Macrophage-specific Transgenic Expression of Cholesteryl Ester Hydrolase attenuates hepatic lipid accumulation and also improves glucose tolerance in ob/ob mice

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Running Title: Kupffer cells regulate hepatic triglyceride accumulation

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Abstract
Cellular cholesterol homeostasis is increasingly being recognized as an important determinant of the inflammatory status of macrophages and a decrease in cellular cholesterol levels polarizes macrophages towards an anti-inflammatory or M2 phenotype. Cholesteryl ester hydrolase (CEH) catalyzes the hydrolysis of stored intracellular cholesteryl esters (CE) and thereby enhances free cholesterol efflux and reduces cellular CE content. We have earlier reported reduced atherosclerosis as well as lesion necrosis and improved insulin sensitivity (due to decreased adipose tissue inflammation) in macrophage-specific CEH transgenic (CEHTg) mice in LDLR-/- background. In the present study we examined the effects of reduced intracellular accumulation of CE in CEHTg macrophages in an established diabetic mouse model namely the leptin-deficient ob/ob mouse. Macrophage-specific transgenic expression of CEH significantly improved glucose tolerance in ob/ob-CEHTg mice compared to ob/ob non-transgenic littermates but with no apparent change in macrophage infiltration into the adipose tissue. However, there was a significant decrease in hepatic lipid accumulation in ob/ob-CEHTg mice. Consistently, decreased [14C]-acetate incorporation into total lipids and triglycerides was noted in precision-cut liver slices from ob/ob-CEHTg mice. In primary hepatocyte-macrophage co-culture system, macrophages from CEHTg mice significantly reduced the incorporation of [14C]-acetate into triglycerides in hepatocytes indicating a direct effect of macrophages on hepatocyte triglyceride biosynthesis. Kupffer cells isolated from ob/ob-CEHTg mice were polarized towards an anti-inflammatory M2 (Ly6Clo) phenotype. Taken together, these studies demonstrate that transgenic over-expression of CEH in macrophages polarizes hepatic
macrophages (kupffer cells) to an anti-inflammatory M2 phenotype that attenuates hepatic lipid synthesis and accumulation.

**Key words:** macrophage phenotype and inflammatory status; cell-to-cell interaction; co-culture
Macrophage cholesterol homeostasis is central to foam cell formation and development of atherosclerotic plaques. While contribution of macrophage foam cells to the growing lipid core of the plaque is well established, role of these foam cells in regulating plaque associated inflammation is increasingly being recognized. Increase in intracellular cholesteryl esters (CE) within macrophages is an important determinant of the inflammatory status of macrophages and Fazio and Linton proposed a feedback loop where defects in cellular cholesterol balance induced changes in production of inflammatory mediators (14). Consistently, an increase in cellular cholesterol content by deficiency of cholesterol transporter ABCA1 leads to an increase in TNF-α secretion from macrophages (22) and cholesterol acceptor ApoA1-mediated increase in removal of cellular cholesterol decreases pro-inflammatory insult by LPS (25). Changes in the inflammatory status of macrophages subsequently lead to systemic inflammation and contribute to other pathologies associated with chronic low-grade inflammation such as Type 2 diabetes mellitus (T2DM). Cholesterol-mediated changes also affect the infiltration of macrophages into other tissues and Subramanian et al recently reported that addition of relatively small amount (0.15%) of dietary cholesterol resulted in marked increase in accumulation of macrophages in adipose tissue (36). Since majority of adipose tissue derived cytokines (TNF-α, IL-6 and IL-1β) actually originate in non-fat cells and among them, infiltrated macrophages play the most prominent role (7), increased activation and recruitment of macrophages into the expanding adipose tissue also leads to increased adipose tissue and systemic inflammation. Collectively, these
studies underscore the importance of macrophage cholesterol homeostasis in regulating diseases associated with chronic inflammation.

Towards the goal of altering macrophage cholesterol homeostasis, we developed transgenic mice with macrophage-specific over-expression of cholesteryl ester hydrolase (CEH). Macrophages from these mice stored less cholesteryl esters (CE) as a result of CEH-mediated CE mobilization and this led to a significant attenuation of diet-induced atherosclerosis in LDLR-/- background (39). We recently also demonstrated attenuated expression of pro-inflammatory mediators and decreased activation of pro-inflammatory transcription factors in macrophages with transgenic over-expression CEH. This led to decreased infiltration of macrophages into the adipose tissue and reduced systemic inflammation together contributing to improved insulin sensitivity (5). The present study was undertaken to test the hypothesis that CEH-mediated decrease in CE accumulation in macrophages will also improve glucose tolerance in an established model of obesity and diabetes, namely the leptin-deficient ob/ob mice. Macrophage-specific CEH transgenic mice (CEHTg) were crossed into ob/ob background and effects on glucose tolerance were determined. The data presented here demonstrate that consistent with the ob/ob mouse model where increased hepatic lipid accumulation is responsible for insulin resistance phenotype (13), CEH-dependent changes in macrophage/kupffer cell phenotype significantly reduced hepatic lipid accumulation and also led to significantly improved glucose tolerance.

Experimental Procedures:

Animals and diets: Development and characterization of macrophage-specific CEH transgenic mice has been described elsewhere (39) and in this model, human CEH is
expressed exclusively in macrophages (circulatory and resident) and leads to a two-fold increase in total intracellular CE hydrolysis. CEHTg mice in C57BL/6 background were crossed into ob/ob background (obtained from Jackson Laboratories). Littermates with or without CEH transgene in ob/ob background (ob/ob-CEHTg and ob/ob) were used for all the studies. Where indicated, mice were fed a 0.2% cholesterol containing diet (TD99399, Harlan Teklad) for 4 weeks.

**Intraperitoneal Glucose Tolerance Tests:** Four weeks old ob/ob and ob/ob-CEHTg littermates were fed 0.2% cholesterol containing diet for 4 weeks. After an overnight fast, a single bolus of glucose (0.5 mg/g body wt) was given intraperitoneally. Blood glucose levels were determined by commercially available glucometer using tail vein blood at 0, 15, 30, 60 and 120 minutes. Data are expressed as Mean±SD for 6 animals per genotype.

**Primary hepatocyte and macrophage co-culture:** Hepatocyte-macrophage co-cultures were used to model hepatocyte-kupffer cell interactions as described by Odegaard et al. (30). Thioglycollate-elicited peritoneal macrophages were harvested and 1X10^6 cells were plated in 0.4μm cell culture Transwells™ (Millipore). Non-adherent cells were removed after 2 h and medium replaced with fresh growth medium (39). Primary hepatocytes were isolated and plated in 12-well cell culture plates coated with collagen (32). After an overnight incubation, Transwells™ containing macrophages were placed in the wells containing hepatocytes and these indirect co-cultures were incubated for an additional 24 h. To monitor the incorporation of [14C]-acetate into triglycerides (TG), medium was supplemented with 2 mM [14C]-acetate (2). Total lipids were extracted at the end of 24h and neutral lipids were separated by TLC using hexane:diethyl
ether:acetic acid::90:10:1 (v/v). Spots corresponding to TG, monoglyceride+diglyceride (MG+DG) and phospholipids (PL) were marked, silica gel scrapped and associated radioactivity determined by liquid scintillation counting. To determine the effect of macrophages on hepatocyte gene expression, indirect co-cultures were set up as described above and at the end of 24 h, total hepatocyte RNA was isolated (using RNeasy Kit from Qiagen) and expression of different genes monitored by quantitative RT-PCR.

Measurement of de novo triglyceride synthesis in liver slices: Livers were harvested from ob/ob and ob/obCEHTg mice and precision cut liver slices were incubated with $[^{14}C]$-acetate for 3h (2). Following three washes in PBS, total lipids were extracted and neutral lipids were separated by TLC using hexane:diethyl ether:acetic acid::90:10:1 (v/v). Spots corresponding to TG were marked, silica gel scrapped and associated radioactivity determined by liquid scintillation counting.

Real Time PCR: Total RNA was extracted using RNeasy kit (Qiagen). Complementary DNA was synthesized using High Capacity cDNA reverse Transcription Kit (Applied Biosystems). Real time PCR was performed on Stratagene Mx3000P machine, using TaqMan Universal PCR Master Mix and optimized probe and primer sets from Applied Biosystems. Following probes were used: CD36-Mm00432403_m1, G6PC - Mm00839363-m1, PCK1 - Mm01247058-m1, Mttp - Mm00435015_m1, Fabp5 - Mm00783731_s1, Fabp1 – Mm00444340_m1, FAS - Mm00662319_m1, Apob - Mm01545156_m1, CD68 – Mm00839636_g1 and Msr1 – Mm00446214_m1.

Histological and biochemical analyses of liver tissue: A small piece of liver was fixed in buffered formalin, paraffin embedded and 3-4 sections (5 μm thick) were transferred to
numbered slides. Slides were then stained with H&E. Images were acquired using a Zeiss Observer A1 inverted microscope and analyzed using the AxioVision Software. About 100 mg of fresh liver tissue was homogenized in PBS and total lipids were extracted by the method of Bligh and Dyer (6). Amount of total cholesterol, cholesteryl esters and TG were determined and normalized to wet weight.

**Kupffer cell isolation and analysis:** Kupffer cells were isolated as described (27). Freshly isolated cells were re-suspended in FACS buffer containing Fc block and incubated with fluorescently labeled antibodies for 20 min at 4°C. After washing, specific immunofluorescent staining of individual cells was detected by flow cytometry (BD Biosciences, Canto II) and the data were analyzed using FlowJo (Tree Star Inc.) software. The following antibodies were used: Anti-mouse CD45-PE (leukocytes), anti-mouse CD11b-PerCP-Cy5.5 (macrophages), anti-mouse Ly6C-APC and the respective isotype controls (all antibodies were obtained from eBiosciences). Distribution of CD45+CD11b+Ly6C+ cells into Ly6C^hi and Ly6C^lo was determined as described before (4).

**TG secretion rates:** Mice were fasted overnight and a baseline blood sample was collected via the tail vein. Mice were subsequently injected with Tyloxapol (Sigma-Aldrich) at a concentration of 500 mg/kg body weight to inhibit lipoprotein lipase. Blood samples were subsequently collected at 1, 2 and 3 h post-injection and plasma TG levels determined (L-Type TG-M kit, Wako Diagnostics). TG production rates were calculated as described (11).

**Plasma analyses:** Fasting plasma was collected and used to determine cytokine levels (BD™ Cytometric Bead Array kit), non-esterified fatty acids (NEFA-HR kit, Wako...
Diagnostics), Insulin levels (Mouse Ultrasensitive Mouse Insulin ELISA kit, Crystal Chem Inc) and lipoprotein profiles as described earlier (39).

Results

Macrophage-specific transgenic expression of CEH does not significantly alter the basal metabolic profile: Table 1 summarizes the basal metabolic profiles for ob/ob and ob/obCEHTg mice. Macrophage-specific transgenic expression of CEH in ob/obCEHTg mice did not affect the body weight, fasting plasma blood glucose or insulin levels. Consistent with our earlier data in LDLR-/- background, fasting plasma lipoprotein profiles were not significantly different and cholesterol associated with the different plasma lipoprotein fractions was also not affected (39). However, in contrast to LDLR-/- background where transgenic expression of CEH significantly attenuated plasma cytokine levels (5), in ob/ob background there was no significant change in circulating cytokine levels indicating no effect on systemic inflammation.

Macrophage-specific transgenic expression of CEH improves glucose tolerance: We have earlier reported an increase in insulin sensitivity in LDLR-/-CEHTg mice where decreased systemic and adipose tissue inflammation was found to be the underlying mechanism (5). To assess the effects of macrophage-specific transgenic expression of CEH in ob/ob background, intraperitoneal glucose tolerance tests were performed in 0.2% cholesterol containing diet-fed ob/ob and ob/ob-CEHTg mice. As shown in Figure 1, significant improvement in glucose tolerance was noted in ob/ob-CEHTg mice. However, unlike LDLR-/-CEHTg where a significant reduction in infiltration of macrophages into the adipose tissue was observed and was established as the underlying mechanism for the improved insulin sensitivity in these mice, no change in
macrophage infiltration was noted between ob/ob and ob/ob-CEHTg mice as assessed by macrophage-specific gene expression (CD-68 or SR-A) in adipose tissue (See Figure 7, Panel D) or by direct histological examination (data not shown). Taken together with no change in plasma cytokine levels, these data suggest that macrophage-specific transgenic expression of CEH in ob/ob background did not alter systemic or adipose tissue inflammation.

CEH over-expression leads to attenuation of hepatic lipid accumulation: The most noticeable difference between ob/ob and ob/obCEHTg mice was the gross appearance of the liver and, therefore, histological and biochemical methods were used to determine changes in hepatic lipid accumulation. H&E stained sections of livers were imaged and representative images are shown in Figure 2. Lipid accumulation is dramatically attenuated in livers of ob/obCEHTg mice compared to those of ob/ob and individual cells appear to be completely filled with lipid in liver from ob/ob mice (Figure 2, bottom panel). The images were analyzed by Axiovision software to determine the area occupied by lipid and as shown in Figure 3, the percent area occupied by lipids was significantly reduced in ob/ob-CEHTg mice. Consistently, there was a significant decrease in hepatic triglyceride (TG), total cholesterol (TC) and CE content (Figure 3) in these animals. These data suggest that transgenic expression of CEH in Kupffer cells (or hepatic macrophages) attenuates lipid accumulation in liver/hepatocytes.

Attenuation of hepatic TG synthesis by CEHTg Kupffer cells/macrophages: To evaluate the effects of macrophage-specific transgenic expression of CEH on hepatic TG synthesis, two different approaches were used. First TG synthesis was examined in precision-cut liver slices by monitoring [14C]-acetate incorporation. In liver slices from
ob/ob-CEHTg mice, there was a significant attenuation of [$^{14}$C]-acetate incorporation in total lipids as well as TG (Figure 4, Panel A) demonstrating reduced lipid synthesis. To examine whether increased secretion of TG may also contribute to the observed decrease in hepatic TG levels, TG secretion rates were monitored. There was no significant increase in TG secretion in ob/ob-CEHTg mice (Figure 4, Panel B). Since hepatic TG synthesis is affected by circulating fatty acids, predominantly released by lipolysis of TG in the adipose tissue, plasma levels of non-esterified fatty acids were also measured. No significant difference was noted between ob/ob and ob/ob-CEHTg mice (Figure 4, Panel B).

To evaluate the direct effects of CEHTg macrophages/kupffer cells on hepatic TG synthesis, freshly isolated hepatocytes were co-cultured with macrophages and incorporation of [$^{14}$C]-acetate into neutral lipids was monitored. There was a significant decrease in [$^{14}$C]-acetate incorporation in PL, MG+DG and TG when hepatocytes were co-cultured with macrophages (Figure 5). However, this decrease was significantly accentuated when hepatocytes were co-cultured with CEHTg macrophages indicating that CEH-dependent changes in macrophage metabolism and/or phenotype attenuate hepatic TG synthesis leading to the observed decrease in observed hepatic lipid content.

Changes in the expression of genes involved in TG synthesis and secretion, FA uptake and transport as well as gluconeogenesis was monitored in hepatocytes co-cultured with macrophages in Transwells™. There was no change in the expression of genes involved in TG synthesis and secretion, namely, fatty acid synthase (FAS), microsomal triglyceride transfer protein (Mttp) and ApoB (Figure 6, Panel A) in hepatocytes co-cultured either with wild type non-transgenic or CEH transgenic
macrophages. Similarly, no difference was noted between the genes involved in fatty
acid uptake and transport, namely CD36, Fabp1 and Fabp5 (Figure 6, Panel B). While
there was a significant increase in the expression of genes involved in gluconeogenesis
(Glucose-6-phosphatase, G6Pase and Phosphoenol pyruvate carboxykinase, PEPCK)
when hepatocytes were co-cultured with macrophages, this increase was not affected
by macrophage genotype (Figure 6, Panel C). Similar results were obtained when
primary hepatocytes were cultured in the presence of macrophage conditioned medium
(data not shown). Consistent with the data obtained with isolated hepatocytes, no
significant differences in expression of these genes were seen in livers from ob/ob and
ob/ob-CEHTg mice (Figure 7, Panels A-C). In addition, kupffer cell or macrophage
number in liver or adipose tissue as assessed by measurement of CD68 and MSR1
mRNA levels (Figure 7, Panel D) remained unchanged between the two genotypes.

Higher numbers of CEHTg Kupffer cells are of anti-inflammatory M2 phenotype:
Macrophages exist in classically activated M1 or alternatively activated M2 phenotype
that are characterized by secretion of pro- and anti-inflammatory cytokines, respectively,
and thus differentially alter the metabolism of surrounding cells. Surface expression of
Ly6C antigen is one of the techniques to determine the relative proportions of M1 and
M2 macrophages; expression of Ly6C being low in M2 macrophages and high in M1
macrophages. Using peritoneal macrophages, we have earlier established this staining
pattern and confirmed the phenotype in the sorted Ly6C(lo) and Ly6C(hi) populations;
Ly6C(lo) population expressed the characteristic markers YM1 and Arginase 1 and
Ly6C(hi) population expressed IL-1β and TNFα (4). To determine whether CEH
overexpression-mediated changes in macrophage phenotype also occurs in resident
macrophages in liver or kupffer cells, using the same staining protocols, freshly isolated kupffer cells were surface stained for Ly6C and distribution of Ly6C(lo) and Ly6C(hi) was determined. There was a significant increase in Ly6C(lo) population in Kupffer cells isolated from ob/obCEHTg mice (Figure 8) suggesting that there is a shift towards a more anti-inflammatory phenotype. Although there was a trend towards a decrease in Ly6C(hi) population, it did not reach statistical significance. These data are consistent with our earlier data demonstrating an M2 polarization of atherosclerotic plaque-associated macrophages in LDLR-/-CEHTg mice (4).

Discussion

Obesity is associated with the development of systemic metabolic derangements characterized by insulin resistance and dyslipidemia and coexistence of this with hypertension is termed as the Metabolic Syndrome. We have earlier demonstrated that despite comparable accumulation of adipose tissue, Western (high fat high cholesterol) diet-fed LDLR-/- mice with macrophage-specific transgenic expression of CEH showed improved glucose tolerance and insulin sensitivity and the underlying mechanism was the attenuated systemic and adipose tissue inflammation (5). Our present study demonstrates that similar to LDLR-/- mice where obesity was diet-induced, macrophage-specific transgenic expression of CEH also improves glucose tolerance in an established mouse model of obesity, namely the leptin-deficient ob/ob mice. Increased hepatic lipogenesis is thought to be responsible for the insulin resistance phenotype of ob/ob mice (13) and the data presented here show that hepatic lipid accumulation and triglyceride synthesis was significantly reduced in ob/ob-CEHTg mice. TG synthesis as measured by [14C]-acetate incorporation was also significantly reduced
in hepatocytes co-cultured with CEHTg macrophages compared to non-transgenic macrophages. Furthermore, resident hepatic macrophages or kupffer cells isolated from ob/ob-CEHTg liver were polarized towards an anti-inflammatory M2 phenotype. Collectively, these data suggest that in leptin-deficient ob/ob background, macrophage-specific transgenic expression of CEH polarizes kupffer cells towards an anti-inflammatory M2 phenotype and decreases hepatic lipid accumulation or hepatic steatosis.

Lipid content of non-adipocytes is under tight regulation and the burden of buffering excessive fatty acids in circulation falls on adipocytes. However, in obesity, while there is significant expansion of subcutaneous and visceral adipose tissue, lipid is also stored in ectopic depots (i.e., outside of these two recognized locations) such as liver, skeletal muscle and heart and determining the role of these depots in development of insulin resistance and metabolic syndrome is being extensively pursued (24). The importance of storing fat in the correct depot, i.e., adipose tissue, is best exemplified by severe hepatosteatosis, diabetes, elevated glucose, insulin, triglyceride and free fatty acids in A-ZIP/F-1 fatless mice with no adipose tissue (subcutaneous or elsewhere) and that transplantation of wild-type adipose tissue into these mice reverses this phenotype (15). Analogous human condition is represented by patients with HAART-associated lipodystrophy characterized by loss of subcutaneous fat and accumulation of fat in the liver resulting in cardiovascular complications (26). Clinical studies evaluating the role of ectopic lipid storage have established correlation between plasma indices of insulin resistance and ectopically stored lipid levels (33). For example, in the NHANES III survey, adults with non-alcoholic fatty liver disease (NAFLD) with ectopic lipid
accumulation in the liver, were twice as likely to have type 2 diabetes (T2DM) compared to subjects without NAFLD (9). Further, accumulation of lipid in the liver is well correlated with hepatic insulin resistance in both T2DM and in non-diabetic individuals (34). Van Herpen and Hinderling showed that accumulation of TG and more specifically, the intermediates in TG biosynthesis namely DG decrease insulin signaling (37). Significant reduction in ectopic lipid accumulation in livers of ob/ob-CEHTg mice as well as reduced synthesis of DG, therefore, likely represents one of the mechanisms underlying the observed improvement in glucose tolerance in these mice. It is noteworthy that in addition to liver, ob/ob-CEHTg mice also have significantly reduced lipid accumulation in skeletal muscle (unpublished observations). Studies are in progress to define the mechanisms by which macrophage-specific transgenic expression of CEH regulates ectopic lipid accumulation in skeletal muscle and how it affects muscle function (e.g. glucose utilization). Since increase in local inflammation due macrophage infiltration into the muscle is known to affect insulin sensitivity (31), contribution of changes in skeletal muscle to the observed improvement in glucose tolerance in ob/ob-CEHTg mice cannot be ruled out based on the data presented here.

The mechanism(s) responsible for increased intra-hepatic triglyceride accumulation are not completely understood. It has been suggested that dysfunctional adipose tissue, characterized by adipocyte hypertrophy, macrophage infiltration, impaired insulin signaling, and insulin resistance, releases a host of inflammatory adipokines and excessive amounts of free fatty acids (FFA) that promote ectopic fat deposition in liver (12). Recent studies have established that upregulation of hepatic CD36 is associated with insulin resistance and hepatic steatosis in humans (29) and
alterations in FFA uptake regulated at the level of fatty acid transporter CD36 expression is thought to be involved in increased triglyceride accumulation in patients with NAFLD (17). In addition, role of intracellular fatty acid binding proteins such as FABP5 in metabolic syndrome and/or diabetes is also established by several studies (3, 8). However, in mouse models with disrupted leptin signaling such ob/ob or db/db mice, fatty acid uptake does not play a causal role in hepatic steatosis (16). Consistently, no significant differences were noted in plasma non-esterified fatty acid levels as well as mRNA expression of CD36 or FABP5 in hepatocytes co-cultured with wild type or CEH transgenic macrophages and livers from ob/ob and ob/obCEHTg mice. Although direct measurement of FFA uptake by hepatocytes was not performed in the present study, these data suggest that transgenic expression of CEH did not affect availability and/or uptake of FFA by the liver.

Another factor that potentially regulates triglyceride accumulation in hepatocytes is its microenvironment namely the effect of resident liver macrophages or Kupffer cells and the role of kupffer cell-hepatocyte interaction is not completely defined. Depletion of kupffer cells is used as a strategy to evaluate their role in modulating hepatic metabolism and these studies, in contrast to our results, indicate that depletion of kupffer cells leads to attenuation of hepatic steatosis (2, 18). However, treatment with gadolinium or liposome-encapsulated clodronate, not only depletes kupffer cells but also depletes macrophages from other tissues including spleen and adipose tissue (19) thereby confounding the inferences regarding the specific role of kupffer cell-hepatocyte interactions. Lanthier et al recently demonstrated that treatment with gadolinium chloride after a period of high fat diet feeding that leads to significant infiltration of
macrophages into the adipose tissue, had no effect on hepatic lipid accumulation or insulin sensitivity (23). However, preventive and prolonged treatment prevents adipose tissue inflammation and ameliorates insulin sensitivity demonstrating that the observed effects of gadolinium treatment are not solely due to macrophage depletion in liver but depletion of macrophages in adipose tissue is also a significant contributor. Pro-inflammatory cytokines (namely, IL-1β, IL-6 or TNFα) secreted by Kupffer cells activate hepatocytes (35), affect gluconeogenesis (38), increase the expression of acute phase proteins (21) and enzymes involved in xenobiotic metabolism (28). On the other hand, anti-inflammatory cytokine IL-10 secreted by Kupffer cells is essential for hepatocyte homeostasis and its loss under conditions of Kupffer cell depletion is associated with increased STAT3-dependent signaling and steatosis leading to decreased insulin signaling (10). Consistently, induction of IL-10 expression in kupffer cells decreases activation of hepatic stellate cells (1). Secretion of pro- or anti-inflammatory mediators by macrophages is determined by the polarization of macrophages towards either M1 or M2 phenotype and M1 or M2 polarization itself is regulated by exposure to Th1 or Th2 cytokines. Kang et al recently established the role of adipocyte as well as hepatocyte-derived Th2 cytokines in regulating macrophage polarization and thus, regulating insulin sensitivity (20). We have recently reported a significant shift in the polarization of CEHTg macrophages towards an M2 phenotype (4) and also demonstrated reduced activation of NF-κB in CEHTg macrophages resulting in a decrease in the secretion of pro-inflammatory mediators (5). Consistently, Kupffer cells isolated from ob/ob-CEHTg livers were polarized towards an M2 phenotype (Figure 7) and significantly higher attenuation of triglyceride synthesis was observed
when primary hepatocytes were co-cultured with CEHTg macrophages (Figure 5). Huang et al recently reported an increase in hepatocyte TG synthesis when co-cultured with M1-polarized (LPS stimulated) kupffer cells (18). Although effects of untreated or M2-polarized cells were not evaluated, these data provide support for the concept that increased M1 polarization enhances TG synthesis in hepatocytes and conversely CEH-mediated M2 polarization of macrophages phenotype beneficially alters hepatocyte metabolism leading to reduced lipid accumulation.

In conclusion, CEH-mediated changes in macrophage cholesterol metabolism shifts the polarization towards an anti-inflammatory M2 phenotype. This polarization of hepatic macrophage or kupffer cells attenuates TG synthesis in hepatocytes leading to the observed decrease in hepatic lipid accumulation in ob/ob-CEHTg mice. Consistent with the role of increased hepatic steatosis in the development of insulin resistance phenotype in ob/ob mice (23), reduction in ectopic (hepatic) lipid accumulation significantly improved glucose tolerance in these animals. These studies establish the role of macrophage cholesterol homeostasis in regulating hepatic lipid metabolism. Additional studies will define the mechanisms underlying the effects of macrophage phenotype and/or cholesterol content-dependent changes in kupffer cells (or hepatic macrophages) on hepatocyte metabolism.
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Author contributions:

Jinghua Bie and Bin Zhao performed the glucose tolerance tests and harvested peritoneal macrophages; Kathryn E Marqueen analyzed the liver sections and extracted hepatic lipids; Jing Wang prepared primary hepatocytes and Kupffer cells, set up the co-cultures, performed insulin ELISA and monitored gene expression; Barbara Szomju was responsible for generating animals for these studies and plasma as well as FACS analyses, and Shobha Ghosh performed $[^{14}\text{C}]-\text{acetate}$ incorporation studies in liver slices and co-cultures and also supervised this project.


**Figure Legends:**

**Figure 1:** Intraperitoneal Glucose tolerance test: Four weeks old ob/ob and ob/ob-CEHTg mice (littermates) were fed chow diet supplemented with 0.2% cholesterol for additional 4 weeks. After an overnight fast, blood glucose levels at time zero were determined using a commercial glucometer. Subsequently, mice were given an intraperitoneal bolus of glucose (0.5 mg/g body weight) and blood glucose levels were determined at indicated times. Data are expressed as Mean±SD, n=6, *P<0.05.

**Figure 2:** Reduced lipid accumulation in ob/ob-CEHTg mice – Histological Analyses: A portion of liver was fixed, paraffin embedded and 5 mm thick sections were stained with H&E. Representative images are shown in Panel A. White unstained region within the cell represent the area occupied by the lipid. Panel B shows magnified images of single cells.

**Figure 3:** Reduced lipid accumulation in ob/ob-CEHTg mice – Biochemical Analysis: Total lipids were extracted from a known amount of liver tissue (~100 mg). Triglyceride (TG) content was determined enzymatically and total cholesterol (TC), free cholesterol (FC) and cholesterol ester (CE) content was determined by GC. Lipid content was normalized to the weight of liver used and expressed as μg/mg tissue weight. Magnified H&E stained images (Figure 2, Panel B) were analyzed to determine the percent area of the cell occupied by lipid. Data are shown as Mean±SD, n=6 and individual P values for the differences between groups are indicated.

**Figure 4:** Decreased de novo triglyceride synthesis in liver slices from ob/ob-CEHTg mice: Panel A: Incorporation of $[^{14}C]$-acetate into total lipids and triglycerides was determined as described under “Experimental Procedures”. Incorporation into each
lipid fraction was normalized to total protein and data are expressed as percent ob/ob-CEHTg control (Mean±SD, n=6) and individual P values for the differences between groups are indicated. Panel B: Non-esterified fatty acid (NEFA) levels in plasma and TG secretion rates were determined as described under “Experimental Procedures”. Data are presented as Mean±SD, n=6 and individual P values for the differences between groups are indicated.

Figure 5: Decreased [14C]-acetate incorporation in hepatocytes co-cultured with CEHTg macrophages: Primary hepatocytes were isolated from 8 week old ob/ob mice and plated in 12-well plates coated with collagen. Thioglycollate elicited macrophages were harvested from wild type or CEHTg mice and 1X10^6 cells were plated in Transwells™. After an overnight incubation, Transwells™ were placed in wells containing hepatocytes and these co-cultures incubated for additional 24 h. During this incubation time, the hepatocyte culture medium was supplemented with 2 mM [14C]-acetate. Total lipids were extracted after 24 h and analyzed as described under “Experimental Procedures”. Data are expressed as percent no macrophage control (Mean±SD, n=6) and individual P values for the differences between groups are indicated.

Figure 6: Changes in the expression of genes involved in Triglyceride synthesis and secretion, fatty acid uptake and transport and gluconeogenesis in hepatocytes co-cultured with macrophages: Primary hepatocytes were isolated and co-cultured with thioglycollate elicited macrophages as described under Methods. Total RNA was extracted from the hepatocytes after 24 h and expression of indicated genes monitored by Real Time qPCR using specific Taqman Assays. β-actin was used as the
Figure 7: Changes in the expression of genes involved in Triglyceride synthesis and secretion, fatty acid uptake and transport and gluconeogenesis in liver: Total liver RNA was extracted and expression of indicated genes monitored by Real Time qPCR using specific Taqman Assays. β-actin was used as the housekeeping gene and data are expressed as percent ob/ob control. (Mean±SD, n=5). In Panel D, expression of genes in adipose tissue is also shown.

Figure 8: Increased number of Kupffer cells in Ly6C$^{lo}$ or M2 phenotype in ob/ob-CEHTg mice: Isolated kupffer cells were stained for leukocytes (CD45), macrophages (CD11b) and Ly6C and analyzed by FACS. The distribution of Ly6C$^{lo}$ and Ly6C$^{hi}$ is plotted as the percent of total CD45+CD11b+Ly6C+ cells. Data are presented as Mean±SD, n=5, *P<0.05.
Table 1: Comparison of metabolic parameters of ob/ob and ob/ob-CEHTg mice

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<td>TC</td>
<td>233.58 ± 14.01</td>
<td>235.33 ± 15.86</td>
<td>0.82</td>
</tr>
<tr>
<td>VLDL</td>
<td>4.98 ± 1</td>
<td>5.82 ± 1.48</td>
<td>0.63</td>
</tr>
<tr>
<td>LDL</td>
<td>43.41 ± 8.93</td>
<td>50.57 ± 10.15</td>
<td>0.57</td>
</tr>
<tr>
<td>HDL</td>
<td>193.46 ± 7.14</td>
<td>178.76 ± 7.30</td>
<td>0.27</td>
</tr>
</tbody>
</table>
Figure 1

Blood Glucose (mg/dl) vs. Time (min)

- ob/ob
- ob/ob CEHTg

* Indicates significant difference.
Figure 2

A: H&E Stained Liver Sections

Ob/ob

Ob/ob-CEHTg

B: Magnified Images of Single Cells

Ob/ob

Ob/ob-CEHTg
Figure 3
Figure 4

A

B

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ob/ob</th>
<th>Ob/ob-CEHTg</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma NEFA (mEq/L)</td>
<td>1.18±0.44</td>
<td>1.08±0.40</td>
<td>0.58</td>
</tr>
<tr>
<td>TG secretion rates (nmoles/kg/h)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0 – 1 h</td>
<td>70.58±18.67</td>
<td>35.83±15.51</td>
<td>0.07</td>
</tr>
<tr>
<td>0 – 2 h</td>
<td>69.83±31.11</td>
<td>50.70±15.46</td>
<td>0.39</td>
</tr>
<tr>
<td>0 – 3 h</td>
<td>64.27±27.59</td>
<td>46.76±1.65</td>
<td>0.33</td>
</tr>
</tbody>
</table>
Figure 5

Incorporation of $[^{14}\text{C}]$-acetate (% Control)

- No Macrophages
- WT Macrophages
- CEHTg Macrophages

- PL: P=0.3
- MG+DG: P=0.03
- TG: P=0.04
Figure 6
Figure 7

Gene Expression (% ob/ob Control)

Gene Expression (% Control)

Gene Expression (% ob/ob Control)

Gene Expression (% ob/ob Control)