RAGE Suppresses ABCG1-Mediated Macrophage Cholesterol Efflux in Diabetes

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Abstract

Diabetes exacerbates cardiovascular disease, at least in part via suppression of macrophage cholesterol efflux and levels of the cholesterol transporters, ATP binding cassette transporters A1 (ABCA1) and ABCG1. The receptor for advanced glycation end products (RAGE) is highly expressed in human and murine diabetic atherosclerotic plaques, particularly in macrophages. We tested the hypothesis that RAGE suppresses macrophage cholesterol efflux and probed the mechanisms by which RAGE downregulates ABCA1 and ABCG1. Macrophage cholesterol efflux to Apolipoprotein A1 (ApoA1) and High Density Lipoprotein (HDL) and reverse cholesterol transport to plasma, liver and feces were reduced in diabetic macrophages via RAGE. In vitro, RAGE ligands suppressed ABCG1 and ABCA1 promoter luciferase activity and transcription of ABCG1 and ABCA1 through PPARγ-responsive promoter elements, but not through Liver X Receptor (LXR) elements. Plasma levels of HDL were reduced in diabetic mice in a RAGE-dependent manner. Laser capture microdissected CD68+ macrophages from atherosclerotic plaques of Ldlr<sup>−/−</sup> mice devoid of Ager (RAGE) displayed higher levels of Abca1, Abcg1 and Pparg mRNA transcripts vs. Ager-expressing Ldlr<sup>−/−</sup> mice, in a manner independent of glycemia or plasma levels of total cholesterol and triglyceride. Antagonism of RAGE may fill an important therapeutic gap in the treatment of diabetic macrovascular complications.

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Accelerated atherosclerosis is a leading cause of morbidity and mortality in types 1 and 2 diabetes (1,2). Important roles for accelerated vascular inflammation have been demonstrated in diabetic atherosclerosis in human and animal subjects. In particular, increased numbers of macrophages together with pro-inflammatory ligands of the receptor for advanced glycation end products (RAGE) populate human atherosclerotic lesions, especially in diabetes (3).

In human diabetes, serum cholesterol efflux capacity and reverse cholesterol transport are impaired (4,5), and are mirrored in animal models of diabetes (6). The overall importance of these mechanisms is inferred from the inverse relationship between cholesterol efflux capacity to both carotid-intima thickness (7), a surrogate marker of atherosclerosis, at least in non-diabetic human subjects, and to incident cardiovascular events (8), underscoring the relevance of high-density lipoprotein (HDL) function to atheroprotective mechanisms. In diabetic human subjects, macrophage levels of two key cholesterol transporters, the ATP binding cassette transporters A1 (ABCA1) and ABCG1, are reduced, contributing to increased macrophage cholesterol accumulation (9,10). Despite the importance of these processes to vascular homeostasis, the precise mechanisms underlying these observations in diabetes are not delineated.

Advanced glycation end products (AGEs) form through the nonenzymatic glycation and oxidation of proteins and lipids. AGEs accumulate in diabetic plasma and tissues. One of the principal means by which AGEs exert their pathological effects is via ligation of RAGE. RAGE and its ligands AGEs, S100/calgranulins and high mobility group box 1 (HMGB1), are highly expressed in human and murine diabetic atherosclerotic lesions and co-localize, at least in part, with macrophage markers (11). In animal models, global deletion of Ager, or administration of soluble RAGE (sRAGE), the RAGE ligand decoy, which binds to and sequesters RAGE ligands, thereby inhibiting their ability to engage cell surface RAGE, is strongly protective against
acceleration of diabetic atherosclerosis, at least in part via reduction in lesional macrophage content and vascular inflammation (12-15). Furthermore, bone marrow transplantation experiments affirmed important roles for Ager expression in myeloid cells in progression of atherosclerosis in murine models (14). Here, we tested the hypothesis that RAGE contributes to impaired macrophage cholesterol efflux and reverse cholesterol transport, particularly in the RAGE ligand-enriched environment of diabetes.

**Research Design and Methods**

**Reagents**

The following materials were purchased: Apolipoprotein A1 (ApoA1), high density lipoprotein (HDL) and acetylated low density lipoprotein (Biomedical Technologies Inc.); fatty acid free bovine serum albumin (Equitech-Bio); T0901317, (TOCRIS Bioscience); human plasma LDL (Sigma-Aldrich); assays for measurements of HDL, total cholesterol and triglycerides (Wako Diagnostics); Rosiglitazone (Sigma-Aldrich); and U0126 and PD98509 (Cell Signaling).

**Animal studies**

Male homozygous Ager<sup>-/-</sup> (RAGE) mice (C57BL/6 Ager<sup>-/-</sup> mice backcrossed >12 generations into C57BL/6J (Jackson Laboratory)) and littermate Ager-expressing control mice were used. Male mice (WT and devoid of Ager) were rendered diabetic with streptozotocin (stz) (Sigma-Aldrich). For high-fat diet/low-fat diet (HFD/LFD) experiments, mice at the age of 6–8 weeks were fed HFD with 60% of calories from lard (D12492; Research Diets, Inc.) or LFD with 13% of calories from fat (5053 PicoLab Rodent Diet 20; LabDiet) for at least 3 months. Ldlr<sup>-/-</sup> mice devoid of or expressing Ager were rendered diabetic at age 7 weeks and then placed on Western
diet (0.15% cholesterol) (D01061401C, Research Diets, Inc) for 15 weeks followed by harvest of
the aortic arches at age 22 weeks. All animal procedures were approved by the Institutional
Animal Care and Use Committees of Columbia University and New York University and
performed in accordance with the National Institutes of Health Animal Care Guidelines.

Cell culture
Human THP-1 peripheral blood monocytic cells, L929 cells and HEK293T cells were obtained
from American Type Culture Collection (ATCC) and cultured as per the manufacturer’s
instructions. THP-1 cells were used as suspension cells throughout this study.

Isolation of murine macrophages
Primary murine bone marrow derived macrophages (BMDMs): Upon sacrifice, bone marrow
was retrieved from bilateral femora and BMDMs were cultured as previously described (16) and
used on day 7 incubation. BMDMs retrieved from non-diabetic mice were subsequently grown in
5.5 mM D-glucose and BMDMs retrieved from diabetic mice were grown in 25 mM D-glucose
to mimic their condition of origin. In other studies, BMDMs from non-diabetic mice were
cultured in 5.5 mM or 25 mM D-glucose for 7 days post-isolation and prior to studies. Peritoneal
macrophages were isolated as previously described, without the use of thioglycollate (17).

RNA isolation and quantitative real-time PCR
Quantitative RT-PCR was performed on isolated RNA using the TaqMan Fast Universal Master
Mix 2X with premade primer sets (Life Technologies) (Table S1).
RNAi silencing

siRNA to reduce levels of AGER was purchased from Life Technologies (ID#110857; sense (5’ to 3’: CGGCUGGUGUUCCAAUAAtt) and antisense (5’ to 3’: UUAUUUGGAACACCAGCCGtg)) and scramble negative control siRNA (ID#AM435).

Over-expression of PPARG and ABCG1 in THP-1 cells

Human PPARG cDNA cloned into pCMV-SPORT6 plasmid was obtained from Thermo Scientific and human ABCG1 cDNA cloned into pCMV6-AC-GFP plasmid was purchased from Origene.

Cholesterol efflux assays

Primary murine BMDMs or THP-1 cells were labeled with 5 μCi/ml of 3H-cholesterol (Perkin Elmer) in the presence of acetylated LDL (50 mg/l) and fatty acid free bovine serum albumin (1%) for 24 h. After washing the cells and achieving equilibrium for 1 h with fatty acid free bovine serum albumin (1%), cells were treated with either ApoA1 or HDL. After 6 h incubation at 37°C, the supernatant was collected. Cells were washed and lysed; aliquots of both supernatant and the medium were measured in a multi-purpose scintillation counter (BECKMAN, LS 6500). The percent cholesterol efflux was calculated by dividing the radioactivity in the supernatant by the sum of the radioactivity measured in the supernatant and the cell lysates.

Western blot analysis
The following antibodies were used: rabbit anti-ABCG1 (NB400-132, 1:500, Novus Biologicals); rabbit anti-ABCA1 (NB400-105, 1:500, Novus Biologicals); mouse anti-tubulin (clone KMX-1, catalog MAB3408, 1:500, Millipore); mouse anti-β-actin (A1978, 1:4000, Santa Cruz Biotechnology); rabbit anti-PPAR Gamma (ab41928, 1:1000, Abcam); rabbit anti-RAGE (GTX23611, 1:1000, GeneTex); rabbit anti-ApoA1 (ab20453, 1:1000, Abcam); rabbit anti-p44/42 MAPK (Erk1/2) (9102, 1:1000, Cell Signaling); and mouse anti-Phospho-p44/42 MAPK (Erk1/2) (9106, 1:1000, Cell Signaling). After incubation with indicated secondary antibodies, protein signals were visualized with the ECL detection system (Thermo Scientific) using the Odyssey FcDual Mode Imaging system (LI-COR); or IRDye 680RD Goat anti-mouse (1:25,000) (LI-COR) and IR Dye 800CW Goat anti-rabbit (1:10,000) (LI-COR) and protein signals were visualized using the Odyssey Infrared Imaging System Model 9120 (LI-COR).

Luciferase Reporter Assays

Human ABCG1 promoter A region spanning -1104 to +37 bp (upstream of exon 1) cDNA was generated following published methods by Uehara et al. (18) and cloned into pCR-script vector. Subsequently, this cDNA for human ABCG1 promoter A region (-1104/+37) was cloned into the pGL3-basic vector using restriction sites KpnI and HindIII. Prior to transfection, HEK293T cells were grown to 50-80% confluence on 12-well plates in DMEM supplemented with 10% FBS + 1% penicillin-streptomycin. Cells were transiently transfected with 1 µg of mouse Ager cDNA in pcDNA3 on Day 1, then on Day 2 cells were transiently transfected with 1 µg of pGL3 luciferase firefly vector (Promega) containing cDNA of the human ABCG1 promoter A or human ABCA1 promoter cDNA (gift from Dr. Antonio Castrillo), and 1ng pRL-TK-Renilla (Promega) (to normalize transfections) using Lipofectamine 2000 (Invitrogen). Luciferase activities were
measured using the Dual-Luciferase Assay System (Promega) and were divided by Renilla activity to obtain a normalized value (Relative Luciferase Units (RLU)). *ABCG1* deletion mutants were prepared as described in Uehara et al. (18). Site-directed mutations to PPRE 1 (sense) and PPRE 2 (antisense) sites (single mutations and a double mutation) on the human *ABCG1* promoter were constructed using the QuikChange II XL (Agilent Technologies).

**Bioinformatics**

The -413 to -303 region of the *ABCG1* promoter was compared to the high quality vertebrate subset of TransfacPro 2013.2 (19) using the Match program (20) set to minimize false positives. Binding sites of 19 unique transcription factors (TFs) were found. Among these were binding sites for Pparg (V$PPARG_02) (21,22). There are two V$PPARG_02 sites, both of which were at -330, one of which is in the sense direction and the other in the antisense direction. The most conserved regions of the site core sequence were used to design single base pair substitutions to interfere with binding. In the following text, the core recognition element is capitalized and the replacement nucleotide is indicated in boldface.

On the sense strand, the following substitutions were made:

**S1: -325G->C**

gagatGGTAgattttcctactt

gagatCGGTAgattttcctactt

For antisense, the following substitutions were made:

**A1: -313C->G**

gagatgggtagATTCCTactt

gagatgggtagATTCCTactt
Electrophoretic-mobility shift assays (EMSA)

Nuclear protein extracts (NPEs) were prepared from THP-1 cells and EMSAs were performed using the Odyssey EMSA Buffer Kit (LI-COR) and 10X Orange Loading Dye (LI-COR) according to manufacturer’s instructions. Gels were visualized using LI-COR Odyssey Imager. The PPARG DNA probe sequences used were:

Sense strand: 5-'/5IRD700/GAGATGGGTAGATTTTCCTACTT x3
Antisense strand: 5-'/5IRD700/CTCTACCCATCTAAAAGGATGAA x3

HDL-cholesterol, total cholesterol and triglycerides measurements

HDL-cholesterol and total cholesterol concentrations were measured using colorimetric assays (Wako Diagnostics); triglyceride levels were measured enzymatically using a colorimetric assay (L-Type TG M Enzyme Color A) (Wako Diagnostics).

Chromatin Immunoprecipitation (ChIP)

ChIP assays were performed using the ChIP-IT Express Enzymatic kit (Active Motif). ChIP-enriched DNA (2 µl) was used for quantitative RT-PCR for the ABCG1 promoter using SYBR Green reagents and the following primers: forward primer, 5’-TTTGCCGTAATTGTTTTCAATG-3’; reverse primer, 5’-GCAGGGTTACTAAAGGCGAGT-3’.

Laser capture microdissection (LCM)
Aortic arches from the indicated \( Ldlr^{+/+} \) or \( Ldlr^{-/-}/Ager^{-/-} \) mice were subjected to laser-capture microdissection of CD68-expressing cells (detected by rat anti-mouse CD 68 clone FA-11 (MCA1957, AbD Serotech) using Leica LMD6500 laser microdissection system and quantitative RT-PCR for measuring \( Abca1, Abcg1 \) and \( Pparg \) mRNA transcripts.

\textit{Oil Red O staining}

Oil Red O staining of the atherosclerotic lesions was performed according to the manufacturer’s instructions (American MasterTech) (23).

\textit{Macrophage Reverse Cholesterol Transport}

Male wild-type C57BL/6 or littermate \( Ager^{-/-} \) mice were rendered diabetic with stz for 2 months and bone marrow was collected. BMDMs were prepared and radiolabeled with 5 \( \mu \)Ci/ml \(^3\)H-cholesterol and with acetylated LDL (50 \( \mu \)g/ml) for 24 h. BMDMs were washed and injected into WT C57BL/6 non-diabetic mice. On the day of injection, recipient mice were housed individually with unlimited access to food and water. \(^3\)H-cholesterol/acetylated LDL-loaded BMDMs (2.5-3.5x10^6/ml) were injected by IP injection. Blood was collected and plasma was subjected to counting in a beta counter. Feces were collected continuously through 64 total h collection. At 64 h, mice were humanely sacrificed and liver tissue collected for further analyses. Results were expressed as percentage of the cpm injected as previously described (24).

\textit{Statistics}

All data are reported as mean ± the standard error of the mean (SEM) unless otherwise stated (“n” is noted in figure legends). Data were analyzed with the Student’s t-test or by ANOVA.
followed by 2-tailed distribution t-test. p-values ≤0.05 were considered statistically significant. Cholesterol efflux and RCT were log transformed prior to analysis to assure approximate normality and analyzed in R. Experiments at each time point were analyzed separately to characterize the effect of each genotype/disease state combination on RCT at that time. Also, experiments on each genotype/disease state combination were analyzed separately at different times to characterize the effect of time on RCT for that genotype/disease state combination. Both the Fligner-Killeen (25) test and visual inspection showed heteroscedasticity, so Welch’s test for the comparison of several means (26) was used instead of standard ANOVA to estimate significance. Significance of comparisons was estimated using Westfall’s method (27,28) as implemented in Multcomp (29) with heteroscedasticity accounted for by a Sandwich estimator (30) as implemented in Sandwich (31). The Figures display values of means and standard errors back-transformed to percentages. Table S2 presents the Welch test p-value, which is the probability that at least one of the conditions is not the same as the other. This table also gives the probability of \((Ager^{+/} \text{DM} - Ager^{+/} \text{NDM}) - (\text{WTDM} - \text{WTNDM})\) differing from 0 by chance, that is to say the probability that the effect of diabetes on RCT in \(Ager^{+/}\) mice differs from the effect of diabetes in WT mice. By simple algebraic manipulation, is seen, that this expression is also equal to \((Ager^{+/} \text{DM} - \text{WTDM}) - (Ager^{+/} \text{NDM} - \text{WTNDM})\), which is the effect of deletion of \(Ager\) in diabetic mice relative to the effect on non-diabetic mice. This later expression shows whether comparison of the effect of deletion of \(Ager\) in diabetic mice, differs from the corresponding effect in non-diabetic mice.

**Results**
**RAGE Impairs Macrophage Cholesterol Efflux to ApoA1 and HDL in diabetes**

Primary BMDMs were retrieved from wild type (WT) male C57BL/6 mice after two months of hyperglycemia induced by streptozotocin (stz). Controls were age- and gender-matched non-diabetic mice treated with intraperitoneal injections of vehicle for stz, citrate buffer. BMDMs were cultured in concentrations of glucose consistent with the glycemic state of the mice from which they were retrieved. WT BMDMs retrieved from diabetic animals displayed significantly reduced cholesterol efflux to ApoA1 and HDL vs. BMDMs from non-diabetic WT mice (p<0.01 and p<0.001, respectively) (Figure 1A-B, Supplementary Table 2). In non-diabetes, although deletion of *Ager* imparted no significant effect on cholesterol efflux to ApoA1 vs. WT BMDMs, significantly higher cholesterol efflux to HDL was observed (p<0.0001) (Figure 1A-B, Supplementary Table 2). In diabetes, deletion of *Ager* resulted in significantly higher cholesterol efflux to both ApoA1 and HDL compared to WT BMDMs (p<0.001) (Figure 1A-B, Supplementary Table 2).

As it has been reported that mRNA transcript and protein levels of *Abca1* and *Abcg1*, principal regulators of cholesterol efflux to ApoA1 and HDL, respectively, are reduced in murine and human diabetes (9,10,32,33), we tested the role of RAGE. The mRNA transcript and protein levels of *Abca1* were lower after two months hyperglycemia in WT BMDMs (Figure 1C-D). In non-diabetes, *Abca1* mRNA transcript levels did not significantly differ between WT and *Ager*<sup>−/−</sup> BMDMs, but ABCA1 protein levels were significantly higher in *Ager*<sup>−/−</sup> vs. WT BMDMs. In diabetes, higher levels of *Abca1* mRNA transcripts and protein were observed between *Ager*<sup>−/−</sup> vs. WT BMDMs (Figure 1C-D).

Diabetes resulted in a significant reduction in *Abcg1* mRNA transcripts and ABCG1 protein in WT BMDMs vs. the non-diabetic state (Figure 1E-F). In both non-diabetic and
diabetic BMDMs, deletion of Ager resulted in significantly higher levels of Abcg1 mRNA transcripts and ABCG1 protein vs. WT BMDMs (Figure 1E). These data indicate that diabetes exerted significant effects on down-regulation of ABCA1 and ABCG1 transporters through RAGE.

To directly test the effects of glucose, we incubated non-diabetic mice BMDMs from WT mice in high glucose (25 mM) for 7 days versus normal levels of glucose (5.5 mM) and determined Abca1 and Abcg1 mRNA levels. No significant differences were observed in mRNA levels in non-diabetic WT BMDMs cultured for 7 days in 5.5 mM D-glucose or 25 mM D-glucose (Supplementary Figure 1A-B).

Additionally, we retrieved primary peritoneal macrophages (without thioglycollate treatment) and prepared mRNA immediately upon retrieval. Compared to WT peritoneal macrophages from non-diabetic mice, WT diabetic peritoneal macrophages displayed significantly lower levels of Abca1 and Abcg1 mRNA transcripts. In the non-diabetic state, a significant increase in Abcg1 mRNA transcripts was observed in Ager<sup>x/x</sup> versus WT peritoneal macrophages, and in the diabetic state, Ager<sup>x/x</sup> peritoneal macrophages displayed significantly higher levels of both Abca1 and Abcg1 mRNA transcripts (Supplementary Figure 1C-D). These results are completely analogous to those observed in primary murine BMDMs (Figure 1C and 1E).

*AGE-RAGE interaction suppresses cholesterol efflux to ApoA1 and HDL in human THP-1 cells*

To ensure that our results were applicable to cells that normally expressed RAGE and were of human origin, we treated THP-1 cells with carboxymethyllysine (CML)-AGE, which was previously shown to be a specific AGE ligand of RAGE that accumulates in human
atherosclerotic lesions (34). Compared to vehicle/scramble siRNA treatment, CML-AGE resulted in significantly reduced cholesterol efflux to ApoA1 and HDL (p<0.0001) (Figure 2A-B, respectively). RNAi-knockdown of AGER in CML-AGE-treated THP-1 cells resulted in significantly higher cholesterol efflux to ApoA1 (p<0.01) and to HDL (p<0.0001) compared to scramble siRNA/CML treatment (Figure 2A-B, respectively).

Treatment of THP-1 cells with CML-AGE in the presence of scramble siRNA resulted in significant suppression of ABCA1 and ABCG1 mRNA transcripts (p<0.01) (Figure 2C-D). RNAi-knockdown of AGER resulted in significantly higher levels of ABCA1 and ABCG1 mRNA transcripts vs. scramble siRNA/CML-AGE treatment (p<0.001 and p<0.01, respectively) (Figure 2C-D, respectively).

At the protein level, Western blotting revealed a trend to lower levels of ABCA1 protein in CML-AGE-treated THP-1 cells; in the presence of RNAi knockdown of AGER, ABCA1 protein levels were similar to those in non-CML-AGE-treated cells (Figure 2E). In the case of ABCG1, treatment with CML-AGE resulted in a significant suppression of ABCG1 protein levels vs. vehicle (p<0.01) (Figure 2F). RNAi knockdown of AGER, in the presence of CML-AGE, resulted in significantly higher levels of ABCG1 protein compared to CML-AGE treatment (p<0.0001) (Figure 2F).

Thus, in human RAGE-expressing THP-1 cells, RAGE ligand CML-AGE reduces cholesterol efflux to ApoA1 and HDL, at least in part through regulation of ABCA1 and ABCG1.

*RAGE mediates impaired reverse cholesterol transport in diabetic macrophages*
Based on these *in vitro* findings that RAGE contributed to suppression of macrophage cholesterol efflux to ApoA1 and HDL, particularly in diabetes, we tested the effects of RAGE on macrophage reverse cholesterol transport *in vivo*. WT and *Ager*-/- mice were rendered hyperglycemic with streptozotocin or treated with control, citrate buffer (non-diabetes) for two months. BMDMs were isolated, loaded with radiolabelled cholesterol and injected into WT non-diabetic mice. As shown in Figure 3A-B, in WT mice, diabetes resulted in reduced macrophage reverse cholesterol transport to plasma at 43 h and 64 h post-injection of WT BMDMs (p<0.001, and p<0.01, respectively). In non-diabetic BMDMs, no statistically significant effects of *Ager* deletion were noted at 43 h or 64 h post-injection (Figure 3A-B). In diabetic BMDMs, however, deletion of *Ager* resulted in significantly higher reverse cholesterol transport to plasma at 43 h and 64 h post-injection (p<0.001, and p<0.01, respectively) (Figure 3A-B, Supplementary Table 2).

Diabetes resulted in a trend to reduced macrophage reverse cholesterol transport to the liver vs. the non-diabetic state (Figure 3C, Supplementary Table 2). Although deletion of *Ager* had no significant effect on macrophage reverse cholesterol transport to the liver in the non-diabetic state, significantly higher reverse cholesterol transport in diabetic BMDMs devoid of *Ager* was noted compared to diabetic WT BMDMs (p<0.01) (Figure 3C, Supplementary Table 2). In feces of WT mice, diabetes resulted in significantly reduced macrophage reverse cholesterol transport (p<0.01). Whereas deletion of *Ager* in non-diabetic BMDMs resulted in modest but significantly lower reverse cholesterol transport to feces vs. WT non-diabetic BMDMs, trends to higher reverse cholesterol transport to feces were observed in diabetic BMDMs devoid of *Ager* compared to diabetic WT BMDMs (Figure 3D, Supplementary Table 2).
We measured plasma levels of HDL and found that diabetes resulted in significantly lower levels of HDL in WT mice compared to WT age-matched, non-diabetic mice, 35.1±0.8 vs. 41.7±2.3, respectively (p<0.01) (Figure 3E). In the non-diabetic state, levels of HDL did not differ by Ager genotype, as mean HDL levels were 41.7±2.3 vs. 42.3±1.4 in WT non-diabetic vs. Ager<sup>-/-</sup> non-diabetic mice, respectively (p=NS) (Figure 3E). In contrast, diabetic Ager<sup>-/-</sup> mice displayed significantly higher levels of HDL vs. age-matched WT diabetic mice, 47.2±0.4 vs. 35.1±0.8, (p<0.0001) (Figure 3E). Levels of total cholesterol were tested (Supplementary Table 3). In the non-diabetic state, there were no significant effects of Ager genotype. Total levels of cholesterol were higher in diabetic vs. non-diabetic WT mice (120.7±4.0 vs. 96.4±3.2) (p<0.001). Diabetic Ager<sup>-/-</sup> mice displayed significantly lower levels of total cholesterol compared to their diabetic WT counterparts (95.4±2.9 vs. 120.7±4.0) (p<0.001) (Supplementary Table 3).

Based on these findings of higher levels of plasma HDL in diabetic mice devoid of Ager, we tested mRNA transcript and protein levels of Apoa1 and Abca1 in mouse liver tissue, as APOA1 and ABCA1 are critical for HDL production (35). Ager genotype had no effect on mRNA transcript levels of Apoa1 (Supplementary Figure 2A) or Abca1 (Supplementary Figure 2B) in non-diabetic mice. However, diabetes resulted in significant reduction in Apoa1 and Abca1 mRNA transcripts vs. non-diabetic state in WT mice (p<0.05). Levels of Apoa1 and Abca1 mRNA transcripts were lower in diabetic Ager<sup>-/-</sup> liver vs. diabetic WT mice (p<0.001 and p<0.05, respectively) (Supplementary Figure 2A-B). Deletion of Ager resulted in increased APOA1 protein levels in non-diabetic mice livers (p<0.01), with trends to increased protein levels of APOA1 in diabetic mice liver devoid of Ager vs. the WT (Supplementary Figure 2C). No difference was observed in ABCA1 protein levels between non-diabetic Ager<sup>-/-</sup> vs. WT liver
tissue, whereas protein levels were significantly higher in diabetic Ager−/− liver compared to diabetic WT mice (p<0.05) and non-diabetic Ager−/− mice (p<0.05) (Supplementary Figure 2D). These findings suggest that the higher levels of HDL in mice devoid of Ager, particularly in the diabetic state, were likely accounted for by significantly higher levels of ABCA1 protein in the liver.

**AGE-RAGE interaction and molecular regulation of ABCG1**

To define the molecular mechanisms accounting for these findings, human ABCA1 and ABCG1 promoter reporter luciferase constructs were transfected into AGER-transfected HEK293T cells (HEK293T cells do not natively express RAGE); treatment of these cells with CML-AGE resulted in significant suppression of both ABCA1 (p<0.05, Supplementary Figure 3A) and ABCG1 (p<0.001, Figure 4A) promoter luciferase activity. We focused on the effects of RAGE on molecular regulation of ABCG1, as the greatest reduction in transcription of the two transporters by CML-AGE/RAGE in macrophages was on ABCG1 and since macrophage cholesterol efflux to HDL was most potently affected by RAGE. Supporting the RAGE dependence of these findings on CML-AGE-mediated suppression of ABCG1 promoter luciferase activity, in the absence of AGER transfection in HEK293T cells, CML-AGE exerted no suppressive effects on ABCG1 promoter luciferase activity (Supplementary Figure 3B). Further, the effect of CML-AGE treatment on suppression of the ABCG1 promoter luciferase activity in AGER-expressing HEK293T cells was prevented by incubation with the extracellular ligand-binding domain of RAGE, sRAGE (p<0.0001) (Figure 4A).

A series of deletion mutants of the human ABCG1 promoter (1104/+37) was prepared as described by Uehara (18). CML-AGE treatment resulted in significant suppression of promoter
luciferase activity in full-length and deletion mutants A, B and C (Figure 4B). However, in deletion mutants D, E, F and G, there were no suppressive effects of CML-AGE, thereby implicating elements within C, but not D, in the suppressive effects of CML-AGE on promoter luciferase reporter activity. As shown in Supplementary Table 4, multiple putative transcription factor binding sites within -413 to -304 were identified. It was notable that NRLH3 (LXR-alpha) putative binding sites were not identified within this region (36). Consistent with this finding, diabetes exerted no significant effect on mRNA transcript levels of Nr1h3 (Lxr-alpha) or Nrlh2 (Lxr-beta) compared to non-diabetic BMDMs (Supplementary Figure 4A-B). Also, CML-AGE exerted no suppressive effect on LXR agonist T0901317–mediated increase in ABCA1 (Supplementary Figure 5A) or ABCG1 (Supplementary Figure 5B) promoter reporter luciferase activity, suggesting a mechanism of action independent of LXRs.

Given the known potent effects of PPARgamma (PPARG) on macrophage properties (37-39), we determined if putative PPARG responsive elements (PPRE) within the human ABCG1 promoter were linked to the suppressive effects of CML-AGE. Although LXR putative binding sites were not observed in the region of interest, -413 to -304, within the ABCG1 promoter, we identified at least two putative PPRE in this region. First, to directly assess the binding of RAGE-expressing THP-1 cell nuclear extract to the PPRE1 and PPRE2 containing binding elements on the -413 to -304 promoter region, we performed electrophoretic mobility shift assays (EMSAs) using a consensus probe incorporating both PPRE sites. As shown in Figure 4C, CML-AGE reduced nuclear protein binding to the ABCG1 promoter elements containing the two indicated PPRE in a manner prevented by soluble RAGE. Note that supershift experiments demonstrated the specific bands of interest by virtue of shifting (reduced intensity) in the presence of anti-PPARG IgG (Figure 4C).
Second, we performed site-directed mutagenesis of PPRE sense (gagatGGGTAgattttcctactt) and PPRE antisense; gagatgggtagatTTTCCtactt) and tested promoter reporter luciferase activity in CML-AGE vs. vehicle-treated RAGE-expressing HEK293T cells. As shown in Figure 4D, mutation of PPRE antisense (mutation 2) and both PPRE antisense and PPRE sense (mutation 2 and 1) prevented the suppressive effects of CML-AGE on the promoter reporter luciferase activity in the region -413 to -304. Taken together, these results demonstrate that CML-AGE suppresses ABCG1 promoter activity, in a manner that can be prevented by sRAGE, and that CML-AGE mediates suppressive effects on the -413 to -304 region on the ABCG1 promoter in a manner involving the antisense (TTTCC) core motif recognized by PPARγ.

Finally, we performed chromatin immunoprecipitation (ChIP) assays to directly study the effects of CML-AGE on PPARγ transcription factor recruitment to the -413 to -304 ABCG1 promoter region. A robust signal of ABCG1 mRNA transcription by quantitative real-time PCR was noted in THP-1 under basal conditions; when THP-1 cells were treated with CML-AGE, this signal was significantly reduced by >50% (p<0.05) (Figure 4E). Treatment with sRAGE significantly rescued the suppressive effects of CML-AGE on ABCG1 mRNA transcription levels in the ChIP assay (p<0.001) (Figure 4E). Thus, CML-AGE suppresses PPARγ recruitment to the ABCG1 promoter locus in the promoter region -413 to -304 in a RAGE-dependent manner. Consistent with the principal effects of CML-AGE/RAGE on PPARγ-dependent mechanisms, treatment of CML-AGE-treated THP-1 cells with the PPARγ agonist rosiglitazone (10 µM) partially, but significantly, rescued the CML-AGE-mediated suppressive effects on ABCG1 mRNA transcripts (p<0.01) (Supplementary Figure 5C).
CML-AGE/RAGE and modulation of levels of PPARG in BMDMs and RAGE-expressing THP-1 cells

These findings prompted us to test if CML-AGE affected levels of PPARG. First, WT BMDMs treated with CML-AGE displayed a reduction in Pparg mRNA (Figure 5A) and PPARG protein (Figure 5B) compared to vehicle-treated WT cells (p<0.01 and p=0.05, respectively). In contrast, when Ager−/− BMDMs were treated with CML-AGE, no reduction in Pparg mRNA or PPARG protein levels was noted (Figure 5A-B). Similarly, in CML-AGE-treated THP-1 cells, treatment with siRNA-AGER vs. siRNA-Scramble prevented the effects of CML-AGE on reducing levels of PPARG mRNA and PPARG protein (p<0.05 and p<0.01, respectively) (Figure 5C-D).

To examine the molecular mechanisms by which CML-AGE suppresses PPARG binding to the ABCG1 promoter, we assessed the mitogen-activated protein kinase (MAPK) pathway since it has been previously demonstrated to be an important regulator of PPARG expression (40). Treatment of THP-1 cells with CML-AGE resulted in increased phospho/total ERK vs. treatment with vehicle (p<0.05) (Figure 5E). Pretreatment of THP-1 cells with the selective MAPK inhibitor U0126 or PD98059 prevented the effects of CML-AGE on suppression of PPARG mRNA vs. vehicle (Figure 5F). Thus, the molecular mechanism by which CML-AGE suppresses PPARG is, at least in part, via the MAPK pathway.

Rescue of CML-AGE modulation on THP-1 cell cholesterol efflux and expression of ABCG1, and modulation of Pparg mRNA in atherosclerotic plaques in diabetic LDLr−/− mice

To definitively link the suppressive effects of CML-AGE on cholesterol efflux to HDL to regulation of ABCG1, we over-expressed human ABCG1 in THP-1 cells and treated the cells
with CML-AGE vs. vehicle. CML-AGE treatment resulted in reduced cholesterol efflux to HDL (p<0.01) (Figure 6A), which was prevented by ABCG1 over-expression compared to CML-AGE treatment alone (p<0.0001) (Figure 6A). To unequivocally link PPARG reduction to the suppression of ABCG1 transcription, we over-expressed human PPARG in THP-1 cells to determine if this rescued the suppressive effects of CML-AGE on ABCG1 and ABCA1 mRNA transcript levels. Although CML-AGE resulted in reduced ABCG1 and ABCA1 mRNA transcripts vs. vehicle in THP-1 cells (p<0.001 and p<0.0001, respectively) (Figure 6B-C), transient transfection with PPARG cDNA prevented the suppressive effects of CML-AGE, as levels of ABCG1 were about 5-fold higher and ABCA1 were about 4-fold higher in PPARG-overexpressed cells vs. CML-AGE treatment alone (p<0.0001 in each case) (Figure 6B-C).

Next, to test if deletion of Ager modulates lesional macrophage levels of Abca1, Abcg1 and Pparg in diabetic atherosclerosis (12), mice devoid of Ldlr, either expressing or devoid of Ager, were rendered diabetic with stz. Mice were fed Western-type diet for 15 weeks, which has been shown to induce insulin resistance (41). The accumulation of fats, lipids and CD68-expressing macrophages was significantly reduced in atherosclerotic plaques from diabetic Ldlr⁻/⁻ mice devoid of Ager vs. Ldlr⁻/⁻ mice expressing Ager (Figure 7A). Laser capture-microdissected CD68-expressing macrophages from the aortic plaques of diabetic Ldlr⁻/⁻ mice devoid of Ager displayed higher levels of Abca1 mRNA transcripts (p=0.05) and significantly higher levels of Abcg1 and Pparg mRNA transcripts compared to macrophages retrieved from atherosclerotic plaques of diabetic Ager-expressing Ldlr⁻/⁻ mice (p<0.05) (Figure 7B). The beneficial effects of Ager deletion were independent of the levels of glucose, total cholesterol or total triglyceride; levels of plasma HDL were higher in Ldlr⁻/⁻ mice vs. Ager-expressing mice devoid of Ldlr (p<0.05) (Table 1).
Finally, to complement our findings in type 1 diabetes, we performed additional experiments to test the role for RAGE in the regulation of Abca1, Abcg1 and Pparg in BMDMs from mice fed high-fat diet (HFD) versus low-fat diet for three months. Compared to LFD, HFD feeding in WT mice resulted in significant suppression of Abca1 and significant but modestly higher levels of Abcg1 and Pparg mRNA transcripts (Supplementary Figure 7A-C). In BMDMs of HFD-fed Ager⁻/⁻ mice, mRNA transcript levels of Abca1, Abcg1 and Pparg were significantly higher than those observed in WT HFD-fed mice (Supplementary Figure 7A-C).

**Discussion**

Hyperglycemia, pathognomonic of all forms of diabetes, leads to the generation of irreversibly-formed AGEs. AGEs such as CML-AGE, a specific signaling ligand of RAGE (34), accumulate in hyperglycemia and their production is exacerbated by inflammation and oxidative stress (42). Previous work suggested that CML-AGE reduced HDL-mediated cellular cholesterol efflux (43); agents known to reduce levels of CML-AGE, aminoguanidine and metformin prevented the suppressive effects of CML-AGE on reduction of HDL-mediated cellular cholesterol efflux. These data support modulatory roles for AGEs in regulation of cholesterol efflux capacity. Here, we illustrate that these mechanisms are RAGE-dependent.

Our findings uncover important roles for PPARG in the mechanisms of CML-AGE/RAGE-dependent reduction in expression of ABCA1 and ABCG1. Although PPARG agonists may exert their effects at least in part via down-regulation of Ager (44,45), the present findings suggest the converse is also true, that is, RAGE ligands directly suppress PPARG through ERK signal transduction. Previous work suggested that CML-AGE reduced PPARG expression in chondrocytes (40); the present study illustrates new roles for AGE/RAGE in
suppression of PPARG in macrophages and suggests a potential novel role for the AGE/RAGE pathway in distinct effects of PPARG, such as macrophage polarization (37-39). Indeed, preliminary findings reveal that Ager−/− BMDMs (non-diabetic or diabetic) display increased levels of prototypic M2 markers versus WT BMDMs (data not shown).

Our work suggests that the CML-AGE/RAGE axis suppresses ABCA1 and ABCG1 promoter luciferase activity and transcription through elements in the human ABCA1 and ABCG1 promoters containing PPRE but not LXR-dependent elements. Consistent with this concept, our data revealed that CML-AGE imparted no suppressive effect on ABCA1 or ABCG1 promoter luciferase activity as stimulated by the LXR agonist, T0901317. It was previously reported by Ozasa and colleagues that the PPARG agonist pioglitazone enhanced cholesterol efflux through regulation of ABCA1 and ABCG1 (46). Those authors showed that LXR contributed to the effects of pioglitazone transactivation of ABCA1 but was only partially involved in the regulation of ABCG1. Our experimental strategy specifically testing the effect of CML-AGE/RAGE suggests that the suppressive effects of this axis were LXR-independent/PPARG dependent. Precedent for LXRα/LXRβ-independent regulation of Abcg1 was previously demonstrated in mice in which PPARG agonism stimulated regulation of Abcg1 in mice devoid of both Nrlh3 and Nrlh2 (47).

Our studies revealed that deletion of Ager in diabetic mice resulted in higher levels of plasma HDL and reduced total cholesterol. Although the higher expression of Abca1 and Abcg1 in Ager−/− diabetic BMDMs vs. the WT would not be expected to increase plasma levels of HDL (48), our data revealing Ager deletion-dependent significant increases in hepatic ABCA1 protein suggest a plausible mechanism for these observations. It was recently proposed that greater understanding of HDL function through its action on promotion of cholesterol efflux and
reverse cholesterol transport might be more clinically-relevant than the absolute levels of HDL (49). More in-depth studies, including analysis of low-density lipoprotein (LDL) cholesterol, very-low-density lipoprotein (VLDL) cholesterol and triglycerides, in addition to the HDL and total cholesterol measurements and potential modulatory roles for RAGE will be beneficial for a full understanding of the reduced total cholesterol levels observed in Ager<sup>−/−</sup> mice. We suggest that despite our findings that the beneficial effects of blockade/deletion of Ager were largely limited to the diabetic state, especially in an atherosclerosis context, these results are nevertheless meaningful, as accelerated macrovascular disease is a major cause of morbidity and mortality in diabetic subjects. It is important to note that beyond macrophages, studies have shown key roles for the endothelium, particularly with respect to LXR biology, in the pathogenesis of atherosclerosis (50).

We conclude that as large-scale clinical trials indicate that the benefits of statins in regression of atherosclerosis in diabetic subjects may be less prominent than those in subjects without diabetes (51,52), the present work may fill an important therapeutic gap in diabetic cardiovascular disease.

**Acknowledgements**

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**Author Contributions.** G.D. and X.S. co-designed the experiments, performed research, analyzed the data and contributed to the writing of the manuscript. L.S., D.T, R.R., A.A., C.HdP., and F.S. helped perform research and reviewed the manuscript. R.F. and Ravichandran Ramasamy contributed to the experimental design of the research, analyzed the data and reviewed and edited the manuscript. A.M.S. designed the experiments, reviewed data and analysis, wrote the manuscript and edited the manuscript. Ann-Marie Schmidt is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.


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Ikewaki K: Pioglitazone enhances cholesterol efflux from macrophages by increasing ABCA1/ABCG1 expressions via PPARgamma/LXRalpha pathway: findings from in vitro and ex vivo studies. *Atherosclerosis* 2011;**219**:141-150


Table 1

Characteristics of diabetic Ldlr<sup>+</sup> and Ldlr<sup>−/−</sup>/Ager<sup>−/−</sup> mice: plasma analyses

<table>
<thead>
<tr>
<th></th>
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<th>Ldlr&lt;sup&gt;−/−&lt;/sup&gt;/Ager&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Glucose</td>
<td>322±16.26</td>
<td>322±16.24</td>
</tr>
<tr>
<td>HDL</td>
<td>74±11.12</td>
<td>84±12.26*</td>
</tr>
<tr>
<td>TC</td>
<td>1524±2.5</td>
<td>1453±1.9</td>
</tr>
<tr>
<td>TG</td>
<td>274±29.45</td>
<td>301±24.27</td>
</tr>
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</table>

Glycemia, high-density lipoprotein (HDL), total plasma cholesterol (TC), and triglyceride (TG) levels were obtained at sacrifice after 15 weeks on a Western diet and after two months of hyperglycemia induced by streptozotocin. All data are expressed as mean ± SEM (n=6). *p<0.05 vs Ldlr<sup>+</sup> mice.

Figure Legends

Figure 1. Effect of diabetes on cholesterol efflux to ApoA1 or HDL and regulation of cholesterol transporters by Ager in primary murine BMDMs. Primary BMDMs were retrieved from non-diabetic and diabetic WT (2 months of hyperglycemia) and Ager<sup>−/−</sup> mice and were cultured in concentrations of glucose consistent with the glycemic state of the mice from which they were retrieved. BMDMs were labeled with <sup>3</sup>H-cholesterol, treated with acetylated LDL and fatty acid free bovine serum albumin (1%) for 24 h. To mediate cholesterol efflux, cells were treated with ApoA1 (5 µg/ml) (A) or HDL (100 µg/ml) (B) for 6 h and supernatant was collected. Percent cholesterol efflux is the radioactivity in the supernatant divided by the sum of the radioactivity measured in the supernatant and the cell lysates from n≥5 mice/group. (C-D) BMDMs were retrieved from WT or Ager<sup>−/−</sup> mice and subjected to quantitative RT-PCR for detection of Abca1 mRNA transcripts (C) and ABCA1 protein analysis (D) (n=3 mice/group). (E-F) BMDMs were prepared as in C-D and assessed for levels of Abcg1 mRNA transcripts (E) and ABCG1 protein (F) (n=3 mice/group). Error bars represent mean ± SEM. NS, not statistically significant.
Figure 2. CML-AGE suppresses cholesterol efflux and regulation of *ABCA1* and *ABCG1* in human THP-1 cells: effects of *AGER*. Human THP-1 cells were transfected with siRNA-scramble or siRNA-*AGER* and treated with vehicle (PBS) or CML-AGE (10 µg/ml) for 16 h. (A-B) Cholesterol efflux was measured to ApoA1 (25 µg/ml) (A) or HDL (100 µg/ml) (B) for 6 h and supernatant was collected. Percent cholesterol efflux is the radioactivity in the supernatant divided by the sum of the radioactivity measured in the supernatant and the cell lysates (n=5 independent experiments). (C-D) mRNA transcripts were determined for *ABCA1* (C) and *ABCG1* (D) by quantitative RT-PCR (n=3 independent experiments). (E-F) Protein expression levels by Western blotting were determined for ABCA1 (E) and ABCG1 (F) (n=3 independent experiments). Error bars represent mean ± SEM. NS, not statistically significant.

Figure 3. Macrophage reverse cholesterol transport and plasma HDL levels: effects of *Ager*. WT non-diabetic mice were injected subcutaneously with $^3$H-cholesterol-labeled, acLDL-loaded BMDMs from non-diabetic or diabetic (at least 2 months hyperglycemia) WT or *Ager<sup>-/-</sup>* mice. Plasma distribution of $^3$H-cholesterol was measured at 43 h (A) and 64 h (B). Hepatic $^3$H-cholesterol tracer levels were measured after 64 h (C) and feces were collected continuously from 0-64 h after injection (D). Data are expressed as the percentage of the $^3$H-cholesterol tracer relative to that of total cpm tracer injected ± SEM. N≥6 mice/group. In E, plasma HDL-cholesterol levels were measured in non-diabetic and diabetic WT and *Ager<sup>-/-</sup>* mice (n=10 mice/group). Error bars represent mean ± SEM. NS, not statistically significant.

Figure 4. Molecular characterization of CML-AGE modulation on the human *ABCG1* promoter. Luciferase activities were measured in RAGE-expressing HEK293T cells transfected
with 1 µg promoter reporter plasmid (pGL3) and 1 ng pRL-TK-Renilla. Firefly luciferase activity was divided by Renilla activity to obtain normalized value as a relative luciferase unit (RLU). Results represent mean ± SEM. (A) Activity of human \textit{ABCG1} promoter treated with CML-AGE (300 µg/ml) or CML-AGE (300 µg/ml) and sRAGE (6,000 µg/ml) (n=3 independent experiments). (B) Activity of human \textit{ABCG1} promoter deletion mutants treated with vehicle (PBS) (white bars) or CML-AGE (300 µg/ml) (black bars) for 16 h (n=6 independent experiments). (C) Electrophoretic mobility shift assay (EMSA) of nuclear protein extracts (THP1 cells) (5 µg) treated with: anti-PPARG IgG (4 µg) (supershift; second lane from left); vehicle (PBS) (third lane from left); CML-AGE (10 µg/ml) (fourth lane from left); CML-AGE (10 µg/ml) + sRAGE (200 µg/ml) (last lane from left). CML-AGE reduces nuclear protein binding to the \textit{ABCG1} promoter containing PPARG elements, which is prevented by sRAGE treatment. Data are representative of three independent EMSA experiments. (D) Activity of human \textit{ABCG1} promoter containing site-directed mutations at the PPARG responsive elements site, treated with vehicle (PBS) or with CML-AGE (300 µg/ml) (n=4 independent experiments). (E) Chromatin immunoprecipitation (ChIP) analysis of the ABCG1 promoter region to PPARG in human THP-1 cells treated with vehicle (PBS), CML-AGE (10 µg/ml) or CML-AGE and sRAGE (200 µg/ml) for 16 h (n=3 independent experiments). Quantitative RT-PCR analysis of ABCG1 promoter region using primers containing both PPRE1 and PPRE2 was determined. Error bars represent mean ± SEM. NS, not statistically significant.

\textbf{Figure 5. CML-AGE modulates PPARG levels: effect of RAGE.} mRNA transcripts (A) and Western blot (B) analysis of PPARG in primary BMDMs retrieved from diabetic WT and \textit{Ager}⁻/⁻ mice treated with vehicle (PBS) or CML-AGE (10 µg/ml) for 16 h (n=3 mice/group). mRNA
transcripts (C) and Western blot (D) analysis of PPARG and RAGE in human THP-1 cells transfected with siRNA-scramble or siRNA-AGER and treated with vehicle (PBS) or CML-AGE (10 µg/ml) for 16 h (n=3 independent experiments). (E) Western blot analysis of Phospho ERK and total ERK in human THP-1 cells after treatment with CML-AGE (10 µg/ml) for 16 h (n=3 independent experiments). (F) Human THP-1 cells were treated with vehicle or CML-AGE (10 µg/ml) with or without the ERK1/2 inhibitor (20 µM U0126 or 75 µM PD98059) for 30 min prior to treatment with vehicle or CML-AGE (10 µg/ml) for 16 h. mRNA transcript levels for PPARG were determined (n=4 independent experiments). Error bars represent mean ± SEM. NS, not statistically significant.

**Figure 6. Effects of PPARG on CML-AGE-mediated downregulation of ABCA1 and ABCG1.** (A) Human THP-1 cells were transfected with human ABCG1 cDNA and treated with vehicle (PBS) or CML-AGE (10 µg/ml) for 16 h. Cholesterol efflux was measured to HDL (100 µg/ml) for 6 h and supernatant was collected. Percent cholesterol efflux is the radioactivity in the supernatant divided by the sum of the radioactivity measured in the supernatant and the cell lysates (n=3 independent experiments). (B-C) Human THP-1 cells were transfected with human PPARG cDNA and treated with vehicle (PBS) or CML-AGE (10 µg/ml) for 16 h. mRNA transcript levels were determined for ABCG1 (B) or ABCA1 (C) (n=3 independent experiments). Error bars represent mean ± SEM. NS, not statistically significant.

**Figure 7. Laser capture microdissection of diabetic atherosclerotic aortic plaques: effects of Ager.** Diabetic Ldlr−/− mice, either expressing or devoid of Ager, were fed a Western diet for 15 weeks; mice were sacrificed at age 22 weeks. (A) Aortic plaques were stained with anti-CD68
IgG (macrophages) and Oil Red O (fats and lipids). Scale bar = 100 µm. Note that Supplementary Figure 6 displays the much smaller aortic arch lesions in diabetic Ldlr^−/− mice, especially those devoid of Ager. (B) CD68+ macrophages were laser captured from aortic sinus atherosclerotic lesions of diabetic Ldlr^−/−/Ager^+/+ and Ldlr^−/−/Ager^−/− mice, and mRNA transcript levels were determined for Abca1, Abcg1 and Pparg (n=6 mice/group). Error bars represent mean ± SEM.
Figure 2

A. APOA1

B. HDL

C. Relative mRNA expression

D. Relative mRNA expression

E. Relative Plasma Levels

F. Relative Plasma Levels

201x224mm (300 x 300 DPI)
Figure 5

A

Relative mRNA expression

WT + Vehicle  WT + CML-AGE  Ager+ + Vehicle  Ager+ + CML-AGE

p<0.01

NS

p<0.01

NS

B

Relative protein levels

WT + Vehicle  WT + CML-AGE  Ager+ + Vehicle  Ager+ + CML-AGE

PPARG

β-actin

p<0.05

p<0.05

NS

C

Relative mRNA expression

si-Scramble  si-Scramble  si-AGER  si-AGER

p<0.0001

p<0.01

NS

D

Relative protein levels

si-Scramble  si-Scramble  si-AGER  si-AGER

PPARG

β-actin

p<0.05

p<0.01

NS

E

Relative protein expression

Vehicle  Vehicle + CML-AGE

Phospho ERK

Total ERK

p<0.05

F

Relative mRNA expression

Vehicle  U0126  U0126 + CML-AGE  PD08090  PD08090 + CML-AGE

p<0.01

NS

NS
Figure 6

A

% of cholesterol efflux / 6hr

Vehicle + CML + ABCG1 + ABCG1 + CML

NS

p<0.01

p<0.0001

B

Relative mRNA expression (ABCG1/PO8)

Vehicle CML-AGE PPARG PPARG + CML-AGE

p<0.0001

p=0.0001

p<0.001

C

Relative mRNA expression (ABCA1/PO8)

Vehicle CML-AGE PPARG PPARG + CML-AGE

p<0.001

p<0.0001

p<0.0001
Figure 7

A

Ldlr\(^{-}\)-DM

Ldlr\(^{-}\)/Ager\(^{-}\)-DM

Oil Red O

CD68

B

\[ \text{Relative mRNA expression/18S RNA} \]

\[ \begin{align*}
\text{Abca1} & : p=0.05 \\
\text{Abcg1} & : p<0.05 \\
\text{Pparg} & : p<0.05
\end{align*} \]
Supplementary Figure 1. Effects of glucose in BMDMs and the effect of diabetes in peritoneal macrophages on transcription of Abca1 and Abcg1. (A-B) BMDMs were isolated from WT NDM mice and cultured for 7 days in either 5.5 mM D-glucose or 25 mM D-glucose to determine the effects of short-term glucose in culture on mRNA expression levels. mRNA transcript levels for Abca1 (A) and Abcg1 (B) were determined (n=4 independent experiments). (C-D) Peritoneal macrophages from non-diabetic and diabetic, WT and Ager⁻/⁻ mice were isolated and mRNA levels for Abca1 (C) and Abcg1 (D) were assessed (n=4 mice/group). Error bars represent mean ± SEM. NS, not statistically significant.
Supplementary Figure 2. *Apoa1* and *Abca1* in mouse liver: effects of *Ager*. Livers were retrieved from non-diabetic and diabetic WT or *Ager*−/− mice. mRNA transcript levels for *ApoA1* (A) and *Abca1* (B) were determined (n=4 mice/group), and Western blotting was performed for detection of protein levels for APOA1 (C) (n=4 mice/group) and ABCA1 (D) (n=3 mice/group). Error bars represent mean ± SEM. NS, not statistically significant.
Supplementary Figure 3. AGER expression in HEK293T cells is required for CML-AGE suppression of human ABCG1 promoter activity. (A) Activity of human ABCA1 promoter treated with vehicle (PBS) or CML-AGE (300 μg/ml) for 16 h (n=3 independent experiments). (B) Treatment with CML-AGE (300 μg/ml) on non-RAGE-expressing HEK293T cells had no effect on ABCG1 promoter activity (bar 2 vs. bar 1). Overexpression of AGER in HEK293T cells resulted in suppression of the ABCG1 promoter activity with CML-AGE treatment (bar 4 vs. bar 3) (n=4 independent experiments). Error bars represent mean ± SEM. NS, not statistically significant.
Supplementary Figure 4. Diabetes does not affect levels of \textit{Nrlh3} and \textit{Nrlh2}. Primary mouse non-diabetic and diabetic BMDMs were retrieved from WT mice. Quantitative RT-PCR for \textit{Nrlh3} (Lxra) and \textit{Nrlh2} (Lxrb) mRNA transcript levels were determined (n=4 mice/group). Error bars represent mean ± SEM. NS, not statistically significant.
Supplementary Figure 5. CML-AGE suppression of *ABCA1* and *ABCG1* promoter is independent of LXR and partially rescued with Rosiglitazone. *Ager*-expressing HEK293T cells were transfected with the *ABCA1* (A) or *ABCG1* (B) promoter reporter luciferase constructs and cells were treated for 16 h with vehicle (PBS), CML-AGE (300 μg/ml), LXR agonist T0901317 (1 μM) or both CML-AGE and T0901317 at the above doses (n=3 independent experiments) and the indicated promoter luciferase activity determined. (C) *ABCG1* mRNA transcript levels were determined in human THP-1 cells treated with either vehicle (PBS), CML-AGE (10 μg/ml), rosiglitazone (10 μM), or CML-AGE (10 μg/ml) and rosiglitazone (10 μM) for 16 h (n=3 independent experiments). Error bars represent mean ± SEM.
Supplementary Figure 6. Aortic sinus lesions of non-diabetic \(Ldlr^{-/-}\) mice. Non-diabetic \(Ldlr^{-/-}\) mice, either expressing or devoid of \(Ager\), were fed a Western diet for 15 weeks. Aortic plaques were stained with anti-CD68 IgG (macrophages) and Oil Red O (fats and lipids). Scale bar = 100 μm.
Supplementary Figure 7. Effects of high-fat diet on mRNA levels of *Abca1*, *Abcg1* and *Pparg* in BMDMs. BMDMs were retrieved from WT or *Ager<sup>−/−</sup>* mice placed on a low-fat diet (LFD) or a high-fat diet (HFD) for at least three months and subjected to quantitative RT-PCR for detection of *Abca1* (A), *Abcg1* (B) and *Pparg* (C) mRNA transcripts (n=3 mice/group).
### Supplementary Table 1

**Primers for real time PCR experiments**

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Supplementary Table 2

Global significance for macrophage cholesterol efflux and macrophage reverse cholesterol transport

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<th>Figure</th>
<th>Experiment</th>
<th>Welch's test P value</th>
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<td>0.027</td>
</tr>
<tr>
<td>5</td>
<td>RCT-Feces</td>
<td>0.071</td>
<td>0.016</td>
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</table>
Supplementary Table 3

Total cholesterol levels for nondiabetic and diabetic WT and Ager^/- mice.

<table>
<thead>
<tr>
<th></th>
<th>Nondiabetic</th>
<th>Diabetic</th>
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<tr>
<td></td>
<td>mg/dL</td>
<td>WT</td>
</tr>
<tr>
<td>TC</td>
<td>96.4±3.2</td>
<td>89.4±2.2</td>
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</tbody>
</table>

Total plasma cholesterol (TC) levels were obtained after two months of hyperglycemia induced by streptozotocin and at sacrifice. Statistical significance was determined with a one-way ANOVA followed by Tukey's multiple comparison's test. Results are expressed as mean ± SEM (n=10). *p<0.001 vs WT nondiabetic; **p<0.001 vs WT diabetic.
Supplementary Table 4

Predicted transcription factors binding to human ABCG1 promoter region –413 to –304

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<tr>
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<tr>
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</tr>
<tr>
<td>MEF2C</td>
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