

# Reduction in ABCG1 in Type 2 Diabetic Mice Increases Macrophage Foam Cell Formation\*

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Atherosclerosis development is accelerated severalfold in patients with Type 2 diabetes. In the initial stages of disease, monocytes transmigrate into the subendothelial space and differentiate into foam cells. Scavenger receptors and ATP binding cassette (ABC) Transporters play an important role in foam cell formation as they regulate the influx and efflux of oxidized lipids. Here, we show that peritoneal macrophages isolated from Type 2 diabetic *db/db* mice have decreased expression of the ABC transporter ABCG1 and increased expression of the scavenger receptor CD36. We found a 2-fold increase in accumulation of esterified cholesterol in diabetic *db/db* macrophages compared with wild-type control macrophages. Diabetic *db/db* macrophages also had impaired cholesterol efflux to high density lipoprotein but not to lipid-free apo A-I, suggesting that the increased esterified cholesterol in diabetic *db/db* macrophages was due to a selective loss of ABCG1-mediated efflux to high density lipoprotein. Additionally, we were able to confirm down-regulation of ABCG1 using *C57BL/6J* peritoneal macrophages cultured in elevated glucose *in vitro* (25 mM glucose for 7 days), suggesting that ABCG1 expression in diabetic macrophages is regulated by chronic exposure to elevated glucose. Diabetic *KK<sup>ay</sup>* mice were also studied and were found to have decreased ABCG1 expression without an increase in CD36. These observations demonstrate that ABCG1 plays a major role in macrophage cholesterol efflux and that decreased ABCG1 function can facilitate foam cell formation in Type 2 diabetic mice.

Atherosclerosis development is accelerated severalfold in patients with Type 2 diabetes (1, 2). A pivotal event in atherogenesis occurs when monocytes adhere to endothelium and transmigrate into the subendothelial space, where they differentiate into macrophages. Upon differentiation, macrophages express the scavenger receptors CD36 and SR-A. Expression of these scavenger receptors allows macrophages to take up mod-

ified lipoproteins (3, 4). When the net influx of cholesterol supersedes that of efflux, the macrophages become lipid-laden foam cells.

Of particular importance in atherosclerosis is the balance between the influx and efflux of modified low density lipoproteins (LDL),<sup>2</sup> including oxidized LDL (ox-LDL) and minimally modified LDL (MM-LDL) moieties, which lead to foam cell generation. The scavenger receptors CD36 and SR-A have been shown to bind and internalize ox-LDL and are key receptors in the development of atherosclerotic lesions, as mice lacking these receptors show a decrease in atherosclerosis (5–7). Expression of both CD36 and SR-A is regulated by the peroxisome-proliferator activated receptor- $\gamma$  nuclear hormone receptor (8). Modified LDLs are among the ligands for peroxisome-proliferator activated receptor- $\gamma$  activation (8).

Likewise, members of the ATP binding cassette transporter family (ABC transporters) are known regulators of cholesterol efflux (9–11). ABCA1 and ABCG1 have been shown to regulate cellular lipid metabolism in macrophages. Mutations in the ABCA1 gene cause Tangier disease, a condition marked by severely reduced plasma HDL levels (7). ABCG1 is highly expressed in lipid-loaded macrophages and mediates cholesterol efflux to HDL, whereas ABCA1 mediates cholesterol efflux to lipid-free apoA-I (12, 13). Expression of both ABCA1 and ABCG1 is regulated by the liver X receptor (LXR) nuclear hormone receptor (10).

While much is known about the roles these proteins play in macrophage foam cell formation and the development of atherosclerosis, little is known about how cholesterol homeostasis in macrophages is altered in the setting of Type 2 diabetes. In an effort to better understand mechanisms contributing to foam cell formation in Type 2 diabetes, we examined macrophages from two well known models of Type 2 diabetes, the *db/db* mouse and the *KK<sup>ay</sup>* mouse. The *db/db* mouse is obese, hyperinsulinemic, and hyperglycemic due to a defect in the leptin receptor gene (14, 15). The *KK<sup>ay</sup>* mouse is a Type 2 diabetes model that arose spontaneously by crossing the *KK* parent strain with the *A<sup>y</sup>/a* strain (16). The *KK<sup>ay</sup>* mouse is also hyper-

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<sup>2</sup> The abbreviations used are: LDL, low density lipoprotein; ox-LDL, oxidized LDL; MM-LDL, minimally modified LDL; ABC, ATP binding cassette; HDL, high density lipoprotein; LXR, liver X receptor; PBS, phosphate-buffered saline; FBS, fetal bovine serum; siRNA, small interfering RNA; BSA, bovine serum albumin; FPLC, fast protein liquid chromatography; ANOVA, analysis of variance; apo, apolipoprotein A-I.

glycemic, hyperinsulinemic, and obese. In the current study, we show that macrophage foam cell formation is increased in these mouse models of Type 2 diabetes and that this is due, at least in part, to decreased cholesterol efflux. Furthermore, we report for the first time that ABCG1 mRNA and protein levels are significantly reduced in macrophages from Type 2 diabetic mice and that ABCG1 levels in diabetic macrophages are regulated by chronic elevated glucose.

**MATERIALS AND METHODS**

**Reagents**—FBS was ordered from HyClone (Logan, UT). Thioglycollate medium, Oil Red O, and mouse anti-tubulin antibody were obtained from Sigma. Oxidized LDL was generated by copper sulfate oxidation and was a kind gift from Dr. Judy Berliner (UCLA). NuPAGE 4–12% denaturing gels came from Invitrogen. Mouse anti-CD36 antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated anti-mouse and anti-rabbit secondary antibodies came from Amersham Biosciences UK Ltd. (Little Chalfont, Buckinghamshire, UK). An anti-ABCG1 antibody designed to recognize the C-terminal end of murine ABCG1 was synthesized for our laboratory by Washington Biotechnology, Inc. (Columbia, MD).

**Mice**—10-Week-old *Lepr<sup>db/db</sup>* (*B6.Cg-m<sup>+</sup>/+Lepr<sup>db</sup>*) knock-out mice (stock no. 000697) and their controls, *C57BL6/J* (stock no. 000664), *KK.Cg-A<sup>y</sup>/J* (stock no. 002468), and *a/a* control littermates were purchased from Jackson Laboratories (Bar Harbor, ME). Mice were fed a standard rodent chow diet or a Western diet (Teklad TD88137) for 10 weeks and housed in microisolator cages in a pathogen-free facility. All experiments followed University of Virginia Animal Care and Use Committee guidelines, and approval for use of rodents was obtained from the University of Virginia.

**Peritoneal Macrophage Isolation**—Hyperglycemic *db/db* or *KK<sup>ay</sup>* mice and their normoglycemic controls were injected with 2 ml of 3% thioglycollate medium. On day 5 post-injection, mice were anesthetized with isoflurane and injected intraperitoneally with 5 ml cold PBS with 10 mM EDTA. The PBS was then removed with a syringe and the process repeated three times. Macrophages from each mouse were plated separately in RPMI/10% FBS overnight. The following morning, nonadherent cells were removed by aspiration and the remaining macrophages were washed thoroughly with PBS and used for experiments.

**Glucose Studies**—Peritoneal macrophages were isolated from *C57BL/6J* mice and were cultured for up to 7 days in either 5.5 mM D-glucose (normal glucose), 25 mM D-glucose (high glucose), or 25 mM L-glucose in Dulbecco's modified Eagle's medium with 10% FBS. Medium was replaced on days 1, 3, 5, and 7, and glucose measurements were made. Glucose concentrations did not drop below 4.0 mM in the normal glucose samples or below 23 mM in the high glucose samples during the time course. RNA was isolated from the cells at various time points, as indicated in the legend to Fig. 4, using TRIzol. We examined a dose-response curve of glucose and found that ABCG1 expression was altered by concentrations greater than 20 mM glucose (data not shown). We chose 25 mM as our concentra-

**TABLE 1**  
Quantitative real-time PCR primers used in this study

Name	Sequence
ABCG1	Forward 5'-TTC CCC TGG AGA TGA GTG TC-3'
	Reverse 5'-CAG TAG GCC ACA GGG AAC AT-3'
ABCA1	Forward 5'-CGT TTC CGG GAA GTG TCC TA-3'
	Reverse 5'-GCT AGA GAT GAC AAG GAG GAT GGA-3'
CD36	Forward 5'-CCT TAA AGG AAT CCC CGT GT-3'
	Reverse 5'-TGC ATT TGC CAA TGT CTA GC-3'
SR A	Forward 5'-CAT GAA CGA GAG GAT GCT GAC T-3'
	Reverse 5'-GGA AGG GAT GCT ATT GAA-3'
LOX-1	Forward 5'-CCT CTG CCT GGT GTT GTC-3'
	Reverse 5'-CCT GCT CTT TGG ATT TCT CG-3'
Cyclophilin	Forward 5'-TGG AGA GCA CCA AGA CAG ACA-3'
	Reverse 5'-TGC CGG AGT CGA CAA TGA T-3'

tion for studies based on the dose curve and upon previous work in our laboratory (17).

**Immunoblotting for CD36 and ABCG1**—RIPA buffer was added to peritoneal macrophages to generate whole cell lysates. The lysates were collected, briefly sonicated, and quantified using a protein assay kit (Bio-Rad).

Proteins were separated by SDS-PAGE, then transferred to nitrocellulose and blocked for 2 h with Blocker-BLOTTO (Pierce). Fifty µg of whole cell lysate was used for detection of all proteins. For detection of CD36, blots were incubated in Tris-buffered saline + 1% Tween 20 (TBST) containing a 1:200 dilution of anti-mouse CD36 antibody overnight at 4 °C. Blots were then incubated with 1:4000 dilution of horseradish peroxidase-conjugated anti-mouse secondary antibody. To detect ABCG1, blots were incubated with Blocker-BLOTTO containing a 1:250 dilution of anti-mouse ABCG1 antibody overnight at 4 °C. Blots were then incubated with a 1:4000 dilution of horseradish peroxidase-conjugated anti-rabbit secondary antibody. Proteins were visualized using chemiluminescence and normalized to tubulin as a control for gel loading. Densitometry was performed using Stratagene Zero D-Scan densitometry software.

**Macrophage Foam Cell Formation**—To generate foam cells, freshly isolated peritoneal macrophages from Type 2 diabetic and control mice were plated on chamber slides in RPMI/10% FBS and incubated with 100 µg/ml ox-LDL at 37 °C for 72 h. Oil Red O staining was used to visualize lipids (18).

**Foam Cell Formation in Vivo**—Peritoneal macrophages were isolated from control *a/a* and diabetic *KK<sup>ay</sup>* mice fed the Western diet for 10 weeks as described (19). Macrophages were plated onto chamber slides in RPMI/10% FBS at 37 °C for 24 h. Oil Red O staining was used to visualize lipids (18, 19).

**Quantitative Real-time PCR**—Total cellular RNA was collected from macrophages using TRIzol Reagent following the manufacturer's protocol. 2 µg of cDNA was then synthesized using an Iscript cDNA synthesis kit (Bio-Rad). Total cDNA was diluted 1:5 in H<sub>2</sub>O, and 2 µl were used for each real-time condition using a Bio-Rad MyiQ single color real-time PCR detection system and iQ SYBR Green supermix (Bio-Rad). Primer sequences are listed in Table 1. Samples were normalized to cyclophilin using the ΔC<sub>t</sub> method.

**Total and Esterified Cholesterol Determinations**—Macrophages were scraped into tubes and pelleted by low spin centrifugation. After several washes with PBS, the cell pellet was extracted with chloroform:methanol (2:1) containing 5α-cho-

## ABCG1 and Type 2 Diabetes

lestane as internal standard. Total and free cholesterol content was determined by gas-liquid chromatography and normalized to cellular protein as described previously (20). Esterified cholesterol was calculated as the difference between total and free cholesterol.

**ABCG1 siRNA Studies**—The following ABCG1 siRNA sequences were designed and spliced into the pSuper expression vector (Oligoengine, Seattle, WA): 5'-CGTGGATGAG-GTTGAGACA-3' (forward) and 5'-GGTGGACAACAAC-TACA-3' (reverse). 0.5  $\mu$ g of siRNA plasmid vector was used per  $10^6$  cells transfected using Nucleofector technology (Amaxa, Gaithersburg, MD). Transfection efficiency was  $\sim$ 65% as measured by green fluorescent protein transfection. Knock-down was measured using quantitative real-time PCR with sequences designed to cover the ABCG1 siRNA splice sequence: 5'-CAACGTGGATGAGGTGTAGA-3' (forward) and 5'-TTTCCCAGAGATCCCTTTCA-3' (reverse). Samples were normalized to cyclophilin.

**Efflux Assays**—Cholesterol efflux assays were performed as described, with minor modification (21). Thioglycollate-elicited peritoneal macrophages were plated in 12-well plates at a density of  $6 \times 10^5$  cells/well. Cells were radiolabeled with 2  $\mu$ Ci/ml of [ $^3$ H]cholesterol for 24 h in the presence of 10% FBS medium and then equilibrated for 2 h in the presence of serum-free medium containing 0.2% fatty acid free bovine serum albumin (BSA). Cholesterol efflux was conducted for 24 h at 37 °C in medium containing: 1) 0.2% BSA, 2) 0.2% BSA + 15  $\mu$ g/ml lipid-free human ApoA-I, or 3) 0.2% BSA + 50  $\mu$ g of protein/ml of human HDL. Human apoA-I and HDL were isolated as described previously (22, 23). The efflux medium was then removed and a 400  $\mu$ l aliquot was taken for  $^3$ H radioactivity determination. Adherent cells were rinsed twice with cold PBS and 1 ml of isopropyl alcohol was added for overnight extraction at room temperature. A 100- $\mu$ l aliquot of the extract was taken for  $^3$ H radioactivity determination. Results are expressed as [ $^3$ H]cholesterol in medium/mg total cell protein. Specific efflux to apoA-I or HDL was calculated by subtracting nonspecific efflux in the presence of 0.2% BSA only.

**Plasma Lipoprotein Analysis**—Mice were fasted for 4 h prior to obtaining blood via the retroorbital sinus. Plasma glucose levels were measured using a One Touch UltraSmart glucometer (Lifescan, Milpitas, CA). Plasma insulin levels were analyzed using a radioimmunoassay kit (Linco, St. Charles, MO). Plasma lipid levels were determined by the University of Virginia Clinical Pathology Laboratory. For plasma lipoprotein analysis of mice fed a Western diet, plasma samples from 8 mice/group were pooled. Plasma lipoproteins were fractionated using FPLC according to the method of Daugherty *et al.* (24).

**Statistical Analyses**—Data for all experiments were analyzed by analysis of variance (ANOVA) using the Statview 6.0 software program. Comparisons between groups were performed using ANOVA methods. Data are graphically represented as mean  $\pm$  S.E., in which each mean consists of six experiments performed in quadruplicate (unless noted otherwise in the figure legends) using cells isolated from 8 mice per group. Comparisons between groups and tests of interactions were made assuming a two-factor analysis with the interaction term testing each main effect with the residual error testing the interaction.

All comparisons were made using Fisher's least standard difference procedure, so that multiple comparisons were made at the 0.05 level only if the overall F-test from the ANOVA was significant at  $p < 0.05$ .

## RESULTS

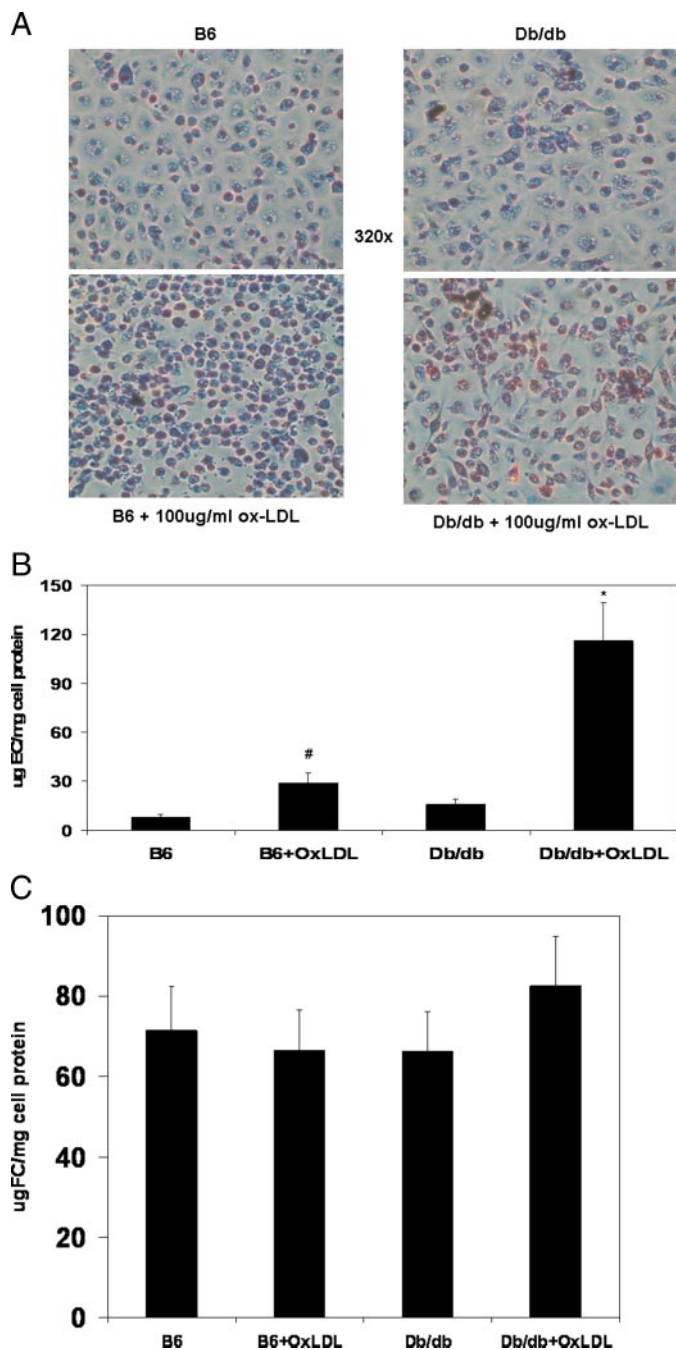
**Foam Cell Formation Is Increased in Diabetic db/db Macrophages**—We examined the ability of diabetic *db/db* macrophages to form foam cells in response to oxidized LDL uptake. Peritoneal macrophages were isolated from mice maintained on a rodent chow diet. We incubated control, nondiabetic *C57BL6/J (B6)* macrophages and diabetic *db/db* macrophages with 100  $\mu$ g/ml oxidized LDL for 72 h and examined foam cell formation using Oil red O staining of lipid. We found increased foam cell formation in *db/db* macrophages compared with *B6* macrophages (Fig. 1A).

**Increased Cholesterol Ester Content of Diabetic db/db Macrophages**—We measured the amount of esterified cholesterol content from both control *B6* and diabetic *db/db* macrophages before and after incubation with oxLDL. Although no difference in esterified cholesterol was observed prior to incubation with oxLDL, we found approximately a 4-fold increase in esterified cholesterol content in diabetic *db/db* macrophages compared with control macrophages after incubation with oxLDL (Fig. 1B). The amount of free cholesterol in the macrophages was similar between *B6* and *db/db* macrophages (Fig. 1C).

**CD36 Expression Is Post-transcriptionally Regulated in db/db Mice**—To examine factors contributing to increased foam cell formation in *db/db* mice, we investigated the expression of candidate molecules known to be important in macrophage cholesterol trafficking. We examined representative members of three scavenger receptor subtypes, SR-A1, CD36, and LOX-1. SR-A and CD36 are responsible for  $\sim$ 75–90% of oxidized-LDL uptake in macrophages (5). Using real-time quantitative PCR, we found that there were no significant differences in mRNA expression of either SR-A or CD36 in diabetic peritoneal macrophages, although there was a consistent trend toward increased CD36 mRNA expression in diabetic *db/db* macrophages (data not shown). LOX-1 mRNA expression was minimal in *B6* macrophages but was fairly robust in diabetic *db/db* macrophages (data not shown).

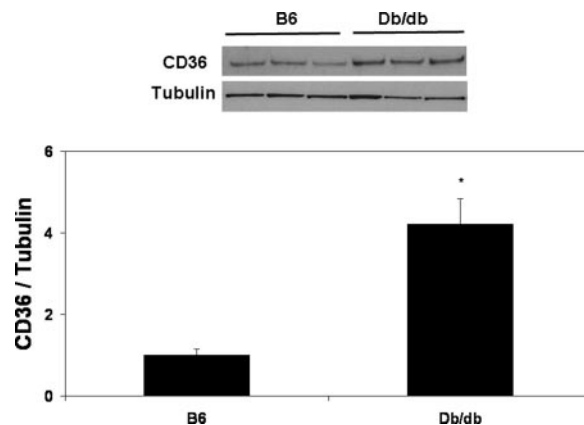
We then examined protein expression of SR-A1, CD36, and LOX-1. We found a significant 3-fold increase in CD36 on diabetic *db/db* macrophages (Fig. 2). These data are consistent with those of Tall and colleagues (25), who recently reported translational regulation of CD36 in *ob/ob* diabetic macrophages. Unfortunately, we were unable to find reliable, commercially available anti-mouse antibodies for either SR-A or LOX-1. Thus, we could not verify whether the increased levels of LOX-1 mRNA in diabetic *db/db* macrophages actually correlated with an increase in LOX-1 protein. However, taken together, our data suggest that cholesterol uptake in *db/db* macrophages is due, in part, to up-regulation of CD36 and possibly LOX-1 in the setting of Type 2 diabetes.

**ABCG1 Expression Is Reduced in Diabetic db/db Mice**—Additional candidate molecules important in regulation of cellular cholesterol trafficking include members of the ABC transporter



**FIGURE 1. Diabetic *db/db* macrophages show increased foam cell formation.** Freshly isolated peritoneal macrophages from control *C57BL/6J* (*B6*) and diabetic *db/db* (*db/db*) mice were isolated and incubated with 100  $\mu$ g/ml ox-LDL for 72 h. *A*, lipid content and morphological differences were observed using Oil Red O and hematoxylin staining. *B*, macrophages were assayed for intracellular esterified cholesterol content as described under "Materials and Methods." Data are normalized to total cell protein. #, *B6* versus *B6* + ox-LDL,  $p < 0.05$ . \*, *db/db* versus *db/db* + ox-LDL,  $p < 0.007$  by ANOVA. *C*, macrophages were assayed for intracellular free cholesterol content as described under "Materials and Methods." Data are normalized to total cell protein. There were no significant differences in free cholesterol content of cells.

family, namely ABCG1, ABCG4, and ABCA1. We measured mRNA expression of these transporters in both control *B6* and diabetic *db/db* macrophages. We found minimal expression of ABCG4 in control and diabetic macrophages (data not shown). Furthermore, we found only a small reduction (~20%) in

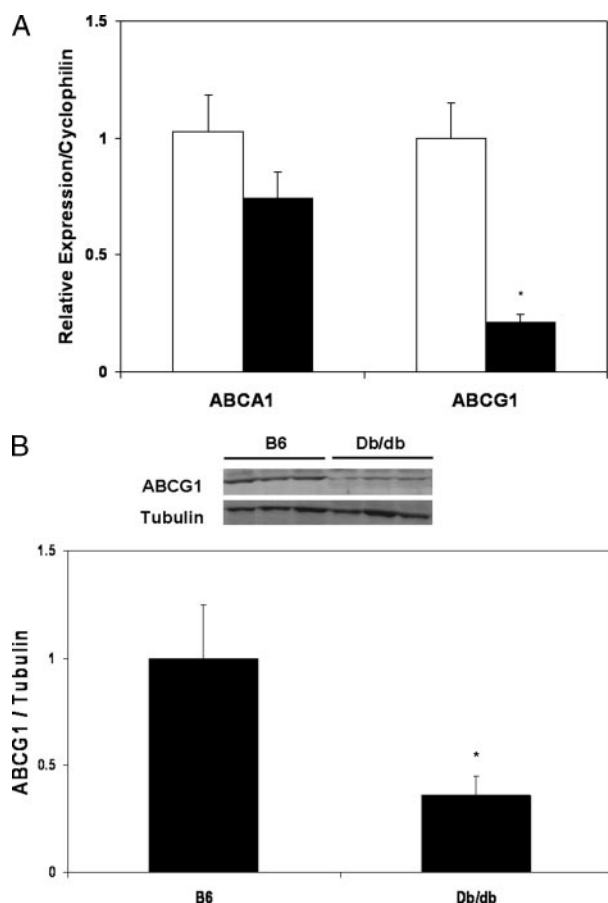


**FIGURE 2. CD36 protein is increased in diabetic *db/db* macrophages.** Lysates were obtained from freshly isolated peritoneal macrophages from control *C57BL/6J* (*B6*) and diabetic *db/db* (*db/db*) mice. SDS-PAGE and Western blot analysis were performed as described under "Materials and Methods." *Top*, immunoblot probed with anti-CD36 and anti-tubulin antibodies. *Bottom*, densitometry of immunoblot normalized to tubulin. \*, *B6* versus *Db/db*,  $p < 0.05$ .

ABCA1 mRNA levels in *db/db* macrophages (Fig. 3A). However, expression of ABCG1 mRNA was significantly reduced by 70% in *db/db* macrophages (Fig. 3A). We examined protein expression of ABCG1 in macrophages and also found a 60% decrease in ABCG1 protein in *db/db* macrophages (Fig. 3B). Taken together, these results suggest that the increased foam cell formation observed in diabetic *db/db* mice is due to both increased surface expression of CD36 and dramatic down-regulation of ABCG1 expression.

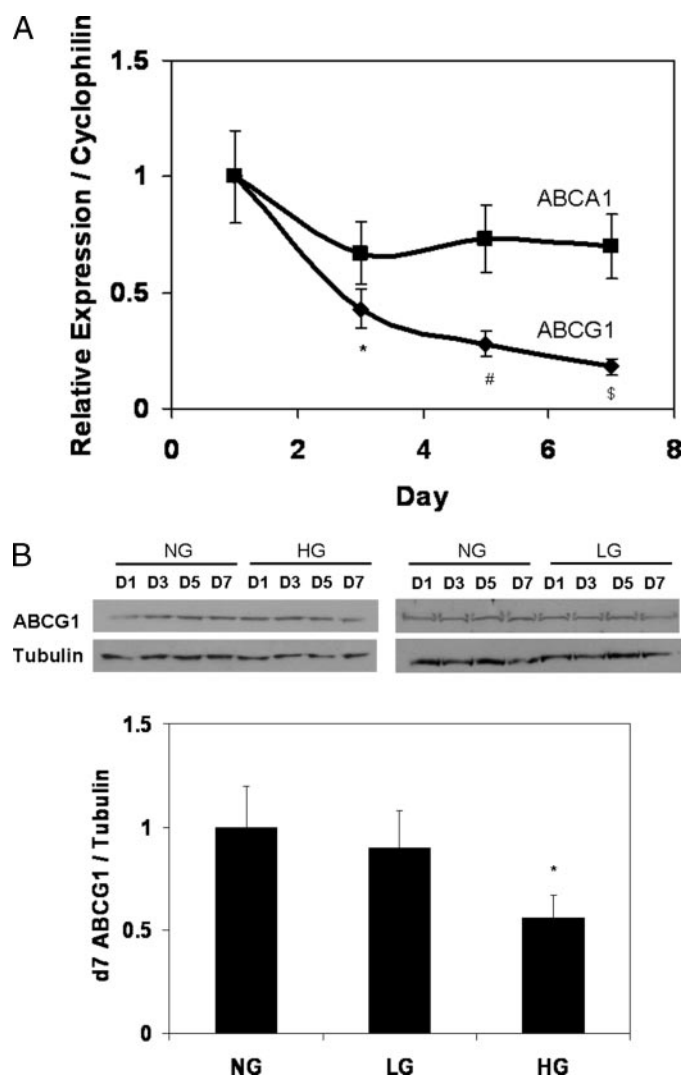
**Glucose Regulates ABCG1 Expression in Macrophages**—We next examined whether the changes in CD36 and ABCG1 expression observed in diabetic *db/db* macrophages were caused by elevated glucose. We cultured freshly isolated peritoneal macrophages from *C57BL/6J* mice in normal glucose (5.5 mM) and 25 mM glucose for 7 days. We then measured mRNA levels for CD36 and ABCG1 over the 7 days time period using real-time quantitative PCR. We observed a 50% reduction in ABCG1 mRNA levels at d3 in macrophages cultured in 25 mM D-glucose (Fig. 4A). Incubation of freshly isolated peritoneal macrophages in L-glucose (which is not taken up by macrophages and serves as an osmotic control) did not reduce ABCG1 mRNA expression (data not shown), suggesting that glucose metabolism was required. Interestingly, there was no significant reduction in ABCG1 expression before 3 days of culture in D-glucose (Fig. 4A). ABCG1 protein expression was also decreased significantly by 25 mM D-glucose at the 7 day time point but not by 25 mM L-glucose (Fig. 4B). In addition, there were no changes found in either LXR $\alpha$  or LXR $\beta$  (data not shown). These data together suggest that glucose may indirectly regulate ABCG1 expression and are similar to those obtained using freshly isolated peritoneal macrophages from diabetic *db/db* mice (Fig. 3). These data indicate that chronically elevated levels of glucose, as occurs in the setting of diabetes, down-regulates expression of ABCG1.

**Decreased Cholesterol Efflux in Diabetic *db/db* Macrophages**—To verify that the cholesterol accumulation that we found was indeed due, in part, to ABC transporter activity and not scavenger receptor activity, we performed cholesterol efflux assays



**FIGURE 3. ABCG1 expression is decreased in diabetic *db/db* peritoneal macrophages.** *A*, total RNA was isolated from control *C57BL/6J* and diabetic *db/db* mouse macrophages. Quantitative real-time PCR for murine ABCG1 (*ABCG1*) and ABCA1 (*ABCA1*) was performed as described under "Materials and Methods." *Open bars* represent control B6 samples. *Solid bars* represent diabetic *db/db* samples. \*, *B6* versus *Db/db*,  $p < 0.05$ . *B*, protein lysates were obtained from freshly isolated peritoneal macrophages from control *C57BL/6J* (*B6*) and diabetic *db/db* (*db/db*) mice. SDS-PAGE and Western blot analysis were performed as described under "Materials and Methods." *Top*, immunoblot probed with anti-ABCG1 and anti-tubulin antibodies. *Bottom*, densitometry of immunoblot normalized to tubulin. \*, *B6* versus *Db/db*,  $p < 0.05$ .

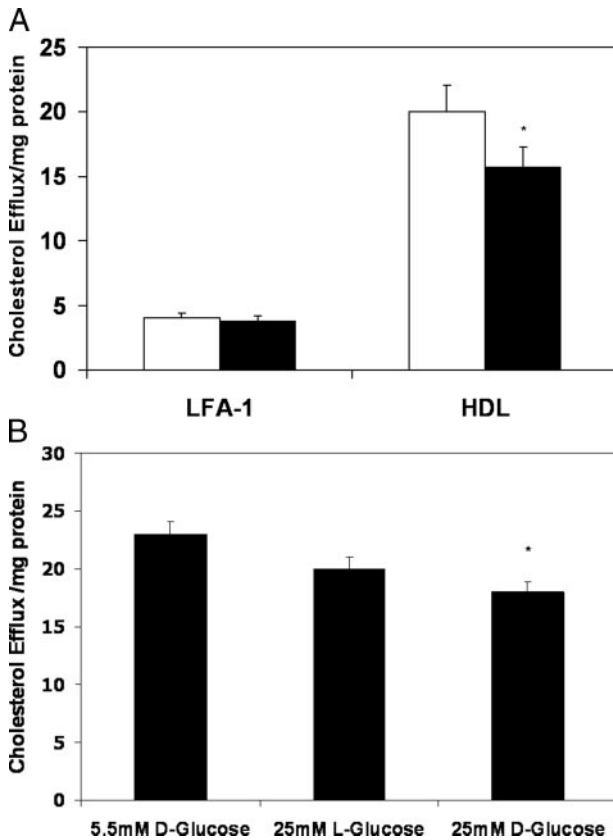
to measure ABC transporter function. We examined the ability of diabetic *db/db* macrophages to efflux cholesterol to either lipid-free apoA-I or to HDL. Tall and colleagues (13) have recently reported that ABCG1 is the key transporter of lipid to HDL, whereas ABCA1 is the key transporter of lipid to apoA-I. Macrophages from control *B6* and diabetic *db/db* mice were incubated with either lipid-free apoA-I or HDL. We observed no changes in efflux of cholesterol to lipid-free apoA-I (Fig. 5A). This suggests that the slight decrease in ABCA1 mRNA observed in *db/db* macrophages (Fig. 3) has no significant impact in modulating ABCA1 protein levels required for efflux of cholesterol to apoA-I. However, we did observe a 25% reduction in the amount of cholesterol effluxed to HDL in diabetic *db/db* macrophages (Fig. 5A). In addition, *B6* macrophages incubated in 25 mM D-glucose, but not L-glucose, for 7 days showed a statistically significant reduction (~25%) in cholesterol efflux to HDL (Fig. 5B). These data support the notion that ABCG1 levels regulate macrophage cholesterol efflux to HDL and that the observed decrease in ABCG1 levels in diabetic



**FIGURE 4. ABCG1 expression in macrophages is regulated by glucose.** Peritoneal macrophages isolated from control *C57BL/6J* mice were cultured in either normal (5 mM), high (25 mM) D-glucose, or high (25 mM) L-glucose medium for up to 7 days. Samples were analyzed at day 1 (D1), day 3 (D3), day 5 (D5), or day 7 (D7). *A*, total RNA was isolated and quantitative real-time PCR for murine ABCG1 (*ABCG1*) and ABCA1 (*ABCA1*) was performed as described under "Materials and Methods." *Diamonds* represent ABCG1. *Squares* represent ABCA1. Data represent the mean  $\pm$  S.E. of six mice. \*, 1-day ABCG1 versus 3-day ABCG1,  $p < 0.05$ . #, 1-day ABCG1 versus 5-day ABCG1,  $p < 0.05$ . \$, 1-day ABCG1 versus 7-day ABCG1,  $p < 0.05$ . *B*, protein lysates were collected before SDS-PAGE and Western blot analysis were performed as described under "Materials and Methods." *Top*, immunoblot probed with anti-ABCG1 and anti-tubulin antibodies. *Bottom*, densitometry of D7 lanes from immunoblot normalized to tubulin. \*, normal glucose (NG) versus high glucose (HG),  $p < 0.05$ ; L-glucose (LG) versus high glucose,  $p < 0.05$ .

*db/db* macrophages contributes to increased foam cell formation observed in these mice.

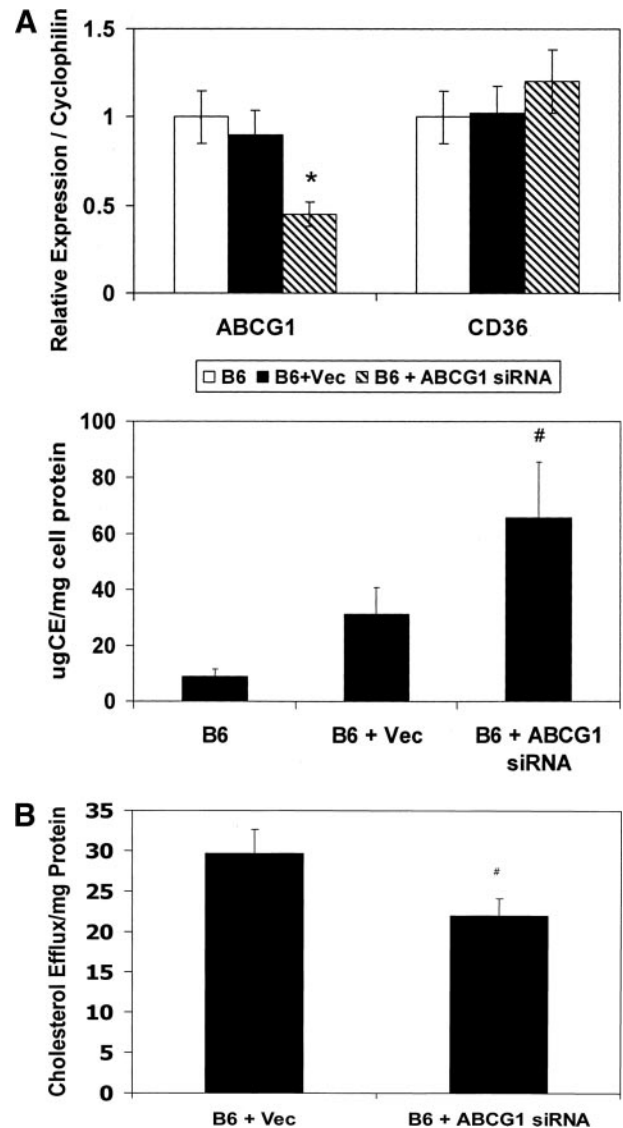
**ABCG1 Reduction Contributes to Foam Cell Formation**—We constructed siRNA to ABCG1 to determine whether ABCG1 levels in the macrophage significantly contributed to cholesteryl ester accumulation and foam cell formation. Using siRNA, we were able to obtain a significant 55% reduction in ABCG1 mRNA expression in primary peritoneal macrophages from *C57BL/6J* mice (Fig. 6A). Incubation of control macrophages with ox-LDL significantly increased intracellular cholesteryl ester accumulation as expected. Reduction of ABCG1 using siRNA increased intracellular cholesteryl ester accumu-



**FIGURE 5. Diabetic *db/db* macrophages show impaired cholesterol efflux to HDL.** A, control *C57BL/6J* (*B6*) and diabetic *db/db* macrophages were isolated and utilized in an efflux assay as described in Methods. Cholesterol efflux to lipid-free apoA-I (*LFA-1*) and to HDL (*HDL*) was assayed. Open bars represent control *B6* samples. Closed bars represent diabetic *db/db* samples. \*, *B6* HDL versus *Db/db* HDL,  $p < 0.001$ . B, *C57BL/6J* (*B6*) macrophages were incubated in either normal (5 mM), high (25 mM) D-glucose, or high (25 mM) L-glucose medium for 7 days and utilized in an efflux assay as described under "Materials and Methods." Cholesterol efflux to HDL was assayed. \*, normal glucose versus high glucose,  $p < 0.05$ ; L-glucose versus high glucose,  $p < 0.05$ .

lation in response to incubation with oxidized LDL by 3-fold compared with control macrophages (Fig. 6A). Furthermore, targeting ABCG1 with siRNA reduced cholesterol efflux to HDL by ~25%, effectively mimicking the *db/db* phenotype (Fig. 6B). Thus, ABCG1 reduction decreases cholesterol efflux to HDL and causes cholesteryl ester to accumulate in the macrophage, contributing to foam cell formation.

**Increased Foam Cell Formation in Vivo in Diabetic Mice—**We used an *in vivo* model for foam cell formation by eliciting peritoneal macrophages in Type 2 diabetic mice that were fed a high cholesterol, Western-type diet. This methodology has been used previously by Glass and colleagues (19) to show increased foam cell formation *in vivo* in LDL receptor-deficient mice fed a high cholesterol diet. To confirm that the differences observed in foam cell formation in diabetic *db/db* mice were common to other models of Type 2 diabetes, we investigated foam cell formation *in vivo* in the *KK<sup>ay</sup>* mouse. Fasting plasma lipids, glucose and insulin levels for both *KK<sup>ay</sup>* and *db/db* mice are listed in Table 2. Plasma lipid values were similar between *KK<sup>ay</sup>* and *db/db* mice. Total cholesterol values increased ~2-fold in diabetic versus control animals of both strains (Table 2). However, when placed on a Western diet for 10 weeks, *KK<sup>ay</sup>* mice did not exhibit greater cholesterol values



**FIGURE 6. ABCG1 siRNA increases cholesteryl ester accumulation in control macrophages.** Peritoneal macrophages from *C57BL/6J* mice (*B6*) were harvested and transfected with either siRNA to ABCG1 (*siRNA*) or pSuper siRNA empty vector (*Vec*) as described under "Materials and Methods." A, top: expression of ABCG1 mRNA after "knockdown" with siRNA is shown. Bottom: macrophages were incubated for 24 h with 100  $\mu$ g/ml oxidized LDL, and intracellular cholesteryl ester accumulation was determined as described under "Materials and Methods." \*, *B6* ABCG1 versus *B6* + siRNA ABCG1,  $p < 0.05$ . #, significantly higher than *B6* or pSuper siRNA empty vector-treated cells,  $p < 0.01$ . B, transfected cells were measured for their ability to efflux cholesterol to HDL as described under "Materials and Methods." #, *B6* + pSuper siRNA empty vector versus *B6* siRNA,  $p < 0.05$ .

than control *a/a* mice. Total plasma cholesterol values averaged  $243 \pm 8$  mg/dl for *a/a* and  $280 \pm 18$  mg/dl for *KK<sup>ay</sup>* on the Western diet. Analysis of plasma lipoproteins using FPLC for mice on the Western diet revealed moderate increases in HDL-C and VLDL-C in *KK<sup>ay</sup>* mice compared with control *a/a* mice (Fig. 7A).

Peritoneal macrophages were isolated from *a/a* and *KK<sup>ay</sup>* mice fed a Western diet for 10 weeks and were stained with Oil Red O to qualify lipid accumulation. In contrast to mice fed a chow diet, macrophages isolated from diabetic *KK<sup>ay</sup>* mice fed a Western diet exhibited dramatic Oil Red O staining (Fig. 7B) compared with *a/a* mice fed a Western diet, indicative of

## ABCG1 and Type 2 Diabetes

**TABLE 2**

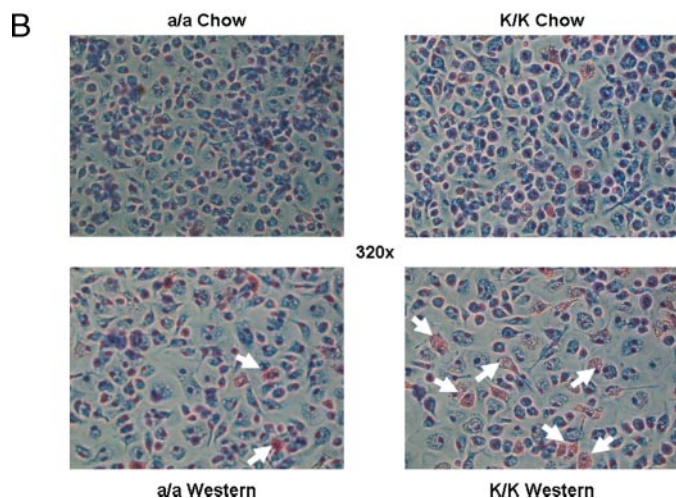
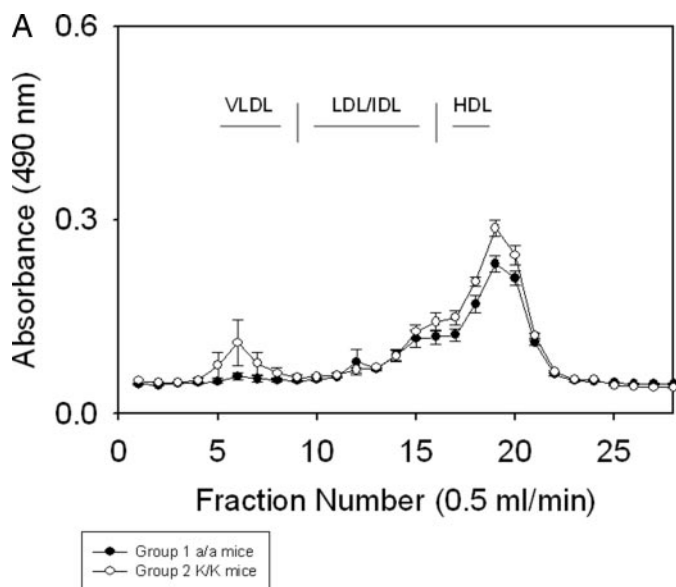
Average fasting plasma lipid, glucose and insulin profiles of mice on a chow diet

Data are represented as mean  $\pm$  S.D.

Mouse strain	Total cholesterol	Triglycerides	HDL	LDL	Glucose	Insulin
	mg/dl	mg/dl	mg/dl	mg/dl	mg/dl	ng/ml
C57BL6/J	81.0 $\pm$ 11.2	64.6 $\pm$ 55.8	55.17 $\pm$ 4.6	15.17 $\pm$ 2.2	173.8 $\pm$ 42.0	0.29 $\pm$ 0.1
Lepr <sup>db/db</sup>	141.5 $\pm$ 11.3 <sup>a</sup>	90.0 $\pm$ 55.9	90.7 $\pm$ 9.4 <sup>a</sup>	35.83 $\pm$ 10.6 <sup>a</sup>	336.0 $\pm$ 123.5 <sup>a</sup>	10.51 $\pm$ 1.9 <sup>a</sup>
AA	80.67 $\pm$ 8.9	163.0 $\pm$ 36.9	53.67 $\pm$ 4.3	0.0 $\pm$ 3.9	161.67 $\pm$ 10.3	2.43 $\pm$ 1.3
KK <sup>ay</sup>	113.0 $\pm$ 11.2 <sup>a</sup>	229.0 $\pm$ 55.9 <sup>a</sup>	66.7 $\pm$ 6.4 <sup>a</sup>	8.33 $\pm$ 12.9 <sup>a</sup>	449.83 $\pm$ 26.1 <sup>a</sup>	17.03 $\pm$ 0.8 <sup>a</sup>
AA Western	223.3 $\pm$ 23.2 <sup>a</sup>	139.0 $\pm$ 16.1	133.7 $\pm$ 14.3 <sup>a</sup>	66.7 $\pm$ 18.9 <sup>a</sup>	211.0 $\pm$ 16.1 <sup>a</sup>	ND <sup>b</sup>
KK Western	280.0 $\pm$ 18.2	168.7 $\pm$ 15.3	90.0 $\pm$ 45.9	41.7 $\pm$ 30.6	373.0 $\pm$ 41.9 <sup>a</sup>	ND

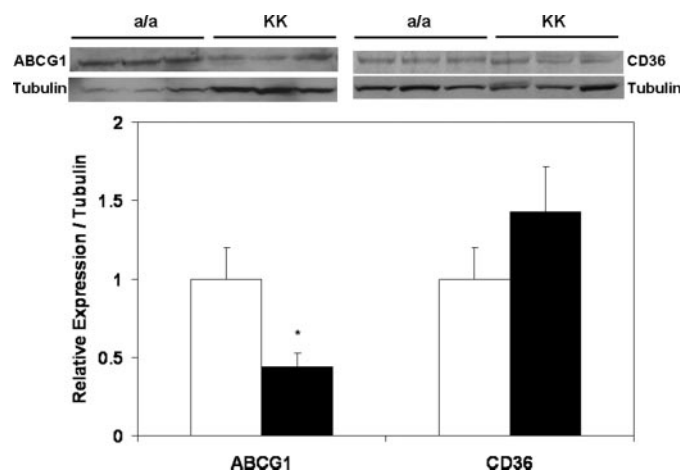
<sup>a</sup> Significantly higher than control, non-diabetic mice,  $p < 0.05$  for each parameter.

<sup>b</sup> ND, not determined.



**FIGURE 7. Increased *In Vivo* foam cell formation in diabetic  $KK^{ay}$  macrophages.** Control  $a/a$  ( $a/a$ ) and diabetic  $KK^{ay}$  ( $KK$ ) mice were fed a Western diet for 10 weeks. **A**, FPLC analysis of plasma lipoproteins. Fasting plasma was analyzed using FPLC according to Methods. The lipoprotein profiles for  $a/a$  and  $KK^{ay}$  mice on the Western diet are shown. Samples represent a pool of eight mice per group. *Closed circles* represent  $a/a$  mice; *open circles* represent  $KK^{ay}$  mice. **B**, foam cell formation *in vivo*. Freshly isolated peritoneal macrophages were isolated from control  $a/a$  and diabetic  $KK^{ay}$  mice fed either a chow or western diet. Lipid content and morphological differences were observed using Oil Red O and hematoxylin staining.

increased foam cell formation. This is the case even though there is not a dramatic difference between total cholesterol values between  $a/a$  and  $KK^{ay}$  mice fed a Western diet (Fig. 7A).



**FIGURE 8. ABCG1 and CD36 expression in control  $a/a$  and diabetic  $KK^{ay}$  macrophages.** Peritoneal macrophages were isolated from control  $a/a$  ( $a/a$ ) and diabetic  $KK^{ay}$  ( $KK$ ) mice. Macrophages were lysed and utilized for Western blotting for ABCG1 ( $ABCG1$ ) and CD36 ( $CD36$ ) as described under "Materials and Methods." *Top left*, immunoblot probed with anti-ABCG1 and anti-tubulin antibodies. *Top right*, immunoblot probed with anti-CD36 and anti-tubulin antibodies. *Bottom*, densitometry of immunoblots normalized to tubulin. *Open bars* represent control  $a/a$  samples. *Closed bars* represent diabetic  $KK^{ay}$  samples.

Indeed,  $a/a$  mice did become mildly diabetic when placed on the Western diet as is evident from their plasma glucose values (Table 2), although the degree of diabetes was not as severe as the  $KK^{ay}$  animals placed on a Western diet. We believe the mild diabetes developed by the  $a/a$  mice on Western diet to be part of the reason why a few foam cells were observed in the  $a/a$  mice *in vivo*.

**ABCG1 Expression Is Decreased in Diabetic  $KK^{ay}$  Mice**—To determine whether ABCG1 expression was reduced in the  $KK^{ay}$  diabetic mouse, we measured ABCG1 mRNA levels by quantitative real-time PCR. We found a significant 60% reduction in ABCG1 mRNA expression in  $KK^{ay}$  mice compared with control, nondiabetic  $a/a$  littermates (data not shown). We also measured CD36, SR-A1, LOX-1, and ABCA1 mRNA expression using quantitative real-time PCR and saw no significant changes (data not shown). We then examined the protein expression of CD36 and ABCG1 by immunoblotting. CD36 expression was not significantly altered, but ABCG1 expression was reduced by 60% (Fig. 8). In addition, we measured cholesterol efflux to HDL in the  $KK^{ay}$  macrophages. Unlike the  $db/db$  macrophages, we found a reduction of only  $\sim$ 10% compared with control  $a/a$  macrophages in their ability to efflux cholesterol to HDL (data not shown). The reason for this remains unclear, but we speculate that this could be due to the fact that

CD36 expression was not increased in the  $KK^{ay}$  macrophages. This could mean that the amount of [ $^3$ H]cholesterol influxed by the macrophages in the assay was not as high, and therefore the resulting efflux was not as high. Despite having similar plasma cholesterol values on the Western diet, the  $KK^{ay}$  mice still exhibited increased lipid accumulation (Fig. 7) due to ABCG1 down-regulation and not due to changes in CD36.

## DISCUSSION

Type 2 diabetes significantly increases risk for the development of atherosclerosis (1, 2). We show for the first time that foam cell formation is increased in mouse models of Type 2 diabetes and that diabetic mouse macrophages have a dramatic reduction in levels of ABCG1. Furthermore, we found that increased foam cell formation in Type 2 diabetes is caused by decreased macrophage cholesterol efflux to HDL. Finally, we show that ABCG1 expression can be down-regulated by glucose *in vitro* by culturing primary macrophages chronically in high glucose.

There are three primary scavenger receptors expressed on macrophages, SR-A, LOX-1, and CD36. These macrophage receptors internalize oxidized lipids and are considered pro-atherogenic (5, 26). Previous work by Liang *et al.* (25) using diabetic *ob/ob* mouse macrophages found increased CD36 protein expression, without increased mRNA expression. In the current study, we found a significant 4-fold increase in CD36 protein expression on diabetic *db/db* macrophages compared with control macrophages (Fig. 2). Using *C57BL/6J* peritoneal macrophages cultured chronically in elevated glucose (25 mM for 7 days), we also found that CD36 mRNA expression was not significantly increased in response to chronic elevated glucose levels (Fig. 4B), thus, further supporting the data by Liang *et al.* (25). However, in diabetic  $KK^{ay}$  mice, neither mRNA nor protein levels of CD36 were altered (Fig. 8), yet these mice still exhibited increased macrophage foam cell formation *in vivo* when placed on a Western diet (Fig. 7).

Recent studies have shown that glucose increased both SR-A1 and LOX-1. Fukuhara-Takaki *et al.* (27) showed increases in both mRNA and protein expression of SR-A1 in human monocyte derived macrophages incubated in high glucose and in STZ induced diabetic mice. In addition, Li *et al.* (28) have shown that LOX-1 mRNA and protein are increased in human monocytes following incubation in high glucose. We found no change in SR-A mRNA expression in the setting of Type 2 diabetes. We did, however, find that diabetic *db/db* macrophages had robust expression of LOX-1 mRNA compared with control *B6* macrophages. Unfortunately, we were unable to explore protein expression of either SR-A1 or LOX-1 due to the lack of a commercially reliable antibody.

Our work shows for the first time a dramatic reduction in ABCG1 expression in diabetic macrophages. Interestingly, it takes chronic incubation of macrophages in elevated glucose for down-regulation of ABCG1 to occur *in vitro*. This would be the likely scenario in Type 1 and Type 2 diabetes. The time course of ABCG1 down-regulation by glucose also suggests the possibility that other factors mediated by glucose, such as reactive oxygen species or AGE product production, may be the cause of ABCG1 down-regulation. While it is widely known that ABCG1 expres-

sion is primarily regulated by LXR, little is known about other possible regulatory mechanisms of ABCG1 levels (29–31). However, ABCA1 mRNA, which is also regulated by LXR, was not significantly reduced, but Oram *et al.* (32) have shown that ABCA1 function can be decreased by addition of AGE products. In addition, recent experiments by Walczak *et al.* (33) revealed that macrophages from LXR null mice were still able to transcribe ABCG1, suggesting that LXR is not the sole regulator of ABCG1 transcription.

The exact mechanism by which ABCG1 is down-regulated by glucose remains unclear. We investigated whether changes in ABCG1 levels in Type 2 diabetic mice could be caused by changes in mRNA stability by performing an actinomycin D study to determine the half-life of ABCG1 mRNA in both the non-diabetic *B6* and diabetic *db/db* macrophages. We found that the half-life of ABCG1 from both diabetic and non-diabetic macrophages is similar (data not shown), suggesting that the observed differences in ABCG1 mRNA levels between the two mouse groups are not the result of differences in mRNA stability. Thus, we hypothesize that the mechanism of control occurs via regulation of ABCG1 promoter activity.

Recently, Kennedy *et al.* (34) reported that global deletion of ABCG1 in mice caused significant accumulation of both cholesterol and phospholipids in multiple tissues when the mice were challenged with a high fat diet. Their data provide new evidence for the role of ABCG1 in lipid trafficking. Of interest was their novel finding that ABCG1 was present in macrophages, lymphocytes, endothelial cells, hepatocytes, Kupffer cells, and epithelial cells. Furthermore, absence of ABCG1 resulted in significant increases in lipid accumulation in these cells. These data shed new light on the role of ABCG1 and indicate its importance in maintaining lipid homeostasis in the body. Thus, down-regulation of ABCG1, as we show occurs in the setting of diabetes, could impact lipid trafficking in multiple tissues, thereby exacerbating the vascular complications of this disease, including, most likely, atherosclerosis. Thus, understanding the molecular regulation of ABCG1 in diabetes will be important for developing new therapies to reduce vascular complications.

However, it is important not to underscore the role of scavenger receptor activity in foam cell formation, as these receptors are responsible for bringing oxidized LDL into the macrophage. It is only after scavenger receptors have brought cholesterol into the macrophage that ABC transporters have substrate to remove from the cell. Our data suggest that diabetes effects macrophage foam cell formation at both the cholesterol influx and efflux stages.

In summary, macrophage foam cell formation is increased *in vivo* in mouse models of Type 2 diabetes. The increased foam cell formation is caused, in large part, by down-regulation of ABCG1 and reduced cholesterol efflux by diabetic macrophages. Thus, regulation of ABCG1 expression in diabetes is important for prevention of atherosclerosis in the setting of Type 2 diabetes.

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