACS was used to check whether ApoA-I- and ApoE-rich PBM-derived macrophages (d 6 of differentiation) belong to one population (Fig. 1L, K). The level of membrane-associated ApoE was significantly higher in ApoA-I-rich cells than in ApoA-I-poor cells;  $10.5 \pm 2.7\%$  of CD68-positive cells (macrophages) expressed ApoE on their surface. There were  $7.7 \pm 1.3\%$  CD68<sup>+</sup>ApoA-I<sup>+</sup>ApoE<sup>+</sup> cells *vs.*  $2.8 \pm 0.7\%$  CD68<sup>+</sup>ApoE<sup>+</sup>ApoA-I<sup>-</sup> cells. Therefore, most ( $\sim 71\%$ )

of the ApoE-rich PBM-derived macrophages were ApoA-I rich. However, the population of ApoA-I-rich macrophages is wider than that of the ApoE-rich;  $26.1 \pm 2.3\%$  of CD68<sup>+</sup>ApoA-I<sup>+</sup> cells did not express significant amounts of ApoE on the surface membrane.

### Endogenous ApoA-I is involved in stabilization of ABCA1 protein in human macrophages

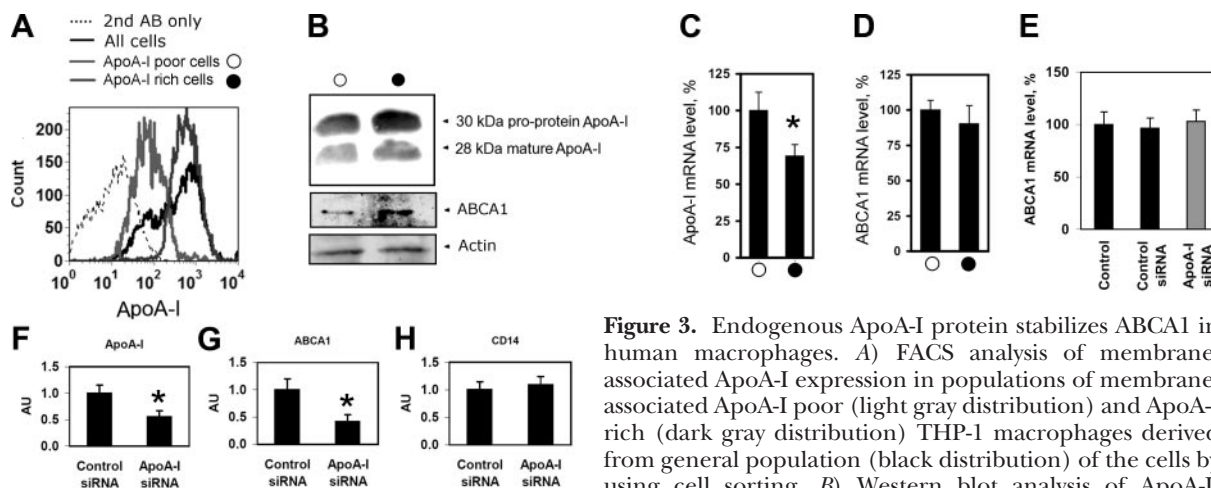
Indirect immunofluorescence/LSCM allows as to show the endogenous ApoA-I protein localization in intracellular vesicular compartments and at the outer surface of THP-1 macrophage (Fig. 2A) and monocyte (data not shown) membranes. The colocalization experiments with ER marker PDI confirm the localization of



**Figure 2.** Localization of ApoA-I protein in human macrophages and interactions of ApoA-I with ABCA1 and lipid rafts. *A*) Indirect immunofluorescence/LSCM analysis of ApoA-I protein localization (green) in THP-1 macrophages. Nuclei are stained with DAPI (blue). *B*) Colocalization of ApoA-I protein (green) with PDI protein (ER marker; red) indirect immunofluorescence/LSCM analysis. Nuclei are stained with DAPI (blue). Arrowheads indicate colocalization. *C*) Immunoprecipitation of membrane proteins of THP-1 macrophages using antibodies against human ApoA-I (AB & ApoA-I) or ABCA1 (AB & ABCA1). *D*) Indirect immunofluorescence/LSCM analysis of ApoA-I (green) and ABCA1 (red) localization in THP-1 macrophages (d 3 of differentiation). Nuclei are stained with DAPI (blue). *E*) Dot plots display results of indirect immunofluorescence/FACS analysis of ApoA-I *vs.* ABCA1 expression in THP-1 macrophages. *F*) Distribution of ABCA1 expression in membrane-associated ApoA-I-rich and ApoA-I-poor THP-1 macrophages. M, median. Results are from 1 of 3 independent reproducible experiments. *G*) FACS analysis of membrane surface localization of ApoA-I in THP-1 macrophages after treatment with methyl- $\beta$ -cyclodextrin (M $\beta$ CD; 10 mM, 1 h); results show percentage of ApoA-I<sup>+</sup> cells. Arrow indicates ApoA-I-rich cells. *H*) Colocalization of surface ApoA-I protein (green) with lipid rafts stained by cholera toxin subunit B labeled by Alexa 647 (red). Arrow indicates direction of serial optical sections toward apical membrane. Arrowheads indicate colocalization of surface ApoA-I with rafts. Nuclei are stained with DAPI (blue). Scale bars = 10  $\mu$ m.

endogenous ApoA-I in ER of THP-1 macrophages (Fig. 2*B*). There are data that exogenous lipid-free ApoA-I directly interacts with cellular ATP-binding cassette transporter ABCA1 (22). To study the probable interaction between macrophage-synthesized ApoA-I and ABCA1, we have checked the association of those proteins from THP-1 macrophages using antibodies against human ApoA-I and ABCA1. It has been shown that endogenous ApoA-I is coimmunoprecipitated with ABCA1 and *vice versa* (Fig. 2*C*). Moreover, at least a part of endogenous ApoA-I is colocalized with ABCA1 at the cell membrane and in intracellular vesicular compartments of THP-1 macrophages, as has been shown by LSCM (Fig. 2*D*). There are previously published data demonstrating that ApoA-I can bind and stabilize ABCA1, resulting in increased cell membrane localization and decreased degradation of ABCA1 by thiol proteases (23, 24). To test the possibility of stabilization of ABCA1 mediated by binding of this protein with macrophage-synthesized ApoA-I, we performed immunostaining of THP-1 macrophages with antibodies

against ApoA-I and ABCA1 and analyzed the levels of the both proteins in the cell population using FACS. Approximately 50% of THP-1 macrophages were double-positive on both ApoA-I and ABCA1 proteins, and dot-plot analysis of ApoA-I *vs.* ABCA1 levels showed a positive linear correlation between these parameters in the double-positive cells (Fig. 2*E*). Analysis of the ABCA1 protein contents in ApoA-I-poor *vs.* ApoA-I-rich THP-1 macrophage populations demonstrated the increased level of ABCA1 protein expression in the ApoA-I rich macrophages (Fig. 2*F*). Similar results have been obtained with human PBM-derived macrophages (data not shown). There are two possibilities to explain these results: ApoA-I stabilizes ABCA1 protein, or both apoA-I and ABCA1 genes tend to be coordinately regulated in a similar manner in macrophages. To test the latter suggestion, we separated membrane-associated ApoA-I-rich and ApoA-I-poor THP-1 macrophages using live cell sorting, as shown in Fig. 3*A, B*. The level of ABCA1 protein was increased in ApoA-I-rich macrophages (Fig. 3*B*). Interestingly, the level of ApoA-I



**Figure 3.** Endogenous ApoA-I protein stabilizes ABCA1 in human macrophages. *A*) FACS analysis of membrane-associated ApoA-I expression in populations of membrane-associated ApoA-I poor (light gray distribution) and ApoA-I rich (dark gray distribution) THP-1 macrophages derived from general population (black distribution) of the cells by using cell sorting. *B*) Western blot analysis of ApoA-I, ABCA1, and  $\beta$ -actin expression in populations of membrane-associated ApoA-I-rich (solid circle) and ApoA-I-poor (open circle) THP-1 macrophages. Data show results from 1 of 3 independent experiments. *C*, *D*) ApoA-I (*C*) and ABCA1 (*D*) gene expression in populations of membrane-associated ApoA-I-rich and ApoA-I-poor THP-1 macrophages. *E*) ABCA1 mRNA level in PBM-derived macrophages treated with ApoA-I siRNA or control siRNA at d 5 of differentiation, determined by real-time RT-PCR.  $y$ -Axis values correspond to relative level of gene expression (100% in control macrophages). Values are presented as means  $\pm$  SE of 3 independent experiments.  $*P < 0.05$ ; unpaired Student's *t* test. *F*–*H*) FACS analysis of ApoA-I (*F*), ABCA1 (*G*), or CD14 (*H*) protein content in PBM-derived macrophages treated with ApoA-I siRNA or control siRNA at d 5 of differentiation. Values are presented as arbitrary units ( $x$ -mean fluorescence of ApoA-I, ABCA1, or CD14 protein expression determined by FACS;  $n=3$ ).

mRNA was lower in ApoA-I-rich THP-1 macrophages than in ApoA-I poor ones (Fig. 3C), which suggest the post-transcriptional regulation of ApoA-I synthesis in human macrophages. There were no statistically significant differences between the levels of ABCA1 mRNA in ApoA-I-poor *vs.* ApoA-I-rich THP-1 macrophages (Fig. 3D). These results suggest that there is no significant correlation between apoA-I and ABCA1 gene expression, so the positive correlation between membrane-associated ApoA-I and ABCA1 protein levels could not be caused by coordinated regulation of these genes and might be explained by ApoA-I-mediated stabilization of ABCA1 in THP-1 macrophages.

To check the latter assumption, apoA-I-knockdown experiments were performed. Treatment of PBM-derived macrophages with siRNA against apoA-I resulted in significant inhibition of both apoA-I mRNA and protein content (Fig. 1E, F). At the same time, apoA-I siRNA had no influence on the level of ABCA1 mRNA (Fig. 3E). We performed FACS analysis of siRNA-treated CD45<sup>+</sup> PBM-derived macrophages and found that ABCA1 protein levels were significantly decreased in cells treated with apoA-I siRNA (Fig. 3G). Simultaneously, the levels of CD14 protein expression were similar in both ApoA-I-deficient and control macrophages (Fig. 3H). FACS analysis showed a decrease in the number of CD45<sup>+</sup>ABCA1<sup>+</sup>ApoA-I<sup>+</sup> cells in apoA-I siRNA-transfected macrophages. Interestingly, apoA-I knockdown had no influence on the number of CD45<sup>+</sup>ABCA1<sup>+</sup>ApoA-I<sup>-</sup> cells (Table 1). Together, these results show that endogenous ApoA-I is involved in stabilization of ABCA1 protein in human macrophages.

### A part of membrane-associated endogenous ApoA-I is localized in plasma membrane lipid rafts in human macrophages

According to previously published data, ApoA-I from circulation can be localized at the outer side of macrophage plasma membrane and is associated with lipid rafts (25). Using CS-removing agent M $\beta$ CD, we tested the association of the macrophage-synthesized ApoA-I protein with lipid rafts in THP-1 macrophages. Treatment of THP-1 cells with M $\beta$ CD led to a 1.8-fold decrease in the number of membrane-associated ApoA-I-rich cells and a 2.5-fold decrease in the level of membrane-associated ApoA-I (Fig. 2G). Moreover, the direct raft visualization experiments showed that membrane-associated macrophage-synthesized ApoA-I is partially associated with lipid rafts (Fig. 2H). Thereby, these data suggest that at least a part of the macrophage-synthesized ApoA-I interacting with the cell membrane is bound to lipid rafts.

**TABLE 1.** FACS analysis of ApoA-I- and ABCA1-positive and -negative PBM-derived macrophages transfected with ApoA-I siRNA or control siRNA

PBM-derived macrophage	Control siRNA (%)	ApoA-I siRNA (%)
CD45 <sup>+</sup> ABCA1 <sup>+</sup> ApoA-I <sup>+</sup>	56.5	35.3
CD45 <sup>+</sup> ABCA1 <sup>-</sup> ApoA-I <sup>+</sup>	8.1	2.1
CD45 <sup>+</sup> ABCA1 <sup>+</sup> ApoA-I <sup>-</sup>	9.1	9.0
CD45 <sup>+</sup> ABCA1 <sup>-</sup> ApoA-I <sup>-</sup>	26.3	53.6

Results are derived from 1 of 3 independent reproducible experiments.

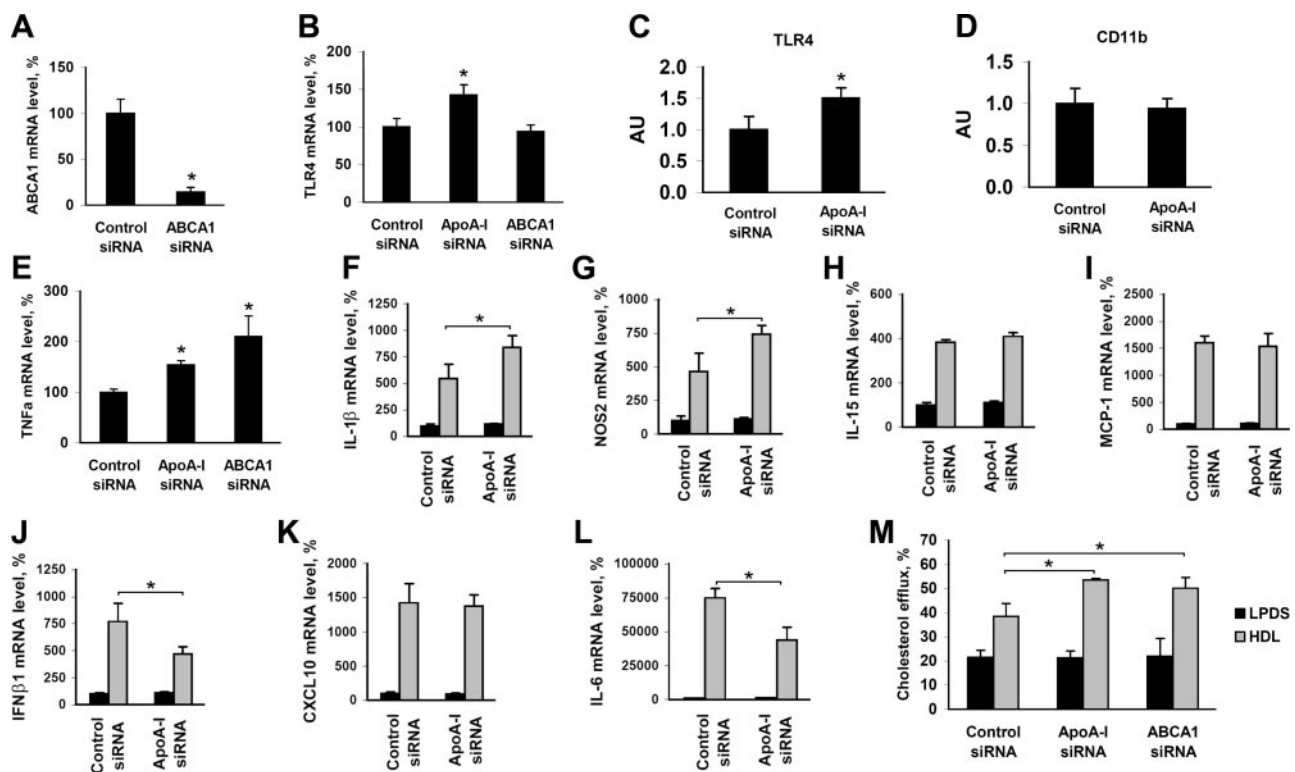
## Inhibition of endogenous synthesis of ApoA-I stimulates TLR4 expression and modulates LPS-induced expression of TNF- $\alpha$ , IL-1 $\beta$ , NOS2, IFN- $\beta$ 1, and IL-6 genes in human macrophages

Because the anti-inflammatory properties of ApoA-I are broadly discussed (4, 5), we measured the levels of expression of several proinflammatory genes in human macrophages transfected with siRNA against apoA-I or control siRNA. Interestingly, TLR4 and TNF- $\alpha$  genes were up-regulated in endogenous ApoA-I-deficient PBM-derived macrophages (Fig. 4B, E). In contrast, the levels of mRNA of TLR2, TLR3, or TLR8 were unaffected by ApoA-I siRNA transfection (data not shown). ApoA-I deficiency also led to an increase of membrane surface TLR4, but not CD14 or CD11b content in macrophages (Figs. 3H and 4C, D). Treatment of PBM-derived macrophages with LPS resulted in an increased LPS-induced activation of IL-1 $\beta$  and NOS2 gene expression in ApoA-I-deficient macrophages (Fig. 4F, G), whereas the levels of IL-15, MCP-1, and CXCL10 mRNAs were similar in control and ApoA-I siRNA-treated macrophages under the effect of LPS (Fig. 4H, I, K). Surprisingly, LPS-induced activation of IL-6 and

IFN- $\beta$ 1 gene expression was abolished in ApoA-I-deficient macrophages (Fig. 4J, L). These results suggest that endogenous ApoA-I may down-regulate stimulation of proinflammatory genes, such as TNF- $\alpha$  and TLR4, in human macrophages, possibly through interaction with ABCA1, which has well-known anti-inflammatory properties (26). To check this assumption, we performed knockdown experiments with siRNA against ABCA1 (Fig. 4A). Down-regulation of ABCA1 led to the up-regulation of the TNF- $\alpha$  gene in a similar manner as the siRNA against ApoA-I, but did not affect the TLR4 gene (Fig. 4B, E). These data suggest that at least a part of the anti-inflammatory properties of macrophage-synthesized ApoA-I can be mediated by its interactions with ABCA1. At the same time, some functions of ApoA-I can be mediated in an ABCA1-independent manner, possibly through ApoA-I association with lipid rafts.

## Down-regulation of ApoA-I in macrophages does not affect CS efflux

To study possible contribution of endogenous ApoA-I in CS efflux from macrophages, we studied the CS efflux from the PBM-derived macrophages transfected



**Figure 4.** Endogenous ApoA-I inhibits TLR4 and TNF- $\alpha$  expression and modulates LPS-induced expression of IL-1 $\beta$ , NOS2, IFN- $\beta$ 1, and IL-6 genes in human macrophages but is not involved in CS efflux in macrophages. A) Down-regulation of ABCA1 gene by siRNA against ABCA1. B–D) Level of TLR4 mRNA (100% in control cells; B) or plasma membrane surface TLR4 (C) and CD11b (D) in human PBM-derived macrophages treated with ApoA-I and/or ABCA1-specific siRNAs, or control siRNA. Values are presented as means  $\pm$  SE (B) or as arbitrary units ( $\times$ mean fluorescence determined by FACS; C, D) of 3 independent experiments. E–L) Levels of TNF- $\alpha$  (E), IL-1 $\beta$  (F), NOS2 (G), IL-15 (H), MCP-1 (I), IFN- $\beta$ 1 (J), CXCL10 (K), and IL-6 (L) mRNAs in human PBM-derived macrophages treated with apoA-I and/or ABCA1 siRNAs, or control siRNA (100% in control cells). Shaded bars represent cells treated with LPS (100 ng/ml, 24 h); solid bars represent untreated cells. M) CS efflux from PBM-derived macrophages treated by ApoA-I or ABCA1 siRNAs to the lipoprotein-deficient FCS (LPDS; solid bars), or HDLs, 50  $\mu$ g/ml (shaded bars). Values are presented as means  $\pm$  SE of 4 independent experiments. \* $P$  < 0.05; unpaired Student's  $t$  test.



by siRNAs against ApoA-I or ABCA1 (Fig. 4M). It was shown that down-regulation of neither ApoA-I nor ABCA1 involves CS efflux from macrophages to lipids of serum. Moreover, there was a stimulation of CS efflux to HDLs in the cells transfected by siRNA against ApoA-I or ABCA-I. These data suggest that endogenously synthesized ApoA-I does not significantly contribute to the CS efflux from macrophages. Rather, it realized antiatherogenic properties by its anti-inflammatory activity. The paradoxical stimulation of CS efflux to HDLs found in the cells treated by siRNAs against ApoA-I or ABCA1 can be explained by the elevation of ABCG1 protein in the response to the ABCA1 down-regulation (27).

### TNF- $\alpha$ stimulates apoA-I gene expression in human monocytes and macrophages

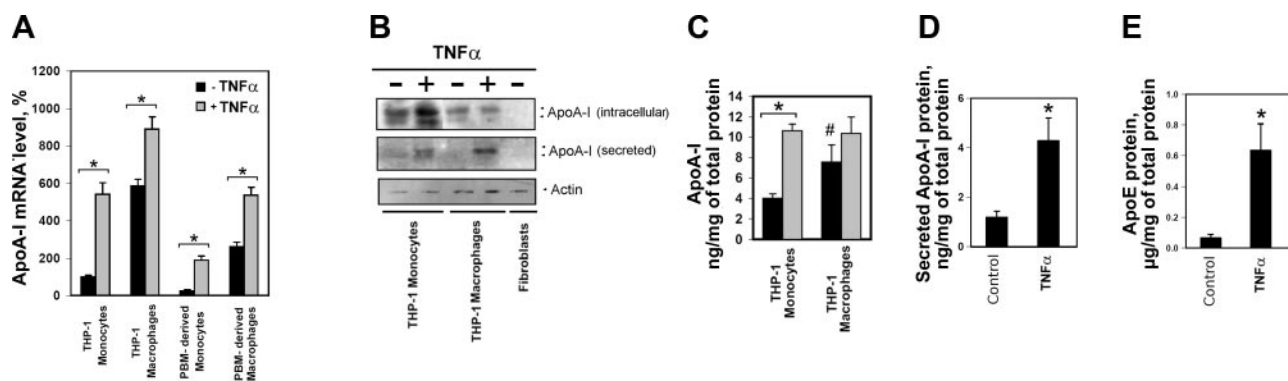
Proinflammatory cytokine TNF- $\alpha$  was found to inhibit the expression of the apoA-I gene in human hepatocytes and enterocytes (17, 28, 29). **Figure 5A** demonstrates apoA-I gene expression in human monocytes and macrophages under the effect of TNF- $\alpha$ . Treatment of THP-1 monocytes with TNF- $\alpha$  (10 ng/ml) led to a  $5.4 \pm 1.3$ -fold increase in the level of apoA-I gene expression in comparison with control cells 24 h after TNF- $\alpha$  administration. TNF- $\alpha$  considerably stimulated apoA-I gene expression in PBM monocytes (~8-fold). At the same time, treatment of PBM-derived or THP-1-derived macrophages at d 3 of differentiation with TNF- $\alpha$  led to a 1.5- to 2-fold activation of apoA-I gene expression, as compared with unstimulated macrophages (Fig. 5A).

According to a Western blot assay and ELISA, TNF- $\alpha$  stimulates ApoA-I level 2-fold over the level in untreated THP-1 monocytes but does not increase ApoA-I content in THP-1 macrophages (Fig. 5B, C). Western blot analysis performed with concentrated culture me-

dium demonstrated very low levels of ApoA-I secretion in unstimulated THP-1 monocytes or macrophages (Fig. 5B). Treatment of THP-1 cells with TNF- $\alpha$  considerably stimulated ApoA-I secretion by both THP-1 monocytes and macrophages (Fig. 5B). To estimate the level of spontaneous and TNF- $\alpha$ -induced secretion of ApoA-I by human PBM-derived macrophages, we used ELISA assay. The basic level of ApoA-I secretion by macrophages at d 3 of differentiation was  $1.2 \pm 0.2$  ng/mg total cell protein (Fig. 5D). Treatment of PBM-derived macrophages with TNF- $\alpha$  for 48 h resulted in an ~4-fold induction of ApoA-I secretion (Fig. 5D). We also determined the level in medium of ApoE protein, secretion of which is known to be stimulated by TNF- $\alpha$  in human macrophages at the early stage of monocyte-macrophage differentiation (30). In the case of control PBM-derived macrophages, the content of ApoE in medium was  $64.2 \pm 18.8$  ng/mg total cell protein, and TNF- $\alpha$  activated ApoE by 10-fold at 48 h after addition (Fig. 5E).

### DISCUSSION

In this work, we have demonstrated for the first time the low expression of ApoA-I on both mRNA and protein levels in human monocytes and macrophages. Attempts to detect apoA-I gene expression in human macrophages were made earlier (9), but the method used (Northern hybridization) seems not to be sensitive enough to identify apoA-I mRNA in those cells. Other groups of researchers have found apoA-I mRNA in human PBM cells by using RT-PCR (10, 11). Recently, we have found two additional human apoA-I gene promoters, which might be responsible for apo A-I gene expression in tissues other than liver and small intestine (29). The activities of these additional alternative promoters have not been found in monocytes and macrophages (data not shown).



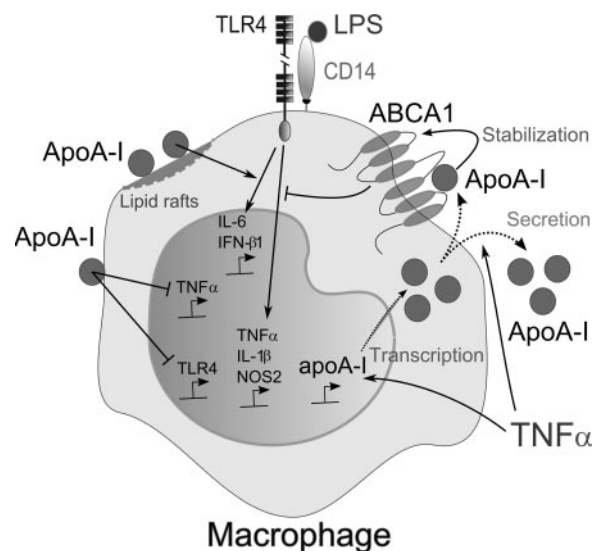
**Figure 5.** TNF- $\alpha$  stimulates ApoA-I gene expression, protein synthesis, and secretion by human monocytes and macrophages. **A)** Levels of apoA-I mRNA in human monocytes and macrophages incubated with or without TNF- $\alpha$  (10 ng/ml, 24 h). Values are presented as means  $\pm$  SE of 4 independent experiments (100% in control THP-1 monocytes). **B)** Western blot analysis of ApoA-I protein content and secretion into culture medium in THP-1 monocytes and macrophages 24 h after treatment of the cells with TNF- $\alpha$  or BSA. ApoA-I protein is not detected in normal human VH-10 fibroblast lysates and fibroblast culture medium (negative control). **C)** ELISA analysis of ApoA-I protein content in THP-1 monocytes and macrophages (control, solid bars; TNF- $\alpha$ -treated, shaded bars). **D, E)** ELISA analysis of ApoA-I (**D**) or ApoE (**E**) protein content in culture medium of PBM-derived macrophages at d 3 of differentiation, after treatment with TNF- $\alpha$  or BSA for 48 h. Values are presented as means  $\pm$  SE of 4 independent experiments. \* $P < 0.05$ , unpaired Student's *t* test; # $P < 0.05$ , Dunnett's criterion.

ApoA-I gene transcription and protein synthesis are activated during monocyte-macrophage differentiation. Similar results were observed for human apoE, expression of which is increased during monocyte-macrophage differentiation (30). Because the both ApoA-I and ApoE proteins participate in reverse CS transport from macrophages (31), coordinated elevation of the expression levels of these genes may lead to a cooperative effect on the CS efflux from macrophages. However, our results show that the level of secreted ApoA-I is 50 times less than that secreted from ApoE content in human macrophages. Moreover, the down-regulation of ApoA-I in PBM-derived macrophages does not affect CS efflux to serum lipids. Therefore, we hypothesize that endogenous ApoA-I has a minor contribution to CS efflux from macrophages. Approximately half of PBM- or THP-1-derived macrophages have a high level of membrane-associated ApoA-I (ApoA-I rich cells), and it has been shown that only a part of the macrophage population synthesizes a high level of ApoE (21). Our data suggest that ApoA-I and ApoE expression correlate and seem to be associated with a common macrophage subpopulation.

We have demonstrated that macrophage-synthesized ApoA-I protein has different intracellular and plasma membrane localizations in THP-1 macrophages. It is well established that ApoA-I interacts with ABCA1 at the outer surface of the macrophage plasma membrane, resulting in CS efflux from the cells (32) and triggering intracellular signaling pathways involved in the inflammatory functions of macrophages (26, 33). Previously published data have demonstrated that exogenous ApoA-I can bind and stabilize ABCA1, resulting in increased cell membrane localization and decreased degradation of ABCA1 (23, 24). We have shown that macrophage-synthesized ApoA-I is bound with and increases the level of ABCA1 by stabilizing this protein in human macrophages, and thereby probably increasing anti-inflammatory properties of ABCA1 (Fig. 6).

In contrast to hepatocytes, which are main producers of plasma ApoA-I (34), secretion of ApoA-I by human monocytes and macrophages is TNF- $\alpha$  induced and is very limited without stimulation. There are publications that showed that anti-inflammatory activity of ApoA-I accumulated at the sites of chronic inflammation during rheumatoid arthritis and lupus erythematosus development (35), where ApoA-I may be involved in inhibition of local production of TNF- $\alpha$  and IL-1 $\beta$  due to the blocking of activated T-lymphocyte-macrophage interactions (36). On the basis of our data, we suggest that monocyte-macrophage cells may realize TNF- $\alpha$ -induced ApoA-I production, controlling development of inflammation in feedback manner or inhibiting the subthreshold levels of inflammation.

What are the consequences of ApoA-I gene expression in macrophages? It was shown that delivery of human apoA-I gene to apoA-I<sup>-/-</sup> mouse macrophages decreases the development of a fat streak at the vessel walls and increases CS efflux from macrophages but does not influence the levels of ApoA-I and CS in serum



**Figure 6.** Hypothetic scheme illustrating a possible role of endogenous ApoA-I in stabilization of ABCA1 and regulation of TLR4 signaling in human macrophages. See Discussion for detailed explanation.

(12–14). In addition, ApoA-I was shown to decrease proinflammatory properties of plaque macrophages in mice (37). It was reported that “lipid-free” ApoA-I reduces human monocyte inflammatory response *via* ABCA1 (38), and the ApoA-I/ABCA1 pathway in macrophages functions as an anti-inflammatory mechanism through activation of JAK2/STAT3 (26). We have shown that siRNA knockdown of endogenous ApoA-I results in a decrease of ABCA1 protein levels as well as a stimulation of TLR4 gene and membrane surface protein expression in human macrophages, thereby activating LPS-induced increase of TNF- $\alpha$ , IL-1 $\beta$ , and NOS2 gene expression (Fig. 6). Recently Zhu *et al.* (39) showed that macrophages from macrophage-specific ABCA1-knockout mice have an enhanced proinflammatory response to LPS due to increased free CS and TLR4 contents in lipid rafts. Our results suggest that endogenous ApoA-I is involved in down-regulation of TLR4 at both transcriptional and protein synthesis levels, and this effect of ApoA-I is ABCA1 independent (Fig. 6). Macrophage-specific ABCA1-knockout mice were found to show enhanced proinflammatory response of macrophages to LPS in terms of TNF- $\alpha$ , MCP-1, NOS2, IL-6, and IL-1 $\beta$  gene expression, which is mediated by increased plasma membrane lipid raft content (40). We speculate that increased LPS-induced activation of TNF- $\alpha$ , IL-1 $\beta$ , and NOS2 genes is mediated by down-regulation of ABCA1 in ApoA-I-deficient human macrophages (Fig. 6). Moreover, association of a part of endogenous ApoA-I with lipid rafts in human macrophages may suggest ABCA1-independent but lipid raft-dependent modulation of inflammatory response mediated by endogenous ApoA-I in macrophages. Interestingly, HDL was found to suppress the type I interferon response, abrogating LPS-induced activation of TRAM/TRIF-dependent genes, such as NOS2, IF- $\beta$ 1, and CXCL10 (also known as IP-10) (27)

Our results show that endogenous ApoA-I abolishes LPS-induced activation of NOS2, but not IF- $\beta$ 1 or CXCL10, which may reflect different anti-inflammatory activities of lipid-free endogenous ApoA-I and HDL. Taking into account these data, we suggest that endogenous expression of apoA-I in human macrophages may stabilize ABCA1 and enhance its anti-inflammatory functions, hereby resulting in down-regulation of atherosclerotic lesions. **FJ**

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