The Role of Different Regions of ATP-Binding Cassette Transporter A1 in Cholesterol Efflux†

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Received March 9, 2007; Revised Manuscript Received June 19, 2007

ABSTRACT: ABCA1 is a key element of cholesterol efflux, but the mechanism of ABCA1-dependent cholesterol efflux is still unclear. Monoclonal antibodies against ABCA1 were used to map functional domains of ABCA1. Two antibodies were directed against a fragment of the first extracellular loop of ABCA1, and the third antibody was directed against a fragment of the fourth extracellular loop. One antibody against the first loop inhibited cholesterol efflux from human macrophages without inhibiting apolipoprotein A-I (apoA-I) binding and internalization. Another antibody against the first loop inhibited apoA-I binding and internalization without inhibiting cholesterol efflux. The antibody against the fourth loop inhibited apoA-I binding to ABCA1 but enhanced cholesterol efflux from macrophages and reduced intracellular cholesterol content. This antibody also increased cholesterol efflux from HeLa cells transfected with ABCA1 but not from cells with ΔPEST-ABCA1. The mechanism of the stimulating effect of this antibody on cholesterol efflux was found to be stabilization of ABCA1 leading to the increase in abundance of cell surface ABCA1. We conclude that a site on the first extracellular loop is required for cholesterol efflux, whereas a site on the fourth extracellular loop may be responsible for ABCA1 stability.

ABCA1[1] is a protein responsible for the initial lipidation of apolipoprotein A-I (apoA-I) and is a key element of reverse cholesterol transport (RCT). ABCA1 plays a dual role in RCT. It mediates the rate-limiting step in cholesterol efflux from a variety of cells, including macrophages, thus protecting against development of atherosclerosis (*1*). ABCA1 is also a key element in the formation of high-density lipoprotein (HDL) (*2*), the anti-atherogenic properties of which are well established. ABCA1 knockout mice do not have HDL and are susceptible to atherosclerosis (*3*). Selective knockout of ABCA1 in macrophages is also atherogenic despite having limited effect on plasma HDL levels (*1*). Overexpression of ABCA1 results in higher plasma HDL levels and enhanced protection against the development of atherosclerosis (*4*). Lack of ABCA1 in humans causes Tangier disease, a disorder characterized by the virtual absence of HDL in plasma and nonexisting reverse cholesterol transport (*5*), while mutations of ABCA1 in humans are major causes of severe hypoalphalipoproteinemia (*6*, *7*).

The natural acceptor of lipids (cholesterol and/or phospholipid) released from cells via the ABCA1-dependent mechanism is apoA-I; however, the mechanisms of ABCA1-dependent cholesterol efflux are yet to be revealed. It has been suggested that binding of apoA-I to ABCA1 is required for loading lipids to apoA-I, which occurs on the plasma membrane (*8*, *9*). Another hypothesis suggested that internalization of the ABCA1−apoA-I complex is required for lipid loading of apoA-I to occur intracellularly (*10*, *11*). A different hypothesis proposes that the presence of ABCA1 on the plasma membrane leads to a redistribution of lipids, making them available for efflux (*12*). Further, there is compelling evidence that apoA-I in addition to being a cholesterol acceptor is essential for the regulation of ABCA1 abundance by preventing its degradation (*13*, *14*). Thus, although binding of apoA-I to ABCA1 is clearly required for the cholesterol efflux pathway to function, it is not clear which step of the efflux pathway is affected by the binding: apoA-I internalization, loading of lipids to apoA-I, or ABCA1 stability. Further, it is not known how these functions of apoA-I are related to each other: e.g., whether apoA-I internalization is required for cholesterol efflux or cholesterol efflux is required for sustaining ABCA1 stability. We have used a panel of three monoclonal antibodies against ABCA1 to discriminate between these possibilities. We have found that distinct sites of ABCA1 are likely responsible for apoA-I binding, internalization, ability to support cholesterol efflux, and stability of ABCA1.

MATERIALS AND METHODS

Monoclonal Antibodies against ABCA1. (A) Antigens. An 18 amino acid peptide corresponding to the sequence of human ABCA1 602−620 was synthesized commercially

† This work was supported by a grant from the National Heart Foundation of Australia (G 04M 1536). D.S. is a fellow of the National Health and Medical Research Council of Australia.

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[1] Abbreviations: ABCA1, ATP-binding cassette transporter A1; apoA-I, apolipoprotein A-I; HDL, high-density lipoprotein; LDL, low-density lipoprotein; RCT, reverse cholesterol transport.

10.1021/bi700473t CCC: $37.00
Published on Web 07/26/2007

(Mimotopes). Cysteine was attached to the amino-terminal end to enable coupling to a carrier. The final sequence was CAIIRVLTGTEKKTGVYMQ. The purity of the peptide was 60−80%. Peptide−KLH and peptide−BSA conjugates were synthesized using Imject maleimide-activated mcKLH and BSA (Pierce, Rockford, IL) according to the manufacturer's instructions.

A recombinant fragment of mouse ABCA1 corresponding to amino acids 1251−1390 of mouse ABCA1 and amino acids 1311−1450 of human ABCA1 was expressed as a fusion protein with MBP using the *Escherichia coli* expression system as described previously (*15*). The sequence used for the expression was MDGKGSYQLKGWKLTQQQFV ALLWKRLLIARRSRKGFFAQIVLPAVFVCILVFSLIVPPF-G K Y P S L E L Q P W M Y N E Q Y T F V S N D A P E D-MGQELLNALTKDPGFGTRCMEGNPIPDTPCLAGEED-WTISPVPQSIVDLFQ.

(B) Immunization and Fusion. Antibody production against the hABCA1 peptide−KLH and mABCA1 fragment−MBP was initiated by intraperitoneally administering of antigen emulsified with Freund's complete adjuvant to 6−8-week-old female BALB/c mice. The amount of antigen administered was 20, 50, and 100 μg per animal. Two booster injections were performed at 2-week intervals using Freund's incomplete adjuvant. Blood samples were taken from the saphenous vein. The bleeds were tested by ELISA. The best responding mice were intravenously injected once more with antigens without adjuvant. Four days later splenectomy was performed, and splenocytes were isolated using Lympholyte M (Cedarlane, Ontario, Canada), collected, and fused with Sp2/0-Ag14 mouse myeloma cells. Fusion and hybridoma selection were performed as described by Kohler and Milstein (*16*).

(C) Cell Culture. Sp2/0-Ag14 myeloma and hybridoma cells were cultured in RPMI medium 1640 supplemented with 10% fetal bovine serum, 50 units/mL penicillin, 50 μg/mL streptomycin, and 2 mM glutamine. Hybridoma cell culture supernantants were tested by ELISA on plates coated with ABCA1 peptide−BSA or ABCA1 recombinant fragment−MBP, with BSA and MBP used as the negative controls. Responding cells were cloned at least twice by limiting dilution. The subclasses of all of the monoclonal antibodies were determined using an isotyping kit (Sigma, St. Louis, MO). Cells were propagated in a CELLine CL350 bioreactor (Integra Bioscience), and antibodies were purified by affinity chromatography with protein G (for IgG) or using a HiTrap IgM purification HP column (Amersham Biosciences) (for IgM).

The hybridoma cells are available for qualified researchers from the Korean Cell Storage Facility at http://cellbank.snu-.ac.kr/ENG/default.htm.

Cells. Mouse macrophages RAW 264.7 were cultured as described previously (*17*). HEK 293 cells were transiently transfected with mouse ABCA1 using Lipofectamine (Invitrogen) according to the manufacturer's instructions. Human macrophages THP-1 were cultured as described previously (*18*). Prior to experiments, THP-1 cells were differentiated by treatment with 100 ng/mL PMA (Sigma) for 72 h, and when indicated expression of ABCA1 was stimulated by treatment with the LXR agonist TO-901317 (final concentration 4 μmol/L) for 18 h. HeLa cells were transfected using Effectene (Qiagen) according to the manufacturer's instructions. Plasmid with wild-type ABCA1 was a kind gift of Dr. G. Chimini, and ΔPEST-ABCA1 plasmid was a kind gift of Dr. A. Tall. Mock transfection was done with the pCMV-β-gal plasmid. HeLa cells stably transfected with ABCA1 were described previously (*19*).

Lipoproteins. Apolipoprotein A-I was isolated from human plasma as described previously (*15*). LDL was purified from human plasma by sequential centrifugation and acetylated as described by Basu et al. (*20*).

Immunoblotting. Cells grown in 75 cm^2 flasks (approximately 15×10^6 cells per flask) were treated as indicated, washed, and harvested. Soluble membrane protein fractions were isolated as described by Yamauchi et al. (*21*). Total protein was determined by the Bradford assay (Pierce). Proteins were separated on a 6% SDS−PAGE followed by immunoblotting. In brief, PVDF membranes were blocked with 2.5% skim milk solution, washed, incubated for 1 h at room temperature with nondiluted hybridoma cell culture supernantants, washed, incubated with biotinylated goat anti-mouse IgG or IgM (Chemicon), washed, and incubated with streptavidin−HRP conjugate (Chemicon). Bands were visualized by the SuperSignal West Pico chemiluminescent substrate kit (Pierce, Rockford, IL), and the relative intensities of the bands were quantitated by densitometry. Analysis of β-actin was used to control loading control.

Confocal Microscopy. THP-1 cells were grown on sterile glass coverslips to approximately 60% confluence and differentiated by incubation for 48 h with 100 ng/mL PMA. ABCA1 expression was activated by incubation for 18 h with 4 μmol/L TO-901317. Cells were then fixed for 10 min with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, washed with PBS, blocked for 30 min with PBS containing 10% goat serum, washed again, and stained. The primary antibodies were either monoclonal anti-ABCA1, polyclonal anti-ABCA1 (Novus Biologicals), or monoclonal or polyclonal anti-calnexin antibodies. Secondary antibodies were either Alexa Fluor 488-labeled goat anti-mouse IgM or IgG antibody or Texas Red-labeled goat anti-rabbit antibody (Molecular Probes). Coverslips were then mounted onto glass slides and viewed using a Zeiss META confocal microscope.

Cholesterol and Phospholipid Efflux. Cholesterol and phospholipid efflux experiments were conducted as described previously (*18*). Briefly, THP-1 or HeLa cells were grown in 12-well plates, and when indicated ABCA1 expression in THP-1 cells was boosted by adding 4 μmol/L LXR agonist TO-901317. Cellular cholesterol or phospholipids were labeled by incubation in serum-containing medium with [1α,2α(n)-^{3}H]cholesterol (Amersham; specific radioactivity 1.81 TBq/mmol, final radioactivity 0.5 MBq/mL) or [methyl-^{14}C]choline (0.2 MBq/mL) for 48 h in a CO_2 incubator. Cells were then washed and incubated for 18 h at 37 °C with serum-free medium containing the indicated concentrations of the antibodies. Cells were washed and incubated for 3 h at 37 °C with serum-free medium containing 30 μg/mL lipid-free apoA-I. For cholesterol efflux analysis, the medium was collected and centrifuged for 15 min at 4 °C at 10000*g*, and aliquots of the supernatant were counted in a β-counter. Cells were harvested, and radioactivity was counted. For phospholipid efflux, phospholipids were isolated from medium and cells by TLC as described previously (*22*), and radioactivity was counted.

Cholesterol and phospholipid efflux was expressed as a proportion of [^{3}H]cholesterol or [^{14}C]phospholipid transferred from cells to medium.

Binding Assay. ApoA-I was iodinated using Iodobeads (Pierce) according to the manufacturer's instructions. THP-1 cells were grown as described above in 12-well plates, and when indicated the expression of ABCA1 was boosted by 4 μmol/L LXR agonist TO-901317. Cells were incubated for 2 h at 37 °C in serum-free medium containing 0.1% BSA (essentially fatty acid free; Sigma) and 5 μg/mL ^{125}I-apoA-I in the presence or absence of 250 μg/mL unlabeled apoA-I. Medium was used to assess ^{125}I-apoA-I degradation. Cells were washed three times with serum-free medium containing 0.1% BSA and once with Ca^{2+},Mg^{2+}-free PBS and treated with 0.05% trypsin/0.002% EDTA for 5 min at 37 °C. The reaction was stopped by adding 100 μL of fetal calf serum. Radioactivity in the medium (binding) and cells (internalization) was determined with a γ-counter. Degradation was determined as non-iodine, trichloroacetic acid-soluble radioactivity in the incubation medium as previously described (*23*).

To measure apoA-I binding specifically to ABCA1, a cross-linking assay was used as described by Wang et al. (*9*). Briefly, cells were incubated with the indicated concentrations of the anti-ABCA1 antibodies for 18 h. ApoA-I was added to the final concentration of 50 μg/mL, incubated for 1 h at 37 °C, and washed out. Dithiobis(succinimidyl)-propionate (DSP; Pierce) was added to the final concentration of 250 μmol/L and incubated for 1 h at room temperature. Cells were then lysed, and ABCA1 was immunoprecipitated with either monoclonal anti-ABCA1 antibody (Novus) (THP-1) or monoclonal anti-GFP antibody (Sigma) (HeLa-ABCA1). Samples were diluted with loading buffer containing 5% 2-mercaptoethanol, boiled for 5 min, and subjected to 12% SDS−PAGE and Western blot; apoA-I was detected using monoclonal anti-apoA-I antibody AI-4.1.

Analysis of Cell Surface ABCA1. For cell surface ABCA1 analysis, cells were first biotinylated with 10 mmol/L sulfo-NHS-SS-biotin (Pierce Chemical Co., Rockford, IL) at 4 °C for 30 min and then lysed with RIPA buffer at 4 °C. After centrifugation, the supernatant of cell lysate was incubated with UltraLink immobilized streptavidin beads (Pierce) overnight at 4 °C. Following centrifugation and washing, the collected beads were resuspended in SDS−PAGE sample buffer with 100 mmol/L 2-mercaptoethanol and boiled for 2 min. ABCA1 was detected by Western blot using NDF4C2 antibody.

In-Cell Western Blot. THP-1 cells were differentiated, and ABCA1 expression was boosted by incubation with TO-901317 (4 μmol/L) for 18 h. Cells were then washed and incubated for the indicated periods of time with serum-free medium (control), fresh TO-901317 (4 μmol/L), apoA-I (50 μg/mL), or NDF6F1 or NDF4C2 antibodies (10 μg/mL). Cells were washed, fixed with 3.7% formaldehyde in PBS for 20 min, and permeabilized with three 10 min washes with 0.1% Triton X-100 in PBS. Li-Cor blocking buffer was added for 2 h followed by overnight incubation with monoclonal NDF4C2 anti-ABCA1 antibodies and rabbit polyclonal anti-β-actin antibodies in 50% blocking buffer in PBS supplemented with 0.2% Tween 20. After three washes, secondary detection was carried out using two species-specific infrared fluorescent dye conjugated antibodies in 1:1 LBB buffer and PBS supplemented with 0.4% Tween 20. After a 1 h incubation, targets were simultaneously visualized and quantitated using the Odyssey infrared imaging scanner (Li-Cor, Lincoln, NB) with the 680 nm fluorophore emitting a red color and the 800 nm fluorophore emitting a green color. ABCA1 abundance was presented relative to abundance of β-actin.

Cholesterol Biosynthesis and Esterification. To assess cholesterol biosynthesis and esterification, macrophages were incubated for 2 h at 37 °C with [^{3}H]acetic acid sodium salt (Amersham; specific activity 370 GBq/mmol; final radioactivity 740 MBq/mL) and [^{14}C]oleic acid (Amersham; specific activity 2.22 GBq/mmol; final radioactivity 0.185 MBq/mL) complexed to BSA (Sigma; essentially fatty acid free). Cells were washed, and lipids were extracted and analyzed by TLC as described previously (*24*). Spots of cholesterol and cholesteryl oleate were identified by standards (Sigma), scraped, and counted in a β-counter.

Statistical Analysis. All experiments were reproduced two to four times, and representative experiments are shown. Unless otherwise indicated, experimental groups consisted of quadruplicates; means ± SEM are presented. The Student's *t*-test was used to determine statistical significance of the differences.

RESULTS

Antibodies. A proposed topography of ABCA1 (*7*) and location of two ABCA1 fragments used for development of the antibodies are shown in Figure 1. The first fragment was an 18 amino acid peptide corresponding to residues 602−620 of human ABCA1 (which corresponds to residues 542−560 of mouse ABCA1). The difference between human and mouse sequences in the selected region is one amino acid (S for T at position 10). The region belongs to the first extracellular domain of ABCA1 (Figure 1). Two monoclonal antibodies produced from mice injected with the 18 amino acid peptide were named NDF4C2 and NDF3F9. NDF4C2 was determined to be IgM, and NDF3F9 was found to be IgG_3. The second fragment was a 140 amino acid peptide corresponding to residues 1311−1450 of human ABCA1 (residues 1251−1390 of mouse ABCA1). Nine residues are different between the human and mouse sequences; the mouse sequence was used. The region belongs to the fourth extracellular domain of ABCA1 (Figure 1). It is adjacent to the PEST sequence (residues 1283−1306 of human ABCA1) (*13*), the key regulatory region of ABCA1 (*13*, *25*). The antibody produced from mice injected with a recombinant 140 amino acid fragment was named NDF6F1; it was found to be IgM.

The antibodies were tested using ABCA1 from the RAW 264.7 mouse macrophage cell line activated or not with 0.3 mmol/L cAMP. cAMP is known to induce ABCA1 expression and to stimulate ABCA1-dependent cholesterol efflux in mouse macrophages (*26*). In an immunoblot analysis all three antibodies gave strong bands corresponding to ABCA1 (Figure 2A). The amount of ABCA1 in cells activated by cAMP was 5−10 times higher compared to nonactivated cells (Figure 2A). A complete image of NDF6F1 immunostaining of ABCA1 from activated and nonactivated RAW 264.7 cells is also presented in Figure 2A (right panel). The most prominent band detected by the antibody NDF6F1 was

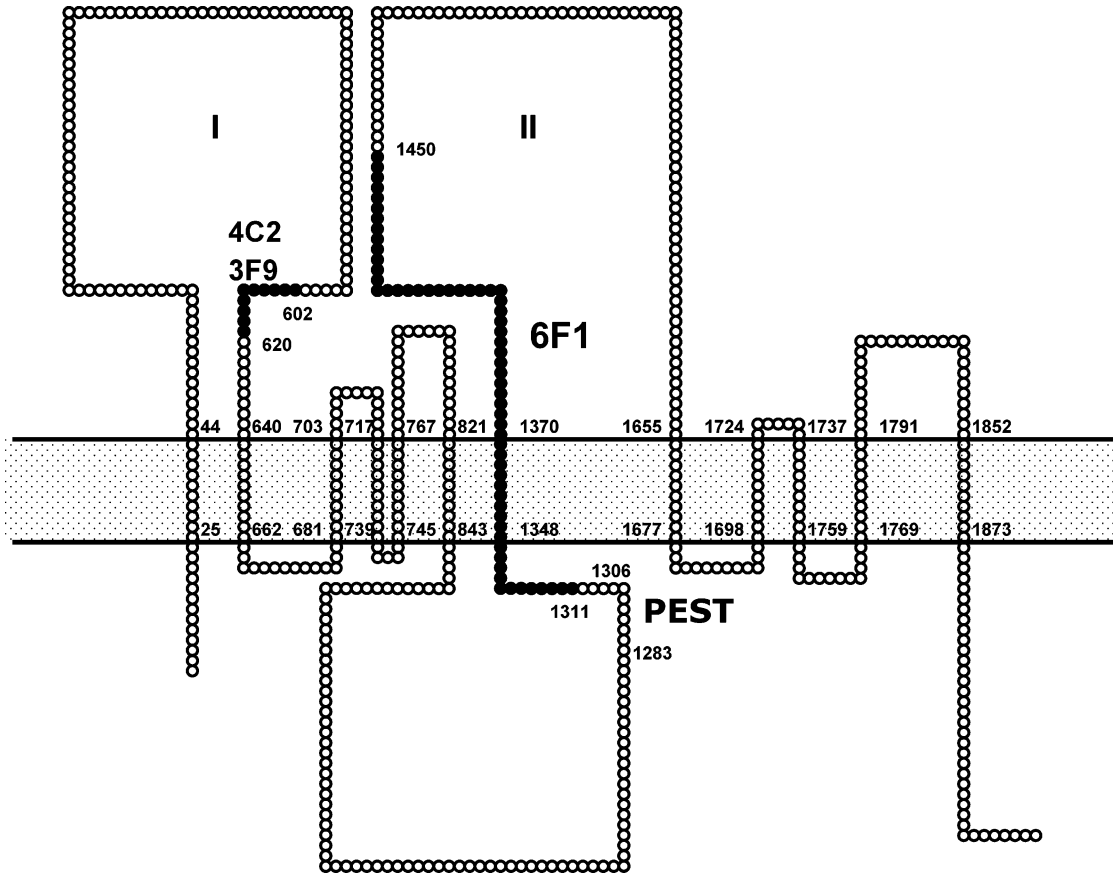

FIGURE 1: 1. Schematic representation of ABCA1 and location of the antibody epitopes. Closed circles denote fragments of ABCA1 chosen as targets for the development of the antibodies. Shaded circles denote the PEST domain.

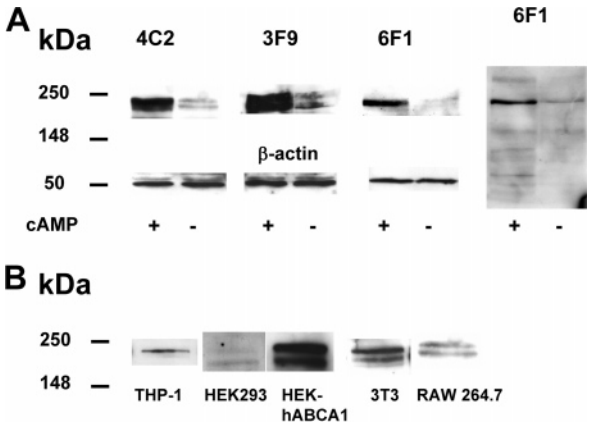

FIGURE 2: 2. Western blotting of activated or nonactivated RAW 264.7 cells (A) or THP-1, HEK 293/hABCA1, 3T3, and RAW 264.7 cells (B) using antibodies against ABCA1. (A) RAW 264.7 cells were activated or not by incubation for 18 h with 0.3 mmol/L cAMP. Cells were then lysed as described in Materials and Methods, and proteins were separated on a 6% SDS−PAGE followed by immunoblotting with the indicated antibodies. The loading control was immunostaining with anti-β-actin antibody. (B) THP-1 cells (differentiated with PMA), HEK 293 cells, HEK 293 cells transiently transfected with human ABCA1, 3T3 cells, or RAW 264.7 cells were grown to confluency. Cells were then lysed as described in Materials and Methods, and proteins were separated on a 6% SDS−polyacrylamide gel followed by immunoblotting with NDF4C2 antibody.

ABCA1. Few weaker bands of lower molecular weight were also seen. Most of these bands were also affected by stimulation with cAMP and most likely represent fragments of degraded ABCA1. Immunostaining with two other antibodies produced similar results (not shown). The antibodies were then used with ABCA1 from various cell types, namely, human monocyte macrophage THP-1, HEK 293 cells transiently transfected with human ABCA1, and mouse fibroblasts 3T3. In all cell types the band similar to that in RAW 264.7 cells was detected by the NDF4C2 antibody (Figure 2B). Two other antibodies also detected similar bands in these cell types (not shown).

The antibodies were further used in immunofluorescence microscopy. Differentiated THP-1 cells were treated with TO-901317 and stained with the antibodies, and the distribution of ABCA1 was studied using confocal microscopy. To assess the localization of ABCA1, cells were also stained with the antibodies against calnexin, a marker for endoplasmic reticulum. All three antibodies stained ABCA1 in both intracellular compartments and plasma membrane (Figure 3A,D,G). There was a partial overlap between the intracellular portion of ABCA1 and calnexin (Figure 3A−I). This distribution is similar to that demonstrated by others (*27*−*29*). The staining was similar to that observed with widely used polyclonal anti-ABCA1 antibodies (Figure 3J−L; note that colors are reversed as polyclonal antibodies were used to detect ABCA1 and monoclonal antibodies were used to detect calnexin). There was only a very weak staining when cells were not activated by TO-901317 and no staining when the first antibodies were omitted (not shown).

Cholesterol and Phospholipid Efflux. To test if the antibodies can affect ABCA1-dependent cholesterol efflux, ABCA1 expression in THP-1 human macrophages was induced by incubation with LXR agonist TO-901317. Cells

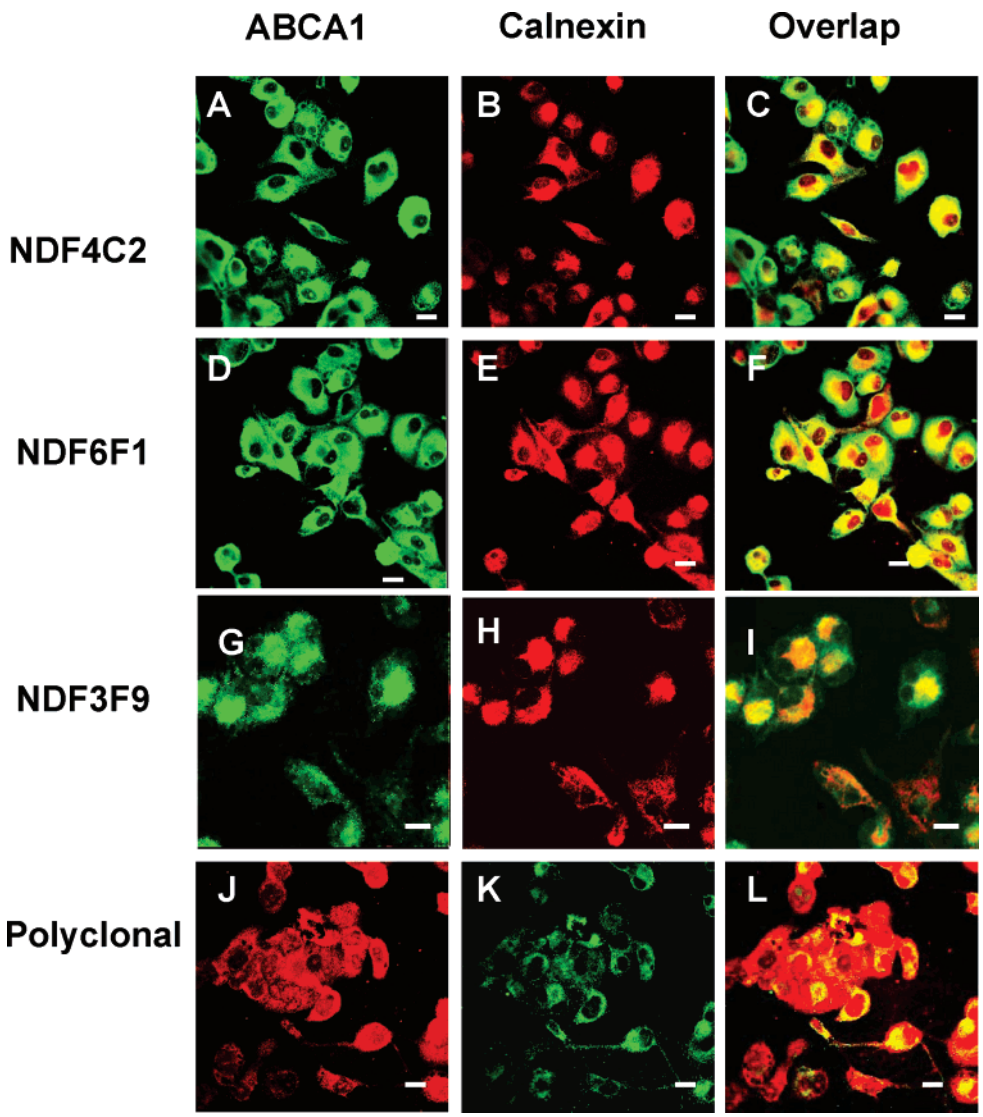

FIGURE 3: 3. Confocal microscopy of THP-1 cells stained with antibodies against ABCA1. THP-1 cells were grown on sterile coverslips to approximately 60% confluence and differentiated by incubation for 48 h with 100 ng/mL PMA, and ABCA1 expression was activated by incubation for 18 h with 4 μmol/L TO-901317. Cells were fixed with 4% paraformaldehyde, permeabilized with 0.2% Triton X-100, washed, and stained. (A) primary antibody, NDF4C2; secondary antibody, Alexa Fluor 488-conjugated goat anti-mouse IgM; (B, E, H) primary antibody, polyclonal anti-calnexin; secondary antibody, Texas Red-labeled goat anti-rabbit antibody; (C) overlap of (A) and (B); (D) primary antibody, NDF6F1; secondary antibody, Alexa Fluor 488-conjugated goat anti-mouse IgM; (F) overlap of (D) and (E); (G) primary antibody, NDF3F9; secondary antibody, Alexa Fluor 488-conjugated goat anti-mouse IgG; (I) overlap of (G) and (H); (J) primary antibody, polyclonal anti-ABCA1 antibody; secondary antibody, Texas Red-labeled goat anti-rabbit antibody; (K) primary antibody, monoclonal anti-calnexin antibody; secondary antibody, Alexa Fluor 488-conjugated goat anti-mouse IgG; (L) overlap of (J) and (K). Bar = 10 μm.

were labeled with [^{3}H]cholesterol and preincubated for 18 h in serum-free medium containing 20 μg/mL each of the antibodies, and cholesterol efflux to lipid-free apoA-I was tested as described in Materials and Methods. Neither nonspecific mouse IgM nor the antibody NDF3F9 affected cholesterol efflux, while the antibodies NDF4C2 and NDF6F1 inhibited cholesterol efflux by approximately 50% ($p < 0.01$) (Figure 4A). The effect of the antibodies on phospholipid efflux was similar to their effect on cholesterol efflux (Figure 4B).

In differentiated THP-1 cells not stimulated with TO-901317 specific cholesterol efflux to apoA-I was 4.5-fold lower compared to stimulated cells, indicating that the abundance of ABCA1 in nonstimulated cells is lower but sufficient to sustain cholesterol efflux to apoA-I. When the effect of the antibodies on cholesterol efflux from THP-1 cells not stimulated with TO-901317 was studied, again NDF3F9 was not active while NDF4C2 inhibited cholesterol efflux (not shown). Surprisingly, however, the antibody NDF6F1 stimulated cholesterol efflux. In a separate experiment a dose dependence of the effect of the antibody NDF6F1 on cholesterol efflux from THP-1 cells, treated or not treated with TO-901317, was studied. In both cases a bell-shaped response was observed (Figure 5). When THP-1 cells were activated, low concentrations of NDF6F1 either slightly inhibited or slightly stimulated cholesterol efflux, while high concentrations clearly inhibited cholesterol efflux (Figure 5A). When ABCA1 in THP-1 cells was not induced, low concentrations of NDF6F1 clearly stimulated cholesterol efflux while higher concentrations did not affect it (Figure 5B). The bell-shaped response suggests that the antibody may have two opposing effects on cholesterol efflux with the

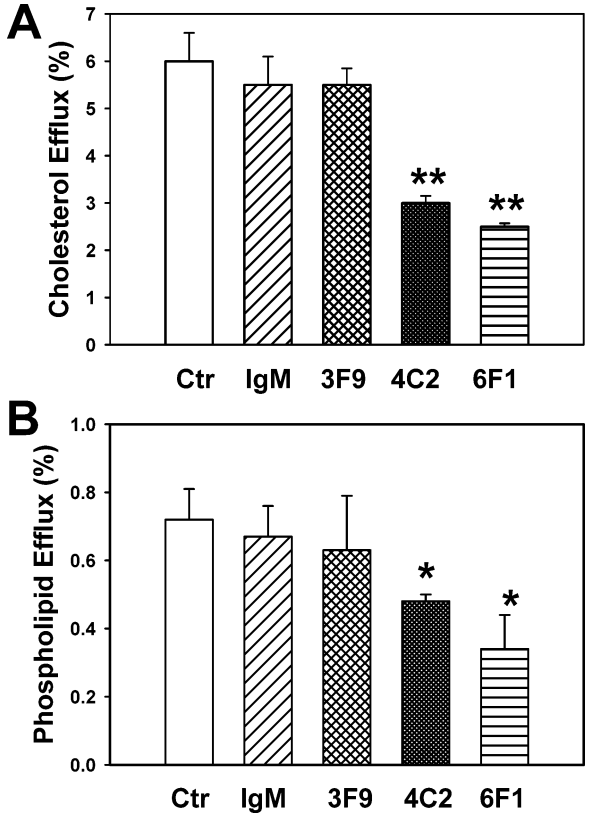

FIGURE 4: 4. Effect of the monoclonal antibodies on cholesterol (A) and phospholipid (B) efflux from THP-1 cells. THP-1 cells were grown in 12-well plates, and ABCA1 expression was boosted by incubation with 4 μmol/L LXR agonist TO-901317. Cellular cholesterol was labeled by incubation of cells in serum-containing medium with [^{3}H]cholesterol or [^{14}C]choline for 48 h in a CO_2 incubator. Cells were then washed and incubated for 18 h at 37 °C with serum-free medium containing the indicated antibodies at the final concentration of 20 μg/mL. Cells were washed and incubated for 2 h at 37 °C with serum-free medium containing 30 μg/mL apoA-I. The medium was then collected and centrifuged for 15 min at 4 °C at 10000*g*, and aliquots of the supernatant and cells were either counted in a β-counter (A) or separated on TLC, and bands corresponding to phospholipid were counted (B). The efflux was expressed as a proportion of [^{3}H]cholesterol or [^{14}C]phospholipid transferred from cells to medium. Means ± SEM of quadruplicate determinations are shown. **, $p < 0.01$; *, $p < 0.05$ versus IgM-treated cells.

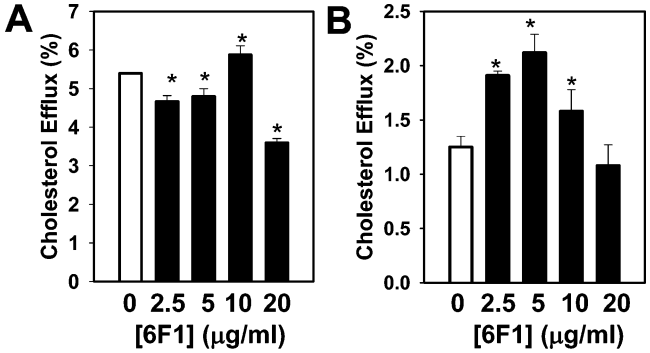

FIGURE 5: 5. Effect of the antibody NDF6F1 on cholesterol efflux from THP-1 cells activated with TO-901317 (A) or nonactivated (B). THP-1 cells were grown in 12-well plates, and when indicated ABCA1 expression was boosted (A) or not (B) by incubation with 4 μmol/L LXR agonist TO-901317. Cellular cholesterol was labeled by incubation of cells in serum-containing medium with [^{3}H]-cholesterol for 48 h. Cells were then washed and incubated for 18 h at 37 °C with serum-free medium containing the indicated concentration of NDF6F1 antibody. Cells were washed and incubated for 3 h at 37 °C with serum-free medium containing 30 μg/mL apoA-I. The medium was then collected and centrifuged for 15 min at 4 °C at 10000*g*, and aliquots of the supernatant were counted in a β-counter. Cells were harvested, and radioactivity was counted. Cholesterol efflux was expressed as a proportion of [^{3}H]-cholesterol transferred from cells to medium. Means ± SEM of quadruplicate determinations are shown. *, $p < 0.01$ versus no antibody.

overall effect depending on a ratio between the concentration of the antibody and abundance of ABCA1. Similar effects were demonstrated when the antibodies were tested using RAW 264.7 mouse macrophages instead of THP-1 human macrophages (not shown).

ApoA-I Binding, Internalization, and Degradation. To test if inhibition or stimulation of cholesterol efflux results from changes in apoA-I binding to cells, we studied the effect of the antibodies on interaction of ^{125}I-apoA-I with activated THP-1 cells. The antibody NDF3F9, which had no effect on cholesterol efflux (Figure 4), inhibited ^{125}I-apoA-I binding and internalization by TO-901317-activated THP-1 cells (Figure 6A,B). The antibody NDF4C2, which inhibited cholesterol efflux (Figure 4), had no effect on ^{125}I-apoA-I binding and internalization by the cells (Figure 6A,B). The antibody NDF6F1, which inhibited or activated cholesterol efflux depending on whether or not ABCA1 expression was activated (Figure 5), inhibited ^{125}I-apoA-I binding to both activated and nonactivated cells (Figure 6A). However, the effect of NDF6F1 on ^{125}I-apoA-I internalization was minimal or absent (Figure 6B). Degradation of ^{125}I-apoA-I was negligible and was not affected by the antibodies (not shown).

ABCA1, however, is not the only binding site for apoA-I, and the majority of cell-bound apoA-I is not bound to ABCA1 (*30*, *31*). To examine the effect of the antibodies on apoA-I binding specifically to ABCA1, THP-1 cells were incubated with apoA-I in the presence or absence of the antibodies; bound apoA-I was then cross-linked to the plasma membrane proteins and immunoprecipitated with anti-ABCA1 antibodies. The amount of ABCA1-bound apoA-I was assessed by Western blot and quantitative densitometry. The amount of ABCA1-bound apoA-I was not reduced by the antibodies NDF3F9 and NDF4C2 but was significantly reduced by the antibody NDF6F1 (20 μg/mL) (Figure 6C). At lower concentration (5 μg/mL) the antibody NDF6F1 reduced the amount of ABCA1-bound apoA-I to a similar extent (not shown). Similar results were obtained when HeLa cells stably transfected with ABCA1 were used instead of THP-1 cells (not shown). These findings suggest that blocking of apoA-I binding to ABCA1 is neither necessary nor sufficient for the inhibition of cholesterol efflux.

ABCA1 Stability. It has been demonstrated in several studies that interaction with apoA-I is essential for ABCA1 stability (*13*). We investigated if the antibody NDF6F1, which competes with apoA-I for binding (Figure 6A), may affect ABCA1 stability similar to the effect of apoA-I. ABCA1 expression in THP-1 cells was boosted by incubation for 18 h with TO-901317 (4 μmol/L); the LXR agonist was then washed out, stopping ABCA1 expression. Cells were then incubated for 18 h in serum-free medium alone or medium containing apoA-I (30 μg/mL) or antibody NDF6F1 (5 μg/mL). Most of the cellular ABCA1 disappeared when cells were incubated with the medium alone, but a significant

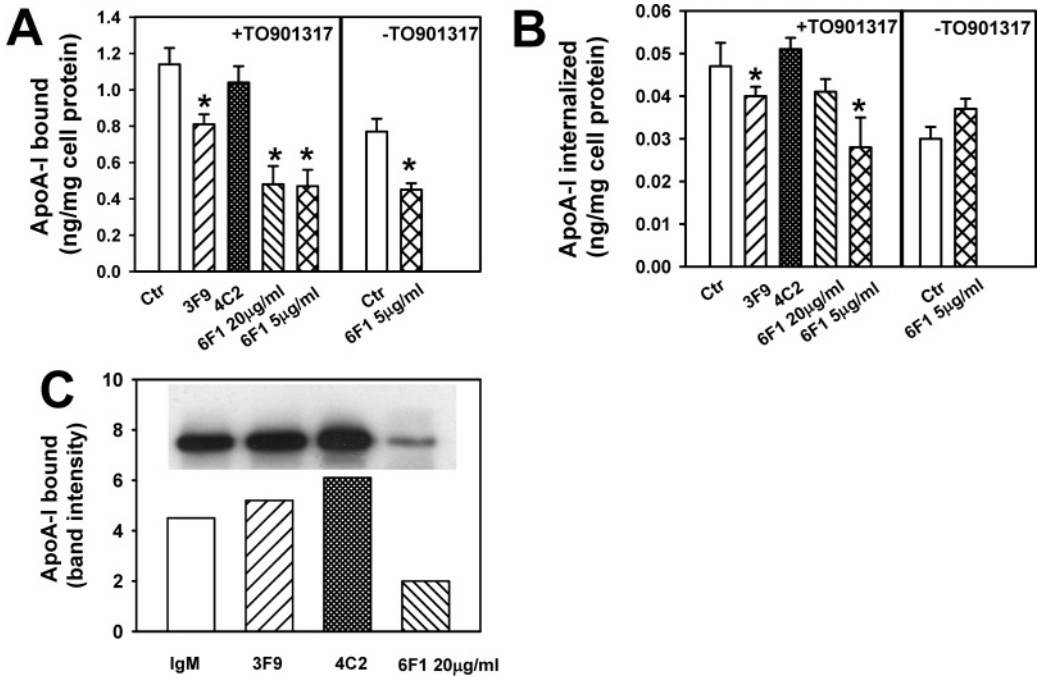

FIGURE 6: 6. Effect of the monoclonal antibodies on ^{125}I-apoA-I binding (A) and internalization (B) by THP-1 cells or cross-linking of apoA-I to ABCA1 in THP-1 cells (C). (A, B) THP-1 cells were grown in 12-well plates, and when indicated the expression of ABCA1 was boosted by incubation with 4 μmol/L LXR agonist TO-901317. Cells were incubated for 2 h at 37 °C in serum-free medium containing 0.1% BSA (essentially fatty acid free; Sigma) and 5 μg/mL ^{125}I-apoA-I in the presence or absence of 250 μg/mL unlabeled apoA-I. Cells were then washed three times with serum-free medium containing 0.1% BSA and once with Ca^{2+},Mg^{2+}-free PBS and treated with 0.05% trypsin/0.002% EDTA. The reaction was stopped by adding 100 μL of fetal calf serum. Radioactivity in the medium (binding) and cells (internalization) was determined in a γ-counter. *, $p < 0.01$ versus no antibody. (C) Quantification of the effect of the antibodies on cross-linking of apoA-I to ABCA1 in THP-1 cells. THP-1 cells were incubated with the indicated antibodies (final concentration 20 μg/mL) for 18 h. ApoA-I was added to the final concentration of 50 μg/mL, incubated for 1 h at 37 °C, and washed out. DSP (Pierce) was added to the final concentration of 250 μmol/L and incubated for 1 h at room temperature. Cells were then lysed, and ABCA1 was immunoprecipitated with polyclonal anti-ABCA1 antibodies. Samples were diluted with loading buffer containing 5% 2-mercaptoethanol, boiled for 5 min, and subjected to 12% SDS−PAGE and Western blot; apoA-I was detected using monoclonal anti-apoA-I antibody AI-4.1 and quantitated by densitometry. Inset: Western blotting with detection with anti-apoA-I antibodies.

proportion of ABCA1 was preserved when cells were incubated in the presence of apoA-I or NDF6F1 (Figure 7A). The antibody NDF4C2 tested in a similar experiment did not protect ABCA1 from degradation (not shown). A similar experiment was conducted to test the effect of the antibodies specifically on the cell surface abundance of ABCA1 using biotinylation technique. THP-1 cells were incubated for 24 h with TO-901317 (4 μmol/L), washed, and incubated for 18 h in the serum-free medium containing TO-901317 (4 μmol/L), antibodies NDF6F1 or NDF4C2 (10 μg/mL), or apoA-I (50 μg/mL) or without additions. Cells were then biotinylated, membrane fractions isolated, and biotinylated proteins precipitated by immobilized streptavidin and analyzed by Western blotting. Considerably more ABCA1 was detected at the cell surface when cells were incubated with TO-901317, the antibody NDF6F1, or apoA-I compared to the medium alone or the antibody NDF4C2 (Figure 7B).

To confirm further the effect of the NDF6F1 antibody on ABCA1 stability, a time course of ABCA1 degradation was investigated using in-cell Western blot (Figure 8). Expression of ABCA1 in THP-1 cells was boosted by TO-901317; the LXR agonist was then removed, stopping ABCA1 expression. The cells were then incubated for various periods of time with serum-free medium alone (control), fresh TO-901317 (4 μmol/L), apoA-I (50 μg/mL), or NDF6F1 or NDF4C2 antibodies (10 μg/mL). Continuous incubation of cells with TO-901317 lead to an additional 30% increase in ABCA1 abundance over 24 h; addition of NDF6F1 instead of TO-901317 had almost the same effect on ABCA1 abundance. In contrast, incubation in the absence of TO-901317 or in the presence of NDF4C2 antibody led to a time-dependent decrease in ABCA1 by 40−60%. Incubation with apoA-I preserved ABCA1 at the initial level (Figure 8). Thus the antibody NDF6F1, but not NDF4C2, can mimic apoA-I in stabilizing ABCA1 at the cell surface, which may be a mechanism for stimulating cholesterol efflux by this antibody.

To investigate the contribution of ABCA1 stabilization to the NDF6F1 effect on cholesterol efflux, we transiently transfected HeLa cells (known not to express ABC transporters) with ABCA1 or an ABCA1 mutant lacking the PEST domain, the site on ABCA1 responsible for its degradation by calpain proteases (*13*). As expected, transfection of HeLa cells with ABCA1 stimulated cholesterol efflux (Figure 9A,B). Similar to observations with macrophages, preincubation of HeLa−ABCA1 cells with NDF6F1 antibody stimulated cholesterol efflux, while preincubation with NDF4C2 antibody inhibited it (Figure 9B). Consistent with the findings of Chen et al. (*32*), transfection of HeLa cells with ΔPEST-ABCA1 increased cholesterol efflux compared to cells transfected with ABCA1 ($p < 0.01$), raising it to the level observed in cells preincubated with NDF6F1. Preincubation of HeLa cells transfected with ΔPEST-ABCA1 with the antibody NDF6F1 did not further increase cholesterol efflux, while antibody NDF4C2 still inhibited efflux (Figure 9B,C). We further tested the effects of NDF6F1

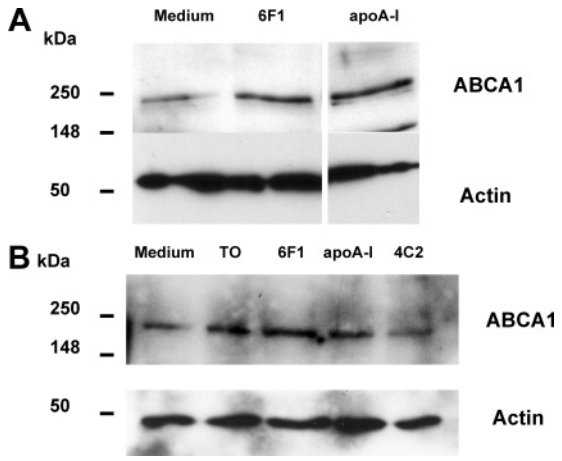

FIGURE 7: 7. Effect of apoA-I and the antibody NDF6F1 on ABCA1 stability (A) and cell surface abundance (B). (A) ABCA1 expression in THP-1 cells was boosted by incubation for 18 h with LXR agonist TO-901317 (4 μmol/L). Cells were then washed and incubated for 18 h in the serum-free medium alone or medium containing antibody NDF6F1 (5 μg/mL) or lipid-free apoA-I (30 μg/mL). THP-1 cells were then lysed as described in Materials and Methods, and proteins were separated on a 6% SDS−PAGE followed by immunoblotting with NDF4C2 antibody or anti-β-actin monoclonal antibody. (B) THP-1 cells were incubated for 24 h with TO-901317 (4 μmol/L), washed, and incubated for 18 h in the serum-free medium, medium containing TO-901317 (4 μmol/L), antibodies NDF6F1 or NDF4C2 (10 μg/mL), or apoA-I (50 μg/mL). Cells were then biotinylated; biotinylated proteins were precipitated and analyzed by Western blot as described in Materials and Methods.

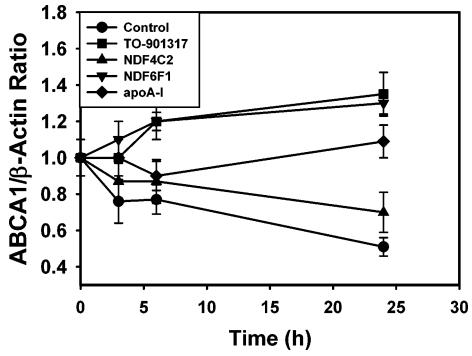

FIGURE 8: 8. Effect of apoA-I and the antibodies NDF6F1 and NDF4C2 on the rate of ABCA1 degradation. THP-1 cells were differentiated, and ABCA1 expression was boosted by incubation with TO-901317 (4 μmol/L) for 18 h. Cells were then washed and incubated for the indicated periods of time with serum-free medium (control), fresh TO-901317 (4 μmol/L), apoA-I (50 μg/mL), or NDF6F1 or NDF4C2 antibodies (10 μg/mL). Cells were then washed, fixed with 3.7% formaldehyde in PBS for 20 min, and permeabilized with three 10 min washes with 0.1% Triton X-100 in PBS. After blocking, cells were incubated overnight with monoclonal NDF4C2 anti-ABCA1 antibodies and rabbit polyclonal anti-β-actin antibodies. Secondary detection was carried out using two species-specific infrared fluorescent dye conjugated antibodies. After 1 h incubation targets were simultaneously visualized and quantitated using the Odyssey infrared imaging scanner (Li-Cor, Lincoln, NB). ABCA1 abundance was presented relative to β-actin abundance.

antibody on ABCA1-dependent cholesterol efflux from HeLa cells stably transfected with ABCA1. ABCA1-dependent cholesterol efflux was defined as the efflux to apoA-I from cells transfected with ABCA1 minus the efflux from mock-transfected cells (the latter was approximately 20% of the former). The absolute level of ABCA1-dependent cholesterol efflux in cells stably transfected with ABCA1 was substantially higher compared to transiently transfected cells, reflecting the higher efficiency of stable transfection (Figure 9D). Similar to observations with macrophages, preincubation of HeLa−ABCA1 cells with 10 μg/mL NDF6F1 antibody significantly stimulated cholesterol efflux, while preincubation with a higher concentration (20 μg/mL) of this antibody was less effective (Figure 9D). Preincubation of mock-transfected cells (both transiently and stably transfected) with NDF6F1 or NDF4C2 did not affect cholesterol efflux (Figure 9A). Thus, preincubation of cells with the antibody NDF6F1 and the deletion of the PEST domain had similar and nonadditive effects on cholesterol efflux, indicating that the mechanisms involved may be similar, e.g., inhibition of ABCA1 degradation.

Intracellular Cholesterol Content. To assess the effect of the antibodies on intracellular cholesterol content, cholesterol and cholesteryl ester synthesis in RAW 264.7 macrophages was measured in the presence of the antibodies (final concentration 5 μg/mL) and apoA-I. RAW 264.7 macrophages were used in these experiments because they are more susceptible than THP-1 cells to cholesterol loading using acetylated LDL (acLDL). Increase of intracellular cholesterol content was expected to trigger compensatory suppression of cholesterol biosynthesis (*33*) and a higher rate of cholesteryl ester synthesis (*34*); the opposite was expected if cell cholesterol content decreased. Consistent with this view, cholesterol loading of cells with acLDL resulted in a significantly lower rate of cholesterol biosynthesis and significantly higher rate of cholesteryl ester formation (Figure 10). Preincubation of cells with the antibody NDF6F1 resulted in a higher rate of cholesterol biosynthesis (Figure 10A) and a lower rate of cholesteryl ester formation (Figure 10B) in both cholesterol-loaded and non-cholesterol-loaded cells. This is consistent with lower intracellular cholesterol content most likely due to enhanced cholesterol efflux. Preincubation of cells with the antibody NDF4C2 resulted in a lower rate of cholesterol biosynthesis and higher rate of cholesteryl ester formation in both cholesterol-loaded and non-cholesterol-loaded cells (Figure 10), being consistent with higher intracellular cholesterol content due to inhibition of cholesterol efflux.

DISCUSSION

In this paper we used new monoclonal antibodies against ABCA1 to unravel structure−function relationships of ABCA1. The structure−function relationship of ABCA1 was previously studied using tagged ABCA1 (*35*), natural ABCA1 mutations (*28*, *36*), and site-directed mutagenesis (*35*, *37*). Tagging ABCA1 has demonstrated that regions of ABCA1 between residues 499−710 and 1345−1466 are likely to be important for apoA-I binding (*35*). Natural mutations found in the Tangier pedigree, R587W, W590S, Q597R, and S1506L, as well as generated mutant C1477R strongly inhibited cholesterol and phospholipid efflux (*28*, *35*, *36*) and, with the exception of W590S, also apoA-I binding (*36*). Cholesterol efflux was also inhibited by truncating 46 C-terminal residues of ABCA1 (*37*). Deletion of the PEST domain (residues 1283−1306) inhibited ABCA1 internalization and degradation (*13*). These studies directly implicate two large extracellular loops of ABCA1 as being important for apoA-I binding and/or cholesterol efflux.

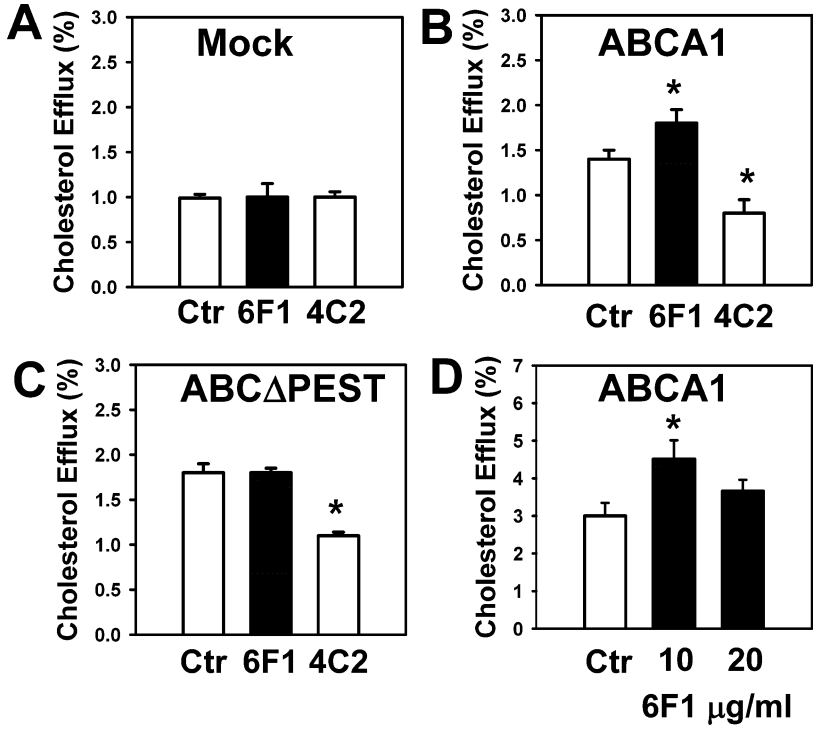

FIGURE 9: 9. Cholesterol efflux from HeLa cells transiently mock-transfected (A) or transfected with ABCA1 (B) or ΔPEST-ABCA1 (C) or stably transfected with ABCA1 (D). HeLa cells were transiently transfected with pCMV-β-gal plasmid (A), ABCA1 (B), or ΔPEST-ABCA1 (C) as described in Materials and Methods. Cellular cholesterol was labeled by incubating the cells in serum-containing medium with [^{3}H]cholesterol for 48 h. Cells were then washed and incubated for 18 h at 37 °C in serum-free medium in the presence or absence of the antibodies (final concentration 10 μg/mL). Cells were washed and incubated for 2 h at 37 °C in serum-free medium containing 30 μg/mL apoA-I. The medium was then collected and centrifuged for 15 min at 4 °C at 10000*g*, and aliquots of the supernatant were counted in a β-counter. Cells were harvested, and radioactivity was counted. Cholesterol efflux was expressed as a proportion of [^{3}H]cholesterol transferred from cells to medium. Means ± SEM of quadruplicate determinations are shown. *, $p < 0.01$ versus no antibody. (D) Experiments were conducted on HeLa cells stably transfected with ABCA1 or mock-transfected. Cholesterol efflux was assayed as described above. ABCA1-dependent cholesterol efflux is shown, i.e., the efflux from ABCA1-transfected cells minus the efflux from mock-transfected cells. Nonspecific IgM was used as a control. *, $p < 0.05$ versus cells treated with nonspecific IgM (Ctr).

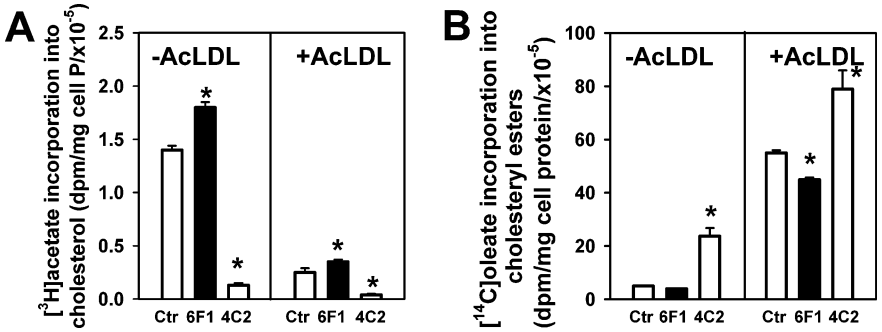

FIGURE 10: 10. Effect of antibodies on cholesterol (A) and cholesteryl ester (B) synthesis in macrophages. RAW 264.7 macrophages were preincubated with the antibodies (final concentration 5 μg/mL) for 18 h at 37 °C. Cells were then incubated for 2 h at 37 °C with [^{3}H]-acetate and [^{14}C]oleic acid (complexed to BSA). Cells were washed, and lipids were extracted and analyzed by TLC. Spots of cholesterol and cholesteryl oleate were identified by standards (Sigma), scraped, and counted in a β-counter. *, $p < 0.01$ versus no antibody.

Another important observation from these studies was that different functions of ABCA1 related to apoA-I binding and lipid fluxes could dissociate. For example, modification of ABCA1 by inserting a HA tag together with Tangier mutations, HA819/1466 and HA819/C1477, inhibited apoA-I binding but did not affect or even enhance lipid fluxes (*35*). Other mutations (e.g., W590S) inhibited cholesterol efflux with apoA-I binding intact (*30*, *35*, *36*).

In our study we have used monoclonal antibodies against regions of the two functionally important extracellular loops of ABCA1 to further investigate structure−function relationships of ABCA1. The advantage of this approach is that we were able to use macrophages, the cells that naturally express ABCA1 and play a key role in atherosclerosis. The limitation of this approach is that the large size of the antibody molecules identifies a region of the target molecule but not a specific sequence.

The most important finding of our study was that while two large extracellular loops of ABCA1 were confirmed to be critical for the interaction with apoA-I and cholesterol efflux, the events following apoA-I binding were different for each loop and independent of each other. The first extracellular loop is most likely involved in cholesterol efflux and/or internalization of the apoA-I/ABCA1 complex. Even within the loop, however, the functions of apoA-I binding, internalization, and cholesterol efflux are likely to be

dissociated. As with the ABCA1 mutant W590S (*35*, *36*), the antibody NDF4C2 inhibited cholesterol efflux without affecting apoA-I binding to ABCA1. Similar to modifications HA819/1466 and HA819/C1477 (*35*), the antibody NDF3F9 inhibited cellular binding and internalization of apoA-I without affecting cholesterol efflux and binding of apoA-I to ABCA1. These two antibodies were raised against the same fragment of ABCA1, but they belong to different isotypes, and the way they bind to ABCA1 may differ. Consequently, they probably induce different conformational changes of the transporter and affect different functions of ABCA1. Consistent with the suggestion of Rigot et al. (*35*) and the recent report of Vedhachalam et al. (*31*), this finding indicates that one apoA-I molecule may be required to induce ABCA1-related changes in membrane structure enabling cholesterol to efflux to another molecule of apoA-I, which may not be bound to ABCA1.

The binding of apoA-I to the fourth extracellular loop is most likely required for another apoA-I function: preventing ABCA1 degradation. The antibody NDF6F1 directed against a region of the fourth extracellular loop was able to inhibit apoA-I binding, yet stimulated cholesterol efflux and reduced intracellular cholesterol content. The fourth extracellular loop, as well as an epitope of NDF6F1, is located close to the PEST domain, a region responsible for ABCA1 internalization and degradation (*13*). In our experiments preincubation of cells with NDF6F1 antibodies induced a similar (and nonadditive) effect on cholesterol efflux as deletion of the PEST domain, indicating that a mechanism similar to calpain-mediated degradation may be involved. The effect of the antibody on ABCA1 stability was similar to the effect of apoA-I (*13*) and apoA-I mimicking peptides (*14*), also suggesting the involvement of calpain-mediated degradation. Importantly, at an optimal concentration the NDF6F1 antibody increased ABCA1 stability without inhibiting its function, cholesterol efflux, indicating that the region of ABCA1 responsible for stability is different from that responsible for the efflux. However, at higher concentration the NDF6F1 antibody did inhibit cholesterol and phospholipid efflux, indicating that excess of the antibody blocks another part of this or a different region of ABCA1 involved in cholesterol efflux.

Combining our findings with those of others (*13*, *28*, *35*, *36*), we are proposing the following hypothetical model of ABCA1/apoA-I-mediated lipid efflux. According to this model, three events can happen as a result of apoA-I binding to ABCA1. First, binding of apoA-I to ABCA1 may induce changes in the plasma membrane enabling cholesterol efflux. The antibody NDF4C2 blocks this process and reduces cholesterol efflux without affecting binding of apoA-I. Second, binding of apoA-I to ABCA1 may lead to the internalization of the apoA-I/ABCA1 complex, as suggested by Neufeld et al. (*10*) and Cavelier et al. (*29*); this event is blocked by the antibody NDF3F9. Internalization of the apoA-I-ABCA1 complex may be not related to cholesterol efflux but instead play a different role, e.g., in apoA-I transcytosis (*29*). The third event is binding of apoA-I to the fourth extracellular loop of ABCA1, preventing its calpain-mediated proteolysis. The antibody NDF6F1 likely binds to this site and acts as an agonist, preventing ABCA1 degradation.

While our findings identified the regions of ABCA1 responsible for the various events related to cholesterol efflux, the molecular mechanisms of these events remain unclear. It is possible to hypothesize that binding of apoA-I (or that of an antibody) may change conformation and/or topology of ABCA1 at the plasma membrane which would affect ABCA1 stability, ability to bind additional apoA-I molecules, or alter the rate of internalization. It can also affect exposure of the functional domains of ABCA1 to apoA-I on the outside and to the proteases and/or kinases on the inside of the plasma membrane.

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BI700473T