ABCA1 Promotes the de Novo Biogenesis of Apolipoprotein CIII-Containing HDL Particles in Vivo and Modulates the Severity of Apolipoprotein CIII-Induced Hypertriglyceridemia[†]

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ABSTRACT: In this study, the ability of the lipid transporter ABCA1 and apolipoprotein CIII (apoCIII) to promote the de novo biogenesis of apoCIII-containing HDL in vivo and the role of this HDL in apoCIII-induced hypertriglyceridemia were investigated, using adenovirus-mediated gene transfer in apoE$^{-/-}$ × apoA-I$^{-/-}$ mice or ABCA1$^{-/-}$ mice. Injection of apoE$^{-/-}$ × apoA-I$^{-/-}$ mice with 8 × 10^8 pfu of an adenovirus expressing the wild-type human apoCIII (AdGFP-CIII$_g$) generated HDL-like particles and triggered only a modest increase in plasma cholesterol and triglyceride levels of these mice, 3−5 days postinfection. Plasma human apoCIII was distributed among HDL, VLDL/IDL, and LDL in these mice. In contrast, ABCA1$^{-/-}$ mice treated similarly failed to form HDL particles and developed severe hypertriglyceridemia which could be alleviated by coinfection with an adenovirus expressing human LpL, while their plasma cholesterol levels remained unchanged 3−5 days postinfection with AdGFP-CIII$_g$. Human apoCIII in these mice accumulated exclusively on VLDL. Control experiments confirmed that the differences between apoE$^{-/-}$ × apoA-I$^{-/-}$ and ABCA1$^{-/-}$ mice expressing human apoCIII were not due to differences in apoCIII expression. Overall, these data show that ABCA1 and human apoCIII promote the formation of apoCIII-containing HDL-like particles that are distinct from classical apoE- or apoA-I-containing HDL. Formation of apoCIII-containing HDL prevents excess accumulation of plasma apoCIII on VLDL and allows for the efficient lipolysis of VLDL triglycerides by LpL. Furthermore, the data establish that ABCA1 and apoCIII-containing HDL play key roles in the prevention of apoCIII-induced hypertriglyceridemia in mice.

Apolipoprotein CIII (apoCIII) is a 79-amino acid glycoprotein synthesized by the liver and intestine (*1*). It is currently believed that apoCIII is secreted in a lipid-poor form in the circulation (*2*) where it subsequently associates with preexisting very low-density (VLDL),[1] low-density (LDL), and high-density (HDL) lipoproteins (*3*). Approximately one-half of apoCIII is found in HDL (*3, 4*) and the remainder in VLDL and chylomicrons (*3*). Small quantities of apoCIII are also found in intermediate-density lipoprotein (IDL) and LDL particles (*5, 6*). ApoCIII is also found as a component of particles with defined lipid and apolipoprotein composition such as LpB:E:CIII, LpAI:AII:CIII, and other combinations (*7, 8*). In addition, in vitro experiments have established that association of purified lipid-free apoCIII with egg lecithin forms discoidal particles with a minor axis of 4 nm and a major axis of 20 nm (*9, 10*).

ApoCIII plays a major role in plasma triglyceride metabolism. Numerous epidemiological and animal studies have established a direct correlation of plasma apoCIII levels with plasma triglyceride levels, and an inverse relationship with the rate of postprandial lipoprotein clearance (*11*−*18*). Many in vitro studies suggested an important role of apoCIII in the catabolism of triglyceride-rich lipoproteins. ApoE-mediated uptake of triglyceride-rich emulsions by cultured HepG2 cells and rat hepatocytes was inhibited by apoCIII (*19, 20*). Liver perfusion studies indicated that apoCs, including apoCIII, inhibited the hepatic uptake of chylomicrons and VLDL (*11, 19*−*28*). In addition, apoCIII inhibited the binding of apoB-containing lipoproteins to the LDL receptor probably by masking the binding domain of apoB, as well as the binding of chylomicrons and VLDL to the lipolysis-stimulated receptor (*24*).

Other studies showed that apoCIII inhibits the activity of lipoprotein lipase, the enzyme that hydrolyzes the triglyc-

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[1] Abbreviations: ABCA1, ATP-binding cassette superfamily A, member 1; ABCA1$^{-/-}$, ABCA1-deficient; Ad, adenovirus; GFP, green fluorescence protein; CMV, cytomegalovirus; AdGFP-CIII$_g$, recombinant attenuated adenovirus containing wild-type human genomic apoCIII DNA and the GFP cDNA under the independent control of two CMV promoters; apoA-I, apolipoprotein AI; apoA-I$^{-/-}$, apoA-I-deficient; apoE, apolipoprotein E; apoE$^{-/-}$, apoE-deficient; apoA-II, apolipoprotein AII; TC, total cholesterol; CE, cholesteryl esters; ELISA, enzyme-linked immunoabsorbent assay; FPLC, fast pressure liquid chromatography; HDL, high-density lipoprotein; IDL, intermediate-density lipoprotein; LCAT, lecithin cholesterol acyl transferase; LpL, lipoprotein lipase; LDL, low-density lipoprotein; pfu, plaque-forming unit; VLDL, very low-density lipoprotein; WT, wild-type; moi, multiplicity of infection; EM, electron microscopy.

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erides of chylomicrons and VLDL (*29, 30*). In patients with apoA-I and apoCIII deficiency, a lack of apoCIII facilitates the conversion of triglyceride-rich VLDL to IDL and LDL (*31*). In hypertriglyceridemic patients, apoCIII was found to be a specific inhibitor of lipoprotein lipase (LpL) (*32, 33*) while purified apoCIII acted as a competitive inhibitor of apoCII in the hydrolysis of triolein (*32*). In vitro studies using synthetic peptides suggested that the N-terminal domain of apoCIII is responsible for the inhibitory effect of apoCIII on LpL activity (*34*), though this result was never confirmed in vivo. Overall, there have been three different mechanisms proposed to explain apoCIII-induced hypertriglyceridemia: (a) apoCIII displaces apoCII from VLDL and reduces the level of LpL activation by apoCII (*47*), (b) apoCIII acts as a direct noncompetitive inhibitor of LpL (*16*−*18*), and (c) apoCIII displaces apolipoprotein E (apoE) from the VLDL, thus reducing the rates of apoE- and LDLr-mediated clearance of triglyceride-rich lipoproteins (*14, 15*).

In addition to the role of apoCIII in the development of hypertriglyceridemia, recent studies suggested a direct link between plasma apoCIII levels and the development of other conditions associated with the metabolic syndrome. Specifically, it was found that apoCIII levels in plasma show a direct correlation with body mass index and insulin resistance (*35*). Furthermore, studies in humans showed that accumulation of apoCIII-containing VLDL and chylomicrons in plasma are strong predictors of coronary heart disease (*36*−*38*).

In this study, the ability of apoCIII to promote the de novo biogenesis of HDL particles in vivo, the role of these particles in the development of hypertriglyceridemia, and the potential contribution of the lipid transporter ABCA1 to these processes were investigated. To address these questions, a recombinant adenovirus (AdGFP-$CIII_g$) expressing the wild-type (WT) human apoCIII under the control of the cytomegalovirus (CMV) promoter (*39*) was generated and used in adenovirus-mediated gene transfer studies of human apoCIII in $apoE^{-/-} \times apoA\text{-}I^{-/-}$ mice or $ABCA1^{-/-}$ mice. To prevent nonphysiologically high levels of apoCIII expression, in all experiments we used a moderate dose of apoCIII-expressing adenovirus that does not trigger hypertriglyceridemia in wild-type C57BL/6 mice. The data presented here show that apoCIII promotes the de novo biogenesis of HDL-like particles in $apoE^{-/-} \times apoA\text{-}I^{-/-}$ mice that are distinct from classical apoE- and apoA-I-containing HDL. This process requires the lipid transporter ABCA1, since its deficiency prevented formation of apoCIII-containing HDL. Furthermore, deficiency in ABCA1 promoted the accumulation of plasma apoCIII exclusively on the VLDL and triggered hypertriglyceridemia in $ABCA1^{-/-}$ mice that are infected with the AdGFP-$CIII_g$ adenovirus. Control experiments showed that plasma apoCIII levels and hepatic mRNA expression of apoCIII were similar in $apoE^{-/-} \times apoA\text{-}I^{-/-}$ mice and $ABCA1^{-/-}$ mice infected with AdGFP-CIIIg, confirming that the differences in plasma triglyceride levels between these two mouse groups are not due to differences in apoCIII expression levels. Taken together, the data presented here establish that de novo biogenesis of apoCIII-containing HDL with the participation of ABCA1 is a key process contributing to the prevention of apoCIII-induced hypertriglyceridemia.

MATERIALS AND METHODS

Construction of the Recombinant Adenovirus Expressing the Wild-Type Human ApoCIII. The construction of the pBMT3X-apo$CIII_g$ plasmid containing the genomic apoCIII sequence has been described previously (*40*). Subcloning of the genomic apo$CIII_g$ DNA from the pBMT3X-apo$CIII_g$ plasmid into the XhoI sites of the pBlueskript (pBK) vector created the pBK-apo$CIII_g$-sense and pBK-apo$CIII_g$-antisense plasmids. In the pBK-$CIII_g$-antisense vector, the 5′ end of the apo$CIII_g$ gene is flanked by a unique KpnI restriction site and the 3′ end is flanked by a unique XbaI site of the pBK vector. Subcloning of the KpnI−XbaI fragment of the pBK-apo$CIII_g$-antisense vector into the KpnI and XbaI sites in the polylinker of the pAdTrack-CMV vector (*39*) generated the pAdTrack-CMV-apo$CIII_g$ vector in which the genomic apoCIII sequence is under the control of the CMV promoter.

The recombinant adenovirus was constructed using the AdEasy-1 system in which the complete adenovirus genome is generated by homologous recombination in bacterial BJ-5183 cells (*39*). Specifically, the recombinant vector pAdTrack-CMV-apo$CIII_g$ was linearized via PmeI digestion and then electroporated into BJ-5183 *Escherichia coli* cells along with the pAdEasy-1 helper vector. pAdEasy-1 contains the viral genome and the long terminal repeats of the adenovirus and allows for the formation by homologous recombination of the recombinant virus containing the gene of interest. Recombinant bacterial clones resistant to kanamycin were selected and screened for the presence of the gene of interest by restriction endonuclease analysis and DNA sequencing. The resulting recombinant vector also contained the green fluorescence protein (GFP) gene under the independent control of a second CMV promoter, which enables detection of the infection in cells and tissues by their green fluorescence. The recombinant adenovirus expressing the WT human apoCIII was designated as AdGFP-$CIII_g$. Correct clones were propagated in RecA DH5α cells. The recombinant vector was linearized with PacI and used to infect 911 cells (*41*). The subsequent steps involved in the generation and expansion of recombinant adenoviruses were plaque identification and isolation followed by infection and expansion in 911 cells (*41*). These steps were followed by a purification process involving CsCl ultracentrifugation performed twice, followed by dialysis and titration of the virus. Usually, titers ranging from 2×10^{10} to 5×10^{10} pfu/mL were obtained. A control virus expressing only GFP (AdGFP) (*42*) was also used in this study to correct for nonspecific effects of the infection process.

Cell Culture Studies. HTB13 cells (SW1783, human astrocytoma) were grown to confluence in medium containing 10% fetal calf serum (FCS). Confluent cultures were washed twice with phosphate-buffered saline (PBS), switched to culture medium containing 2% heat-inactivated horse serum, and then infected with AdGFP-$CIII_g$ at multiplicities of infection (moi's) of 3, 6, 12, and 24, as indicated. Twenty-four hours postinfection, cells were washed twice with PBS, and fresh serum-free medium was added. Following incubation for an additional 24 h, medium was collected and analyzed via an enzyme-linked immunoabsorbent assay (ELISA) and Western blot analysis for apoCIII expression.

Animal Studies. Mice were purchased from Jackson Laboratories. ABCA1$^{-/-}$ mice were generated by crossing ABCA1$^{+/-}$ mice (*43*). Female apoE$^{-/-}$ × apoA-I$^{-/-}$ mice [generated in our laboratory by crossing apoE$^{-/-}$ mice (*44*) with apoA-I$^{-/-}$ mice (*45*)] and ABCA1-deficient mice (*43*) that were 8−10 weeks old were used in these studies. Groups were formed after the fasting cholesterol and triglyceride levels of the individual mice were determined, to ensure similar average cholesterol and triglyceride levels among groups. For the adenovirus infections, groups of six to eight mice were injected intravenously through the tail vein with doses of 8×10^8 pfu of the apoCIII$_g$-expressing adenovirus or the control adenovirus AdGFP. Blood was obtained daily following a 4 h fasting period, 3−5 days postinjection. Aliquots of plasma were stored at 4 and −20 °C.

RNA Analysis. To assess the expression of hepatic human apoCIII mRNA, three mice from each group were sacrificed 4 days postinfection. Livers were collected from individual animals, frozen in liquid nitrogen, and stored at −80 °C. Total RNA was isolated from the livers and analyzed for apoCIII mRNA and GAPDH expression by Northern blotting followed by phosphorimaging (*42*).

Plasma Lipid Determination. Plasma triglycerides, total cholesterol, free cholesterol, and phospholipids were assessed spectrophotometrically using the GPO-Trinder kit (Sigma, catalog no. TR0100-1KT), the Infinity Cholesterol kit (Thermo Electron Corp., catalog no. TR13521), the Free Cholesterol E kit (Wako, catalog no. 435-35801), and the Phospholipid C determination kit (Wako, catalog no. 433-36201) respectively, according to the manufacturers' instructions and as described previously (*42*).

Fractionation of Plasma Lipoproteins by Density Gradient Ultracentrifugation. To assess the ability of human apoCIII to associate with different lipoproteins, 0.35 mL pools of plasma from WT C57BL/6, apoE$^{-/-}$ × apoA-I$^{-/-}$, or ABCA1$^{-/-}$ mice infected with the adenovirus expressing the WT human apoCIII were fractionated by density gradient ultracentrifugation, and different density fractions were analyzed for human apoCIII protein levels by Western blotting, as described below. The lipid composition of each fraction was also determined as described above.

Western Blot Analysis of ApoCIII. Western blot analysis of apoCIII was performed as described previously (*42, 46*−*49*), using a goat anti-human apoCIII antibody (Biodesign International, catalog no. K74140G) as the primary antibody and a rabbit anti-goat antibody (Santa-Cruz, catalog no. sc-2768) as the secondary.

Quantification of Human ApoCIII by ELISA. Serum human apoCIII concentrations were measured with a sandwich ELISA (*42, 46*−*49*). A polyclonal goat anti-human apoCIII antibody (Biodesign International, catalog no. K74140G) was used for coating microtiter plates, and a polyclonal goat anti-human apoCIII antibody coupled to horseradish peroxidase (Biodesign International, catalog no. K74140P) was used as the secondary antibody. The immunoperoxidase procedure was employed for the colorimetric detection of apoCIII at 450 nm, using tetramethylbenzidine as the substrate. Different dilutions of plasma from healthy human subjects with an apoCIII level of 10 mg/dL were used as standards.

Rate of Hepatic VLDL Triglyceride Production in Mice Infected with AdGFP-CIII$_g$. To compare the effects of human apoCIII expression on hepatic VLDL triglyceride secretion in apoE$^{-/-}$ × apoA-I$^{-/-}$ and ABCA1$^{-/-}$ mice, six mice from each group were infected with a dose of 8×10^8 pfu of AdGFP-CIII$_g$ adenovirus. Four days postinfection, mice were fasted for 4 h and then treated with Triton-WR1339 at a dose of 500 mg/kg of body weight, using a 15% solution (w/v) in 0.9% NaCl [Triton-WR1339 has been shown to completely inhibit VLDL catabolism (*13*)], as described previously (*42, 46, 49*). Briefly, serum samples were isolated at 5, 10, 20, 40, 60, and 90 min after injection with Triton-WR1339. As a control, serum samples were isolated 1 min after injection with the detergent. Plasma triglyceride levels at each time point were determined as described above, and linear graphs of triglyceride concentration versus time were generated. The rate of VLDL triglyceride secretion expressed in milligrams per deciliter per minute was calculated from the slope of the linear graphs for each individual mouse. Then, slopes were grouped together and reported as the mean ± standard error of the mean in the form of a bar graph.

Electron Microscopy Analysis of HDL. Pools of the HDL fractions isolated from equilibrium density gradient ultracentrifugation were dialyzed against ammonium acetate and carbonate buffer and then stained with sodium phosphotungstate. Particles were visualized in a Phillips CM-120 electron microscope (Phillips Electron Optics, Eindhoven, The Netherlands) and photographed as described previously (*49, 50*). The photomicrographs were taken at a magnification of 75000× and enlarged three times. Size measurements of particles ($n > 200$ from each population) were taken directly from image negatives (75000×) with a 7× measuring magnifier with a metric scale of 0.1 mm divisions. Measured values were classified into 1.3 nm intervals, and the diameter of each value within an interval was represented by the midpoint of that interval. Descriptive statistics of a measured population of at least 200 particles were prepared using the Analysis Tool Pack in Microsoft Excel 2002.

Statistical Analysis. A comparison of data from two groups of mice was performed using the Student's t test. Data are reported as means ± the standard error of the mean. An asterisk indicates $p < 0.05$. Two asterisks indicate $p < 0.005$. ns indicates statistically nonsignificant differences. n represents the number of animals tested in each experiment.

RESULTS

Expression and Secretion of ApoCIII by Cultures of HTB-13 Cells Infected with the Recombinant Adenovirus Expressing Human ApoCIII. Human astrocytoma HTB-13 cells that do not synthesize endogenous apoCIII were infected with the recombinant adenovirus AdGFP-CIII$_g$, at multiplicities of infection of 0, 3, 6, 12, and 24. Western blot analysis of the medium from the infected cultures showed that human apoCIII is secreted efficiently in the culture medium 24 h postinfection (Figure 1). Analysis of the culture medium by a sandwich ELISA confirmed that apoCIII is secreted in the media of the infected cultures at concentrations ranging from 10 to 50 μg of apoCIII per milliliter of culture medium, 24 h after infection.

Expression of ApoCIII in WT C57BL/6 Mice Infected with the Recombinant Adenovirus Containing Human ApoCIII$_g$. To confirm that infection of mice with the AdGFP-CIII$_g$ adenovirus results in the efficient production and secretion

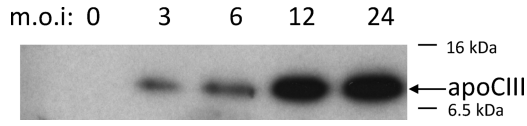

FIGURE 1: Western blot analysis of culture medium of HTB-13 cells infected with different multiplicities of infection (moi's) of the human apoCIII-expressing adenovirus. Cultures of HTB-13 cells that do not express endogenous apoCIII were grown to confluence in six-well plates and then infected with moi's of 0, 3, 6, 12, and 24 of the AdGFP-$CIII_g$ adenovirus. Following infection for 24 h, cultures were washed twice with PBS, and fresh serum-free medium was added for an additional 24 h. Then, 20 μL of culture medium was isolated and analyzed by Western blot analysis using an anti-human apoCIII antibody. The molecular mass of the markers and the position of apoCIII are indicated.

of human apoCIII in the plasma of these mice, WT C57BL/6 mice were treated via the tail vein with a moderate dose of 8×10^8 pfu or a high dose of 2×10^9 pfu of the AdGFP-$CIII_g$ adenovirus, and plasma samples were collected on days 3, 4, and 5 postinfection. Then, plasma levels of human apoCIII, cholesterol, and triglycerides were determined as a function of time.

As shown in Figure 2, infection with 2×10^9 pfu of AdGFP-$CIII_g$ triggered severe hypertriglyceridemia and a significant increase in plasma cholesterol levels ($p < 0.05$) in the infected mice (Figure 2A,B), a finding consistent with previous results on apoCIII overexpression in vivo (*16*−*18*). In contrast, infection of mice with the lower dose of 8×10^8 pfu of AdGFP-$CIII_g$ resulted in only a modest increase ($p < 0.05$) in their plasma triglyceride levels which, however, remained within normal levels (≤150 mg/dL) throughout the duration of the experiment (Figure 2B). This modest increase in plasma triglyceride levels was accompanied by a modest increase in plasma cholesterol levels of these mice (Figure 2A). As control, infection of C57BL/6 mice with 2×10^9 pfu of the empty AdGFP virus had no effect on the plasma triglyceride or cholesterol levels of these mice, confirming that there are no nonspecific effects on plasma lipid levels due to the infection (Figure 2A,B). ELISA analysis of plasma samples isolated on day 5 postinfection showed that steady-state human plasma apoCIII levels were in the range of 50−80 mg/dL in C57BL/6 mice infected with 2×10^9 pfu of AdGFP-$CIII_g$ and 15−25 mg/dL in C57BL/6 mice infected with 8×10^8 pfu of AdGFP-$CIII_g$.

Fractionation of plasma isolated from the infected mice by density gradient ultracentrifugation followed by Western blotting for human apoCIII showed that, in C57BL/6 mice infected with 8×10^8 pfu of AdGFP-$CIII_g$, apoCIII was mainly found in HDL and to a lesser extent in VLDL and IDL (Figure 2C). However, in C57BL/5 mice infected with 2×10^9 pfu of AdGFP-$CIII_g$, apoCIII levels increased in both VLDL/IDL and HDL, while a significant amount of apoCIII was also found in the LDL fractions (Figure 2D), in agreement with previously published studies (*3*−*6*).

Therefore, infection of C57BL/6 mice with AdGFP-$CIII_g$ results in the efficient expression and secretion of human apoCIII in the plasma of the infected mice. In addition, the recombinant adenovirus AdGFP-$CIII_g$ provides a versatile in vivo expression system that depending on the dose produces different phenotypes that are similar to those previously reported in apoCIII transgenic mouse models.

Plasma Lipid and ApoCIII Levels and Hepatic ApoCIII mRNA Expression in ApoE$^{-/-}$ × *ApoA-I*$^{-/-}$ *and ABCA1*$^{-/-}$ *Mice following Adenovirus Infection.* In the following studies, we selected to use the moderate dose of 8×10^8 pfu of AdGFP-$CIII_g$ adenovirus that does not trigger hypertriglyceridemia when administered to WT C57BL/6 mice.

Six to eight apoE$^{-/-}$ × apoA-I$^{-/-}$ or ABCA1$^{-/-}$ mice were infected with 8×10^8 pfu of the human apoCIII-expressing adenovirus or the control AdGFP adenovirus, and fasting plasma cholesterol and triglyceride levels were determined 4 and 5 days postinfection.

In apoE$^{-/-}$ × apoA-I$^{-/-}$ mice, expression of human apoCIII resulted in a modest elevation of their plasma cholesterol levels on days 4 and 5 postinfection ($p < 0.05$) (Figure 3A). However, plasma triglyceride levels remained normal (≤150 mg/dL) during the course of the experiment despite a modest increase on day 5 postinfection ($p < 0.05$) (Figure 3B). In contrast, expression of human apoCIII in ABCA1$^{-/-}$ mice had no significant effects on plasma cholesterol levels of these mice ($p > 0.05$) (Figure 3C), although it triggered severe hypertriglyceridemia on days 4 and 5 postinfection ($p < 0.005$) (Figure 3D).

Treatment of apoE$^{-/-}$ × apoA-I$^{-/-}$ or ABCA1$^{-/-}$ mice with the control adenovirus expressing the green fluorescence protein (AdGFP) did not change significantly the plasma cholesterol and triglyceride levels of these mice (Figure 3A−D). Potential liver damage following adenovirus infection was assessed by measuring levels of serum transaminases using the Reflotron Plus system (Roche), and normal serum transaminase levels were found in mice infected with 8×10^8 pfu of the recombinant adenoviruses.

To confirm that the differences in plasma cholesterol and triglyceride levels between apoE$^{-/-}$ × apoA-I$^{-/-}$ and ABCA1$^{-/-}$ mice infected with 8×10^8 pfu of AdGFP-$CIII_g$ were not due to different levels of apoCIII expression, plasma apoCIII levels and hepatic mRNA expression levels were determined in these mice. Analysis of steady-state plasma apoCIII levels on days 4 and 5 postinfection via an ELISA showed that apoE$^{-/-}$ × apoA-I$^{-/-}$ mice infected with 8×10^8 pfu of AdGFP-$CIII_g$ had higher steady-state plasma human apoCIII levels than ABCA1$^{-/-}$ mice infected with the same dose of the human apoCIII-expressing adenovirus. Specifically, plasma levels of human apoCIII in AdGFP-$CIII_g$-infected apoE$^{-/-}$ × apoA-I$^{-/-}$ mice were 18.22 ± 2.32 mg/dL on day 4 and 19.14 ± 2.69 mg/dL on day 5, while plasma levels of human apoCIII in AdGFP-$CIII_g$-infected ABCA1$^{-/-}$ mice were 16.31 ± 2.31 mg/dL on day 4 and 13.85 ± 1.75 mg/dL on day 5.

Furthermore, Northern blot analysis of RNA from livers of the infected mice 5 days postinfection showed similar average hepatic human apoCIII mRNA expression in C57BL/6, apoE$^{-/-}$ × apoA-I$^{-/-}$, and ABCA1$^{-/-}$ mice infected with 8×10^8 pfu of the AdGFP-$CIII_g$ adenovirus ($p > 0.05$) (Figure 4A−C). However, only infected ABCA1$^{-/-}$ mice developed severe hypertriglyceridemia (Figure 3D), while infected C57BL/6 and apoE$^{-/-}$ × apoA-I$^{-/-}$ mice had normal triglyceride levels (Figures 2B and 3B).

The Hypercholesterolemia of ApoE$^{-/-}$ × *ApoA-I*$^{-/-}$ *Mice Expressing Human ApoCIII Is Due to an Increased Level of Accumulation of Cholesterol in the LDL and HDL, while the Hypertriglyceridemia of ABCA1*$^{-/-}$ *Mice Expressing ApoCIII Is Due to Accumulation of Triglyceride-Rich VLDL in Plasma.* To determine the distribution of phospholipids, triglycerides, and total, free, and esterified cholesterol among

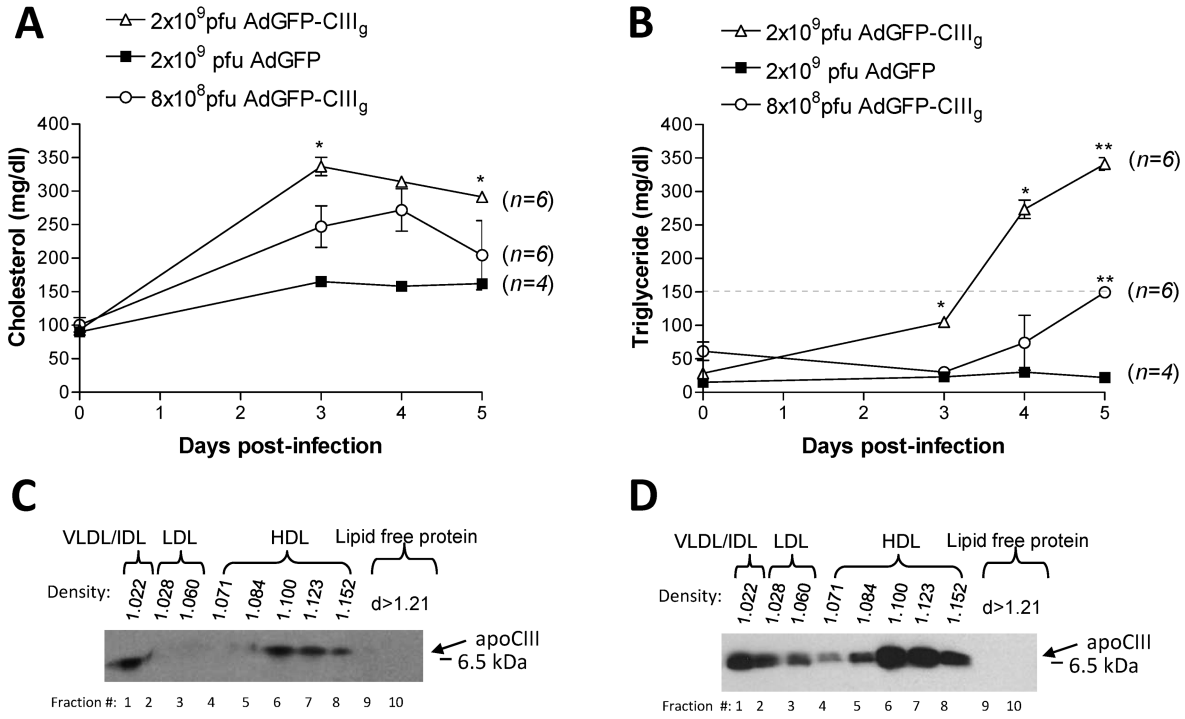

FIGURE 2: Cholesterol and triglyceride levels, and apoCIII distribution among lipoproteins in plasma of WT C57BL/6 mice infected with the adenovirus expressing human apoCIII or the control adenovirus AdGFP. (A) Plasma cholesterol levels and (B) plasma triglyceride levels of C57BL/6 mice infected with a moderate dose of 8×10^8 pfu ($n = 6$) or a high dose of 2×10^9 pfu of AdGFP-CIII$_g$ ($n = 6$) or the control AdGFP adenovirus ($n = 4$). The dotted line indicates the upper physiological plasma triglyceride level of 150 mg/dL. (C and D) Western blot analyses using an anti-human apoCIII specific antibody of plasma lipoprotein fractions isolated by density gradient ultracentrifugation of plasma from C57BL/6 mice infected with 8×10^8 pfu (C) or 2×10^9 pfu (D) of AdGFP-CIII$_g$. The densities and the lipoprotein classes to which they correspond are indicated. The statistical significance of the observed differences among groups at each time point is as indicated (one asterisk corresponds to $p < 0.05$, and two asterisks correspond to $p < 0.005$).

lipoproteins in apoE$^{-/-}$ × apoA-I$^{-/-}$ and ABCA1$^{-/-}$ mice infected with 8×10^8 pfu of AdGFP-CIII$_g$, plasma samples were isolated 5 days postinfection and fractionated by density gradient ultracentrifugation, as described in Materials and Methods. Then, different density fractions were collected and analyzed for total, free, and esterified cholesterol, triglyceride, and phospholipid levels.

This analysis showed that expression of apoCIII in apoE$^{-/-}$ × apoA-I$^{-/-}$ mice resulted in an increase in plasma cholesterol levels of the LDL and HDL fractions while the VLDL cholesterol level remained unchanged, compared to mice infected with the control AdGFP adenovirus (Figure 5A). The increase in LDL and HDL cholesterol levels was accompanied by an increase in free cholesterol levels (compare panels A and B of Figure 6), consistent with the role of apoCIII as an inhibitor of the plasma enzyme lecithin: cholesterol-acyl transferase (LCAT) (*51*). Consistent with the increased cholesterol levels, phospholipid levels were also significantly elevated mainly in LDL/IDL and HDL fractions of these mice, while there was only a modest increase in the level of their VLDL/IDL fraction (Figure 5B).

In contrast, ABCA1$^{-/-}$ mice infected with 8×10^8 pfu of the AdGFP-CIII$_g$ or control AdGFP adenovirus had similar but very low levels of total cholesterol. All cholesterol was found only in the VLDL/IDL fraction (Figure 5D) and was esterified (Figure 6C,D). No cholesterol was found in the LDL or HDL fractions of either mouse group. However, there was a significant increase in phospholipid levels of the VLDL/IDL fraction of the ABCA1$^{-/-}$ mice expressing apoCIII, compared to ABCA1$^{-/-}$ mice infected with the control AdGFP virus (Figure 5E).

Expression of human apoCIII in apoE$^{-/-}$ × apoA-I$^{-/-}$ mice resulted in a modest increase in the triglyceride levels of their VLDL/IDL fraction and to a lesser extent of their LDL fractions, compared to mice infected with the control AdGFP adenovirus which had very low levels of triglycerides present only in the VLDL/IDL fractions (Figure 5C).

However, ABCA1$^{-/-}$ mice infected with the AdGFP-CIII$_g$ adenovirus showed that the increase in total plasma triglyceride levels of these mice was due to a dramatic increase in the triglyceride content of their VLDL/IDL fraction and to a much lesser extent in their LDL fraction, compared to the control AdGFP-infected ABCA1$^{-/-}$ mice (Figure 5F).

The Increased VLDL Triglyceride Content of the ABCA1 Mice Infected with 8×10^8 pfu of AdGFP-CIII$_g$ Correlates with the Exclusive Accumulation of Human ApoCIII in the VLDL of These Mice. To determine the distribution of human apoCIII among different lipoprotein classes, plasma samples were isolated from apoE$^{-/-}$ × apoA-I $^{-/-}$ and ABCA1$^{-/-}$ mice on day 5 postinfection with 8×10^8 pfu of AdGFP-CIII$_g$. Then, lipoproteins were separated by equilibrium density ultracentrifugation, and different lipoprotein fractions were analyzed by Western blotting using an anti-human apoCIII specific antibody, as described in Materials and Methods.

This analysis showed that in apoE$^{-/-}$ × apoA-I$^{-/-}$ mice infected with AdGFP-CIII$_g$, apoCIII was distributed in the VLDL/IDL, LDL, and HDL fractions (Figure 7A), consistent with the distribution of total cholesterol and phospholipids among these fractions (Figure 5A,B). In contrast, in ABCA1$^{-/-}$ mice infected with AdGFP-CIII$_g$, apoCIII accumulated exclusively in the VLDL/IDL fraction (Figure

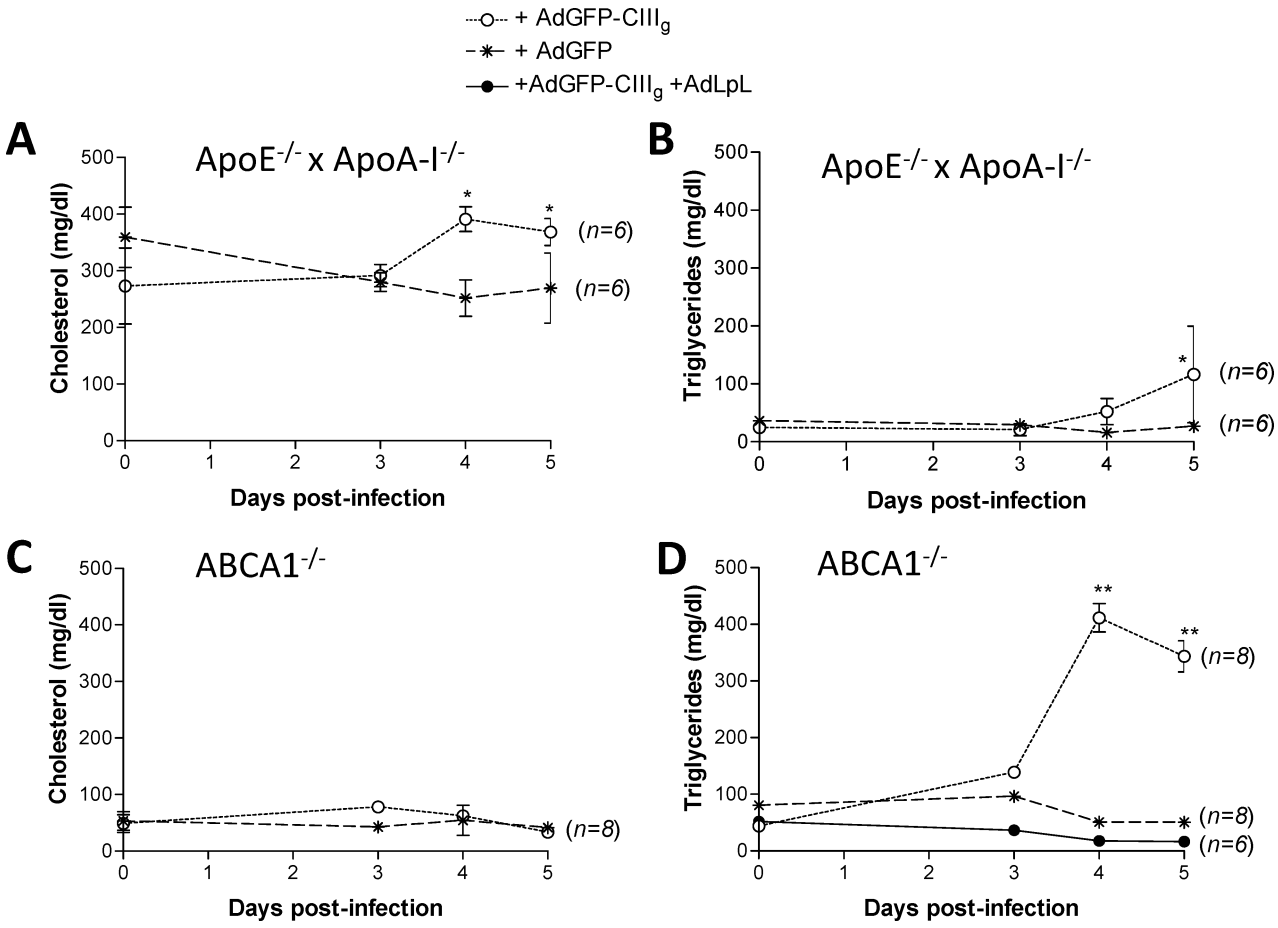

FIGURE 3: Cholesterol and triglyceride levels of apoE$^{-/-}$ × apoA-I$^{-/-}$ mice and ABCA1$^{-/-}$ mice on different days postinfection with 8 × 10^8 pfu of the adenovirus expressing the human apoCIII or the control AdGFP virus. (A and C) Plasma cholesterol levels of apoE$^{-/-}$ × apoA-I$^{-/-}$ mice (n = 6) (A) and ABCA1$^{-/-}$ mice (n = 8) (C) on different days postinfection. (B and D) Plasma triglyceride levels of apoE$^{-/-}$ × apoA-I$^{-/-}$ mice (n = 6) (B) and ABCA1$^{-/-}$ mice (n = 8) (D) on different days postinfection. Panel D also shows plasma triglyceride levels of ABCA1$^{-/-}$ mice infected with a mixture of 8 × 10^8 pfu of the apoCIII-expressing adenovirus and 5 × 10^8 pfu of the adenovirus expressing the human lipoprotein lipase (LpL) (n = 6). The statistical significance of the observed differences among groups at each time point is as indicated (one asterisk corresponds to $p < 0.05$, and two asterisks correspond to $p < 0.005$).

7B), consistent with the accumulation of total cholesterol, phospholipids, and triglycerides in this fraction (Figure 5D−F). ELISA analysis showed that VLDL of apoE$^{-/-}$ × apoA-I$^{-/-}$ mice infected with AdGFP-CIII$_g$ had a human apoCIII content of 3.32 ± 0.37 mg/dL while VLDL of ABCA1$^{-/-}$ mice infected with the same virus had a human apoCIII content of 11.78 ± 0.23 mg/dL.

The hepatic VLDL-TG secretion assay did not reveal any significant differences in the secretion rates of VLDL triglycerides between apoE$^{-/-}$ × apoA-I$^{-/-}$ mice and ABCA1$^{-/-}$ mice infected with 8 × 10^8 pfu of the human apoCIII-expressing adenovirus (secretion rates of 4.3 ± 0.1 mg dL^{-1} min^{-1} for apoE$^{-/-}$ × apoA-I$^{-/-}$ mice vs 4.5 ± 0.3 mg dl^{-1} min^{-1} for ABCA1$^{-/-}$ mice). However, co-infection of ABCA1$^{-/-}$ mice with 8 × 10^8 pfu of AdGFP-CIII$_g$ and 5 × 10^8 pfu of an adenovirus expressing the human lipoprotein lipase (LpL) (*49*) ameliorated the apoCIII-induced hypertriglyceridemia (Figure 3D), suggesting that accumulation of apoCIII in the VLDL of ABCA1$^{-/-}$ mice resulted in inhibition of LpL-mediated lipolysis of VLDL triglycerides and triggered hypertriglyceridemia.

ApoCIII Promotes the Biogenesis of HDL Particles in ApoE$^{-/-}$ × ApoA-I$^{-/-}$ Mice, and This Process Requires the Participation of ABCA1. Since infection of apoE$^{-/-}$ × apoA-I$^{-/-}$ mice with AdGFP-CIII$_g$ led to an increased level of accumulation of human apoCIII in the HDL, the next set of experiments was designed to determine whether expression of apoCIII in these mice promotes the biogenesis of HDL particles. Negative staining electron microscopy analysis of a pool of HDL fractions isolated from apoE$^{-/-}$ × apoA-I$^{-/-}$ mice infected with 8 × 10^8 pfu of the control AdGFP virus for 5 days revealed the presence of only very small and very few particles and no HDL formation (Figure 8A). In contrast, similar analysis revealed the formation of a mixture of discoidal and spherical HDL particles in apoE$^{-/-}$ × apoA-I$^{-/-}$ mice following infection with 8 × 10^8 pfu of the human apoCIII-expressing adenovirus for 5 days (Figure 8B).

To test the involvement of ABCA1 in the biogenesis of apoCIII-containing HDL, electron microscopy analysis was also performed on pools of HDL fractions isolated from plasma of ABCA1$^{-/-}$ mice infected with 8 × 10^8 pfu of either the control AdGFP or the AdGFP-CIII$_g$ adenovirus, 5 days postinfection. As expected, ABCA1$^{-/-}$ mice infected with the control AdGFP adenovirus had few very small (non-HDL-sized) particles (Figure 8C). A similar pattern was also obtained in ABCA1$^{-/-}$ mice infected with AdGFP-CIII$_g$ for 5 days (compare panels C and D of Figure 8), consistent with the absence of apoCIII from the HDL fractions of these mice (Figure 7B).

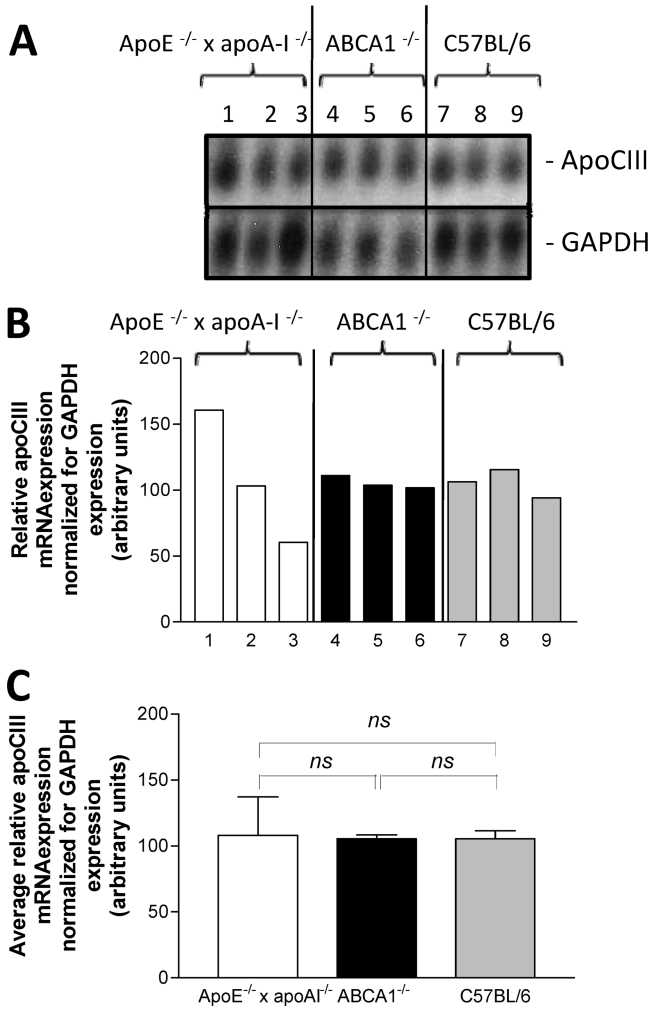

FIGURE 4: Representative mRNA analysis of mice infected with 8×10^8 pfu of the recombinant adenovirus expressing the human apoCIII. Total RNA was isolated from livers of infected mice on day 5 postinfection and analyzed by Northern blotting for human apoCIII and GAPDH mRNA levels. (A) Representative autoradiograms of Northern blot analysis of RNA from the livers of C57BL/6, apoE$^{-/-}$ × apoA-I$^{-/-}$, and ABCA1$^{-/-}$ mice infected with 8×10^8 pfu of AdGFP-CIII$_g$. (B) Relative human apoCIII mRNA expression normalized for GAPDH expression in individual mice. (C) Average relative apoCIII mRNA expression normalized for GAPDH mRNA levels, for each mouse strain (n*s* indicates statistically nonsignificant differences).

DISCUSSION

ApoCIII is an important component of the lipid and lipoprotein transport system and is primarily responsible for modulating plasma triglyceride levels. Excess apoCIII in plasma results in hypertriglyceridemia, one of the components of the metabolic syndrome, mainly due to inhibition of plasma LpL activity (*16, 17, 31*−*33*). In this study, a moderate dose of 8×10^8 pfu of the apoCIII-expressing adenovirus that does not trigger hypertriglyceridemia in WT C57BL/6 mice was used. This moderate level of human apoCIII expression allowed the careful assessment of the sensitivity of ABCA1$^{-/-}$ mice to hypertriglyceridemia in response to apoCIII expression and the contribution of ABCA1 to the formation of apoCIII-containing HDL. The data presented here establish that deficiency in ABCA1 prevents the formation of apoCIII-containing HDL, results in excess accumulation of apoCIII on VLDL, and increases the sensitivity to apoCIII-induced hypertriglyceridemia.

Previous studies (*50, 52*) have shown that lipid-poor apoE and apoA-I interact functionally with ABCA1 and this interaction is required for the formation of apoE- and apoA-I-containing HDL (reviewed in ref *52*). Here, the ability of apoCIII to promote the de novo biogenesis of HDL was tested, using apoE$^{-/-}$ × apoA-I$^{-/-}$ mice that do not form classical apoE- or apoA-I-containing HDL particles. It was found that in apoE$^{-/-}$ × apoA-I$^{-/-}$ mice infected with 8×10^8 pfu of AdGFP-CIII$_g$, a significant amount of human apoCIII was found in HDL, while the rest was distributed among VLDL/IDL and LDL fractions. In agreement with these findings, further analysis of the HDL fraction by electron microscopy revealed the presence of a mixture of spherical and discoidal particles.

It is a common belief that lipid-poor apoCIII associates randomly in the circulation with existing classical apoE- and apoA-I-containing HDL (*2*). However, the data presented here establish that the accumulation of apoCIII on HDL in vivo is not simply the result of a random association of apoCIII with preexisting HDL since infection of apoE$^{-/-}$ × apoA-I$^{-/-}$ mice with AdGFP-CIII$_g$ results in the accumulation of apoCIII in HDL fractions and the formation of a mixture of discoidal and spherical HDL-like particles. Furthermore, this process depends on the action of the lipid transporter ABCA1.

The requirement of ABCA1 in the formation of apoCIII-containing HDL was tested by classical biochemical methods and electron microscopy (EM) analysis of HDL fractions. EM analysis showed that infection of the ABCA1$^{-/-}$ mice with the human apoCIII-expressing adenovirus did not result in either discoidal or spherical particles. In agreement with these findings, expression of human apoCIII in ABCA1$^{-/-}$ mice resulted in the exclusive accumulation of apoCIII on VLDL, while only trace amounts of apoCIII were detectable in the other lipoprotein fractions. It is possible that the presence of trace amounts of apoCIII in HDL or LDL may be an artifact of the fractionation process during density gradient ultracentrifugation. Thus, the findings of this study establish that functional interactions between ABCA1 and apoCIII are essential for the formation of apoCIII-containing HDL-like particles in vivo, which are distinct from classical HDL containing apoE or apoA-I. Furthermore, these findings are in full agreement with the work of Fitzgerald and coworkers (*53*), who showed that in cultures of 293 cells transfected with wild-type ABCA1 cDNA, purified apoCIII interacted with ABCA1, stimulated lipid efflux, and inhibited cross-linking of wild-type apoA-I to ABCA1.

An increased level of accumulation of apoCIII in the VLDL of ABCA1$^{-/-}$ mice correlated with increased VLDL triglyceride and phospholipid content while cholesterol content remained unchanged, as compared to those of ABCA1$^{-/-}$ mice infected with the control adenovirus. The increase in VLDL phospholipid content is consistent with increased VLDL size due to defective lipolysis and impaired clearance of VLDL triglycerides.

In our studies, moderate levels of apoCIII expression that do not trigger hypertriglyceridemia in WT C57BL/6 or apoE$^{-/-}$ × apoA-I$^{-/-}$ mice resulted in significant hypertriglyceridemia in ABCA1$^{-/-}$ mice. The increased sensitivity of ABCA1$^{-/-}$ mice to apoCIII-induced hypertriglyceridemia

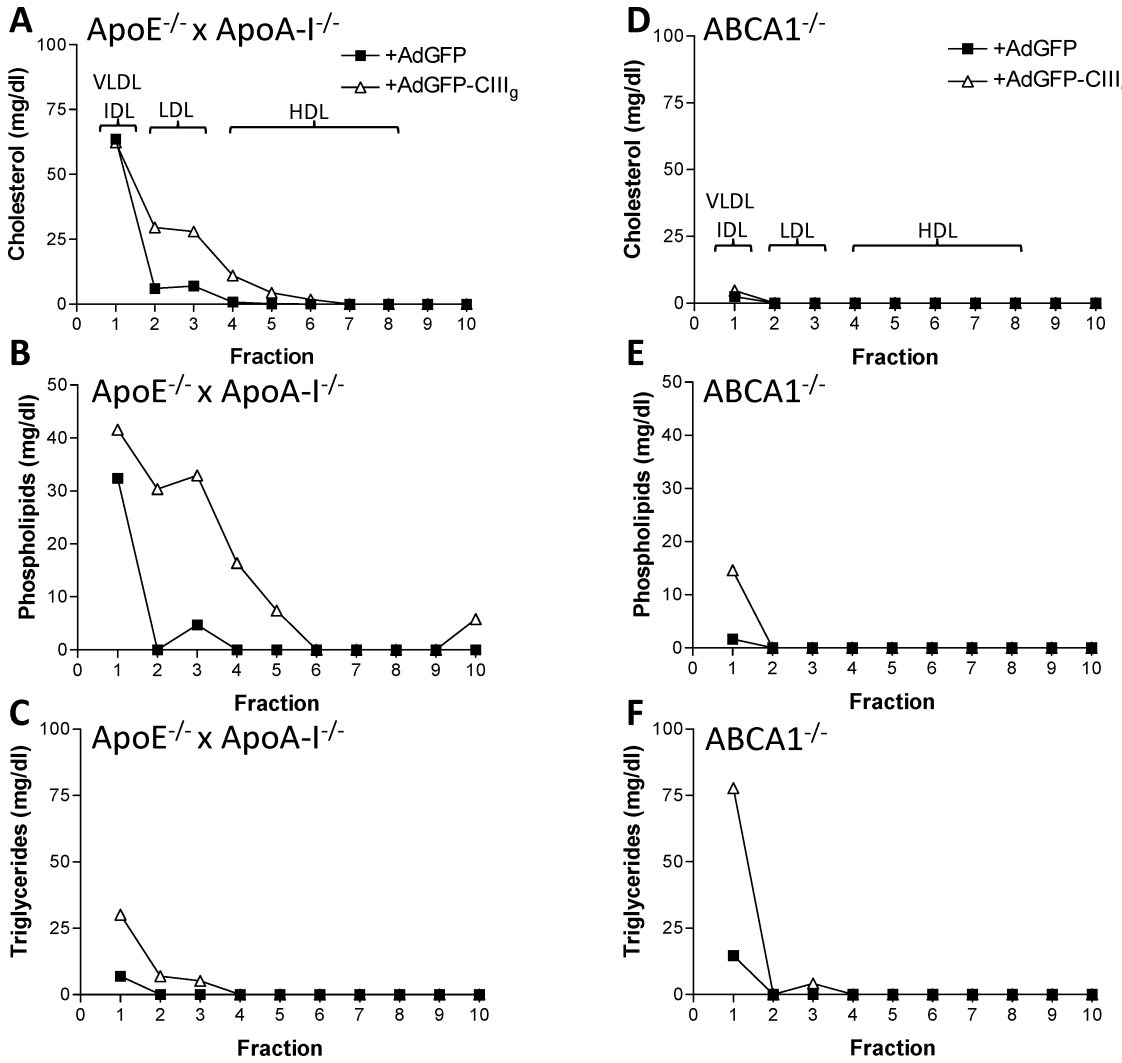

FIGURE 5: Cholesterol (A and D), phospholipid (B and E), and triglyceride (C and F) levels of lipoprotein fractions isolated by density gradient ultracentrifugation analysis of pools of plasma samples from apoE$^{-/-}$ × apoA-I$^{-/-}$ mice (A−C) and ABCA1$^{-/-}$ mice (D−F) infected with 8×10^8 pfu of the human apoCIII-expressing adenovirus or the control adenovirus AdGFP, 5 days postinfection.

correlated with accumulation of excess human apoCIII in the VLDL of these mice. A hepatic VLDL-TG secretion assay did not reveal any significant differences in the rates of hepatic triglyceride secretion between ABCA1$^{-/-}$ and apoE$^{-/-}$ × apoA-I$^{-/-}$ mice infected with 8×10^8 pfu of AdGFP-CIII$_g$. However, co-infection of ABCA1$^{-/-}$ mice with the human apoCIII-expressing adenovirus and an adenovirus expressing LpL ameliorated the apoCIII-induced hypertriglyceridemia. These findings suggest that in the absence of ABCA1, inhibition of LpL activity by excess accumulation of apoCIII in the VLDL of ABCA1$^{-/-}$ mice is responsible for the development of hypertriglyceridemia and the diminished rate of conversion of VLDL into LDL in these mice.

Expression of apoCIII in apoE$^{-/-}$ × apoA-I$^{-/-}$ mice was also accompanied by an increase in the free cholesterol content of mainly their HDLs and LDLs. This finding is consistent with previous reports that have identified apoCIII as an inhibitor of LCAT activity in in vitro LCAT assays (*51*). With an increased level of human apoCIII expression in the apoE$^{-/-}$ × apoA-I$^{-/-}$ mice, LCAT activity is inhibited, resulting in slower esterification of the free cholesterol of discoidal HDL, and a reduced rate of conversion of discoidal HDL particles into spherical. Similarly, in a separate experiment, expression of human apoCIII in wild-type C57BL/6 mice which normally contain classical spherical HDL resulted in a mixture of discoidal and spherical HDL particles (data not shown), further confirming the in vitro data (*51*) for the role of apoCIII as an inhibitor of LCAT.

It has been suggested that increased plasma apoCIII levels lead to an accumulation of apoB- and triglyceride-rich particles (LpB:apoCIII) in the circulation and result in a decrease in the relative HDL-to-VLDL apoCIII ratio (*54*). It is possible that, under conditions of apoCIII overexpression, the ABCA1-mediated pathway of apoCIII-HDL formation becomes saturated and excess lipid-poor apoCIII is now available for binding to VLDL, leading to hypertriglyceridemia. Conditions that promote the accumulation of apoCIII on HDL, such as increased ABCA1 activity, may lead to the preferential accumulation of apoCIII on HDL, reduction in the apoCIII content of VLDL, and prevention of apoCIII-induced hypertriglyceridemia.

Previous studies showed that under conditions of overexpression, apoE (*46, 55*), apoA-I (*56*), and apoA-II (*57*) accumulate on TG-rich VLDL and inhibit LpL activity, thus leading to hypertriglyceridemia. It is possible that, in a fashion similar to that of apoCIII, ABCA1 deficiency may also increase the sensitivity of mice to apoE-, apoA-I-, or

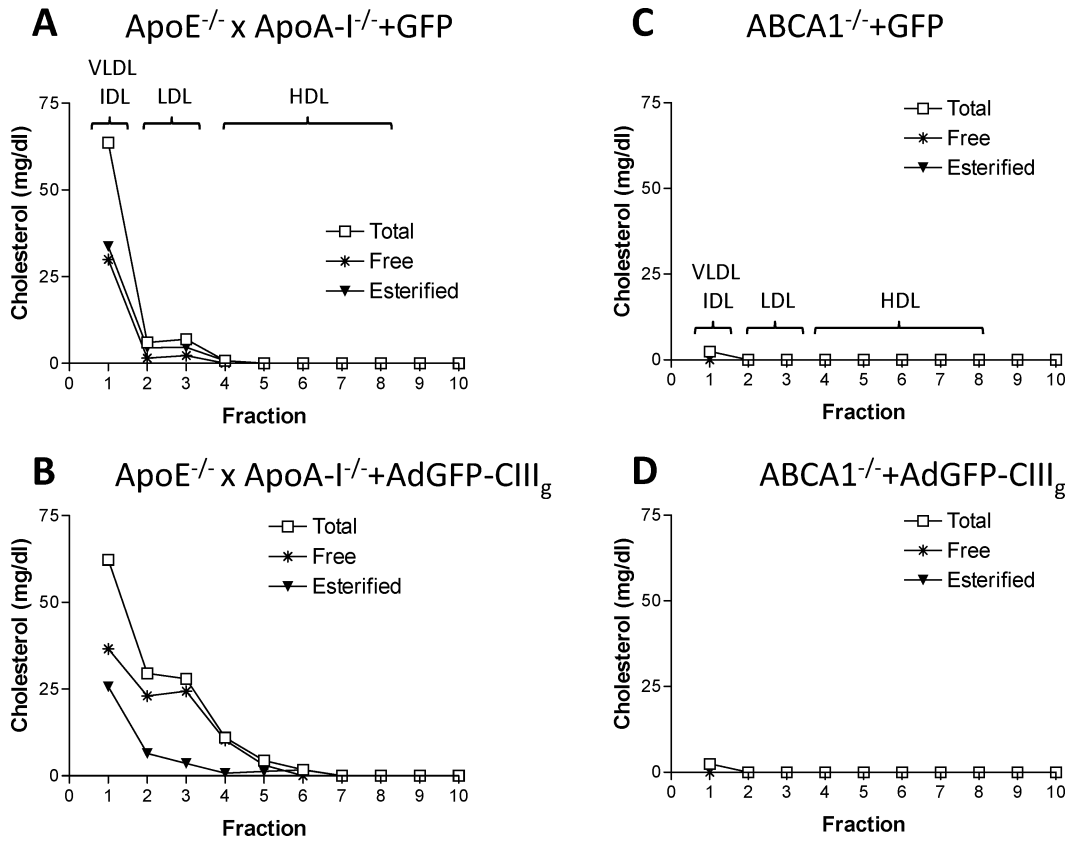

FIGURE 6: Total, free, and esterified cholesterol levels of lipoprotein fractions isolated following density gradient ultracentrifugation analysis of pools of plasma samples from apoE$^{-/-}$ × apoA-I$^{-/-}$ mice (A and B) and ABCA1$^{-/-}$ mice (C and D) infected with 8 × 10^8 pfu of the control adenovirus AdGFP (A and C) or the human apoCIII-expressing adenovirus (B and D), 5 days postinfection.

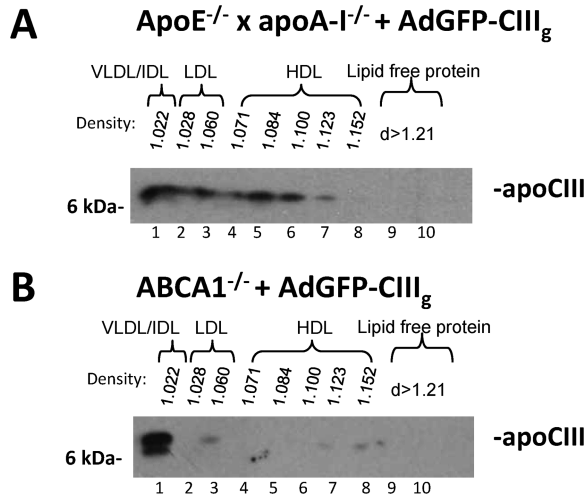

FIGURE 7: ApoCIII distribution in different lipoprotein fractions isolated by density gradient ultracentrifugation of pools of plasma from apoE$^{-/-}$ × apoA-I$^{-/-}$ mice and ABCA1$^{-/-}$ mice infected with the AdGFP-CIII$_g$ adenovirus for 5 days. Plasma samples from apoE$^{-/-}$ × apoA-I$^{-/-}$ mice (A) and ABCA1$^{-/-}$ mice (B) infected with 8 × 10^8 pfu of the AdGFP-CIII$_g$ adenovirus were isolated on day 5 postinfection and fractionated by density gradient ultracentrifugation. Different density fractions were resolved on a 15% SDS−PAGE gel and then analyzed by Western blot analysis for human apoCIII using a specific anti-human apoCIII antibody. The densities of each fraction and the lipoprotein classes to which they correspond are indicated.

apoA-II-induced hypertriglyceridemia by preventing the formation of apoE-, apoA-I-, or apoA-II-containing HDL and promoting the accumulation of an excess of these apolipoproteins in the TG-rich VLDL.

In humans, mutations in ABCA1 that impair its functions cause Tangier's disease (*58*), an autosomal codominant disorder that is characterized by an extremely marked reduction in the levels of plasma HDL cholesterol and mild to moderate hypertriglyceridemia (*59, 60*). In previous studies, analysis of the composition (*59, 61*) and post-heparin lipolytic activities of VLDL (*59*) from patients with Tangier's disease showed that deficiency in ABCA1 results in an abnormal apolipoprotein composition of VLDL, reduced reactivity of VLDL triglycerides with plasma LpL, and hypertriglyceridemia (*59*). The data presented here provide a mechanistic interpretation for the hypertriglyceridemia associated with Tangier's disease. Specifically, the findings of this study suggest that, in addition to its other established properties, HDL may also act as a buffer that prevents accumulation of excess plasma apolipoproteins (such as apoCIII and possibly other apolipoproteins) in VLDL. In the absence of ABCA1, this HDL buffering capacity is eliminated, resulting in the abnormal apolipoprotein composition of VLDL and the hypertriglyceridemia that have been previously documented in patients with Tangier's disease.

In summary, our findings show that in the presence of ABCA1, apoCIII expression promotes the formation of apoCIII-containing HDL and limits the amount of lipid-poor apoCIII that is available for association with TG-rich VLDL (Figure 9, +ABCA1 branch). This results in normal lipolysis of VLDL triglycerides in plasma and the efficient removal of lipoprotein remnants from the circulation. In contrast, deficiency in ABCA1 prevents the de novo biogenesis of apoCIII-containing HDL and promotes accumulation of the vast majority of plasma apoCIII on TG-rich VLDL (Figure

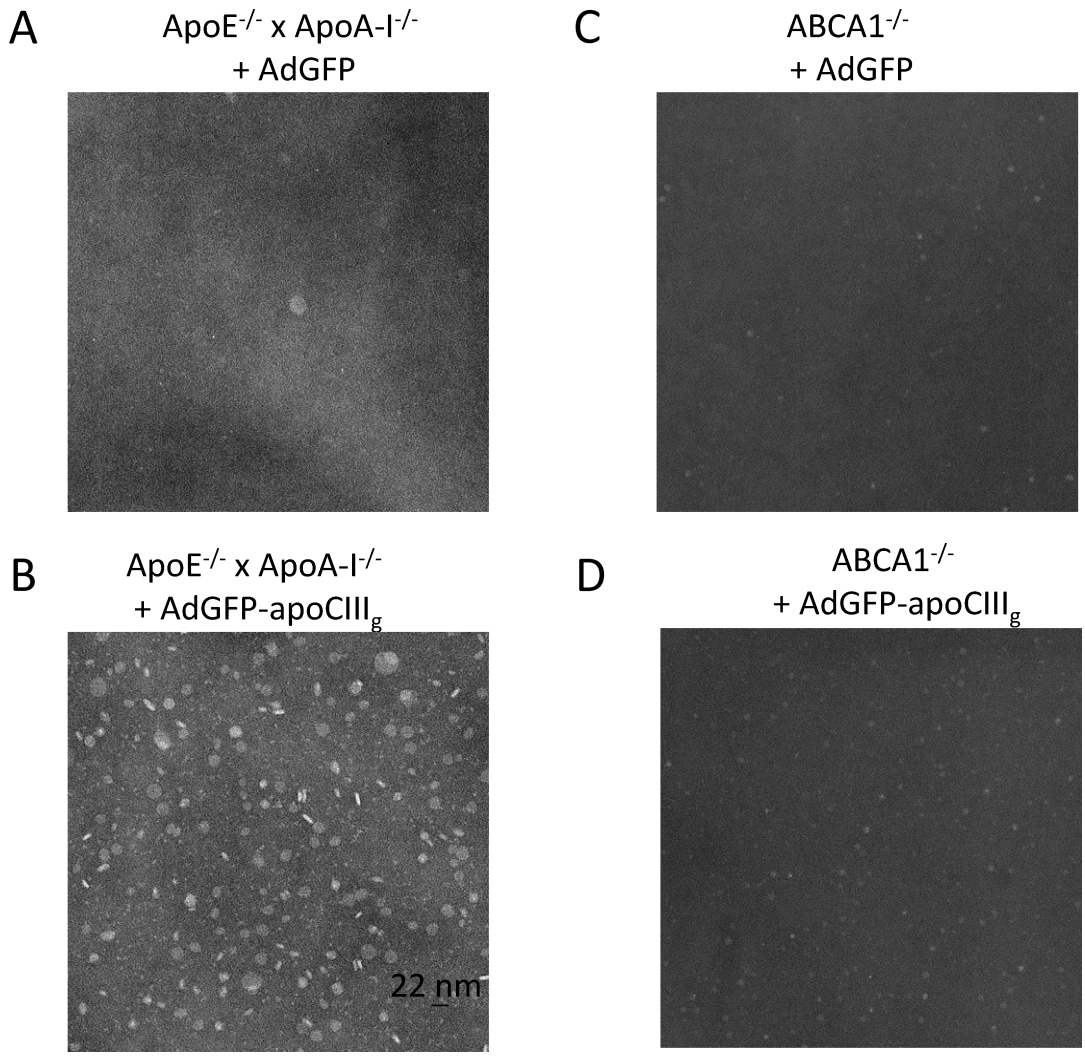

FIGURE 8: Electron microscopy analysis of pools of HDL fractions isolated from apoE$^{-/-}$ × apoA-I$^{-/-}$ mice (A and B) and ABCA1$^{-/-}$ mice (C and D) infected with 8 × 10^8 pfu of the control AdGFP adenovirus (A and C) or the AdGFP-CIII$_g$ adenovirus (B and D) 5 days postinfection.

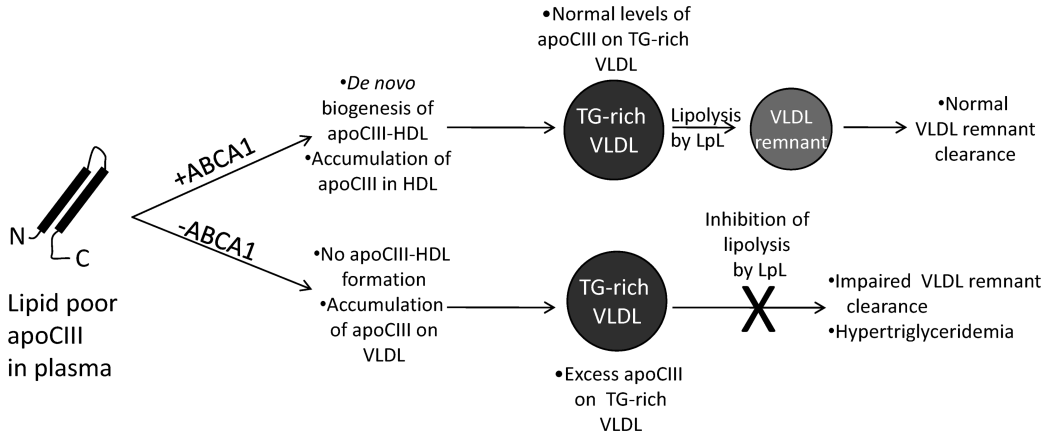

FIGURE 9: Schematic representation summarizing the role of apoCIII and ABCA1 in the biogenesis of apoCIII-containing HDL and the development of hypertriglyceridemia. ApoCIII is synthesized mainly in the liver and secreted in the form of lipid-poor apolipoprotein in the circulation. In the presence of ABCA1 (+ABCA1 branch), apoCIII acquires cholesterol and promotes the de novo biogenesis of apoCIII-containing HDL particles that are distinct from the classical apoA-I- and apoE-containing HDL. The activation of this pathway results in the sequestration of apoCIII mainly in the HDL, thus limiting the amount of apoCIII available for association with TG-rich VLDL. This results in normal LpL activity and lipolysis of VLDL triglycerides and the efficient removal of lipoprotein remnants from the circulation. In contrast, in the absence of ABCA1 (−ABCA1 branch), no apoCIII-containing HDL is formed, and the vast majority of lipid-poor plasma apoCIII accumulates in TG-rich VLDL. This results in inhibition of LpL activity, impaired clearance of triglyceride-rich VLDL from the circulation, and hypertriglyceridemia.

9, −ABCA1 branch). This results in a reduced level of LpL-mediated lipolysis of VLDL triglycerides and the development of hypertriglyceridemia.

Overall, this study identifies apoCIII-containing HDL and the lipid transporter ABCA1 as important contributors to the prevention of apoCIII-induced hypertriglyceridemia. Since plasma apoCIII levels correlate with an increased body mass index and the development of insulin resistance (*35*), it is possible that apoCIII-containing HDL and the lipid transporter ABCA1 may also play important role in the prevention of insulin resistance and type II diabetes.

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