Macrophage-specific de novo synthesis of ceramide is dispensable for inflammasome-driven inflammation and insulin-resistance in obesity

Christina D. Camell1,2, Kim Y. Nguyen1,2, Michael J. Jurczak3, Gerald I. Shulman3,
Gerald S. Shadel4 and Vishwa Deep Dixit1,2

1Section of Comparative Medicine and 2Department of Immunobiology, 3Department of Internal Medicine, 4Department of Pathology and Genetics, Yale School of Medicine, New Haven, CT 06520, USA

Address and Correspondence to:
1Vishwa Deep Dixit, Ph.D
Section of Comparative Medicine and Department of Immunobiology
Yale School of Medicine
310 Cedar St, New Haven CT06520
Email: Vishwa.Dixit@yale.edu
Phone: 203-785-2525
Fax: 203-785-7499

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Capsule

**Background:** Serine palmitoyltransferase-2 (Sptlc2) is required for *de novo* synthesis of ceramide and palmitate-induced cytokine secretion.

**Results:** Myeloid-deficiency of Sptlc2 does not alter macrophage activation, diet induced adipose tissue inflammation or insulin resistance.

**Conclusions:** *De novo* synthesis of ceramide is dispensable for lipid-driven Nlrp3 inflammasome inflammation.

**Significance:** Macrophage-Sptlc2 can be eliminated as a therapeutic target for metabolic syndrome.

**Abstract**

Dietary lipid overload and calorie excess during obesity is a low grade chronic inflammatory state with diminished ability to appropriately metabolize glucose or lipids. Macrophages are critical in maintaining adipose tissue homeostasis, in part by regulating lipid metabolism, energy homeostasis and tissue remodeling. During high fat diet-induced obesity, macrophages are activated by lipid derived “danger signals” such as ceramides and palmitate and promote the adipose tissue inflammation in an Nlrp3 inflammasome-dependent manner. Given that the metabolic fate of fatty acids in macrophages is not entirely elucidated, we have hypothesized that *de novo* synthesis of ceramide, through the rate-limiting enzyme serine palmitoyltransferase long chain (Sptlc)-2, is required for saturated fatty acid driven Nlrp3 inflammasome activation in macrophages. Here we report that mitochondrial targeted overexpression of catalase which is established to mitigate oxidative stress controls ceramide-induced Nlrp3 inflammasome activation in macrophages. We therefore hypothesize that *de novo* synthesis of ceramide, through the rate-limiting enzyme serine palmitoyltransferase long chain (Sptlc)-2, is required for saturated fatty acid driven Nlrp3 inflammasome activation in macrophages. Here we report that mitochondrial targeted overexpression of catalase which is established to mitigate oxidative stress controls ceramide-induced Nlrp3 inflammasome activation but does not affect the ATP-mediated caspase-1 cleavage. Surprisingly, myeloid cell-specific deletion of Sptlc2 is not required for palmitate driven Nlrp3 inflammasome activation. Furthermore, the ablation of Sptlc2 in macrophages did not impact macrophage polarization or obesity-induced adipose tissue leukocytosis. Consistent with these data, investigation of insulin-resistance using hyperinsulinemic-euglycemic clamps revealed no significant differences in obese mice lacking ceramide *de novo* synthesis machinery in macrophages. These data suggest that alternate metabolic pathways control fatty acid derived ceramide synthesis in macrophage and the Nlrp3 inflammasome activation in obesity.

**Introduction**

Diet-induced obesity (DIO) is a growing epidemic and has greatly augmented the number of humans diagnosed with metabolic diseases such as type 2 diabetes, cardiovascular disease or atherosclerosis (1,2). The importance of inflammation in driving metabolic dysregulation during DIO is well-established, and research has highlighted the importance of the adipose tissue and resident immune cells in maintaining glucose homeostasis (3,4).

DIO induces adipose tissue inflammation that is largely characterized by infiltration of immune cells, including pro-inflammatory activated macrophages, T cells and B cells (5-8). In lean adipose, resident macrophages exhibit anti-inflammatory characteristics, including expressing surface markers such as MGL1, CD206 and Arginase1. In contrast, infiltrating macrophages express increased amounts of inflammatory cytokines, such as TNFα, MCP1 and IL1β (7). Infiltrating CCR2+ macrophages surround dying adipocytes, forming crown-like-structures, and contain large lipid droplets (9,10); suggesting they are uniquely sensitive to the lipid-loaded microenviroment. Diet-induced inflammation is mediated through the activation of the NLRP3 (NLR family pyrin domain containing 3) inflammasome, a large cytosolic multiprotein scaffolding complex that activates caspase-1 and leads to the secretion of bioactive IL1β (11,12). Inflammasome activation has been shown to impair insulin sensitivity in adipose tissue, liver and skeletal muscle and increases adipose tissue inflammation (13-16).

In humans and mouse models, DIO is also characterized by alterations in lipid metabolism, excess lipid availability and increased ceramides systemically and in the adipose tissue (17-19). In lipid metabolism, the fatty acid oxidative pathway utilizes palmitoyl-CoA to fuel mitochondrial oxidative phosphorylation; alternatively palmitate enters the non-oxidative pathway to be converted...
into ceramide via irreversible condensation with L-serine by the rate-limiting enzyme, serine palmitoyltransferase (SPT) (20). SPT is a heterodimer, present in the endoplasmic reticulum, and is composed of mainly two subunits, Sptlc1 and Sptlc2 (20). Ceramides and other sphingolipids are structural components of membranes and signaling molecules which serve to mediate cell homeostasis (20), however, dysregulated increase in ceramide content in cells is linked to elevated inflammation and insulin-resistance (21,22). Inhibition of ceramide synthesis prevents lipid induced-insulin resistance, diet-induced insulin resistance and hepatic steatosis (23-25).

In addition to upregulating ceramide synthesis, palmitate treatment of macrophages inhibits AMPK activation, generates reactive oxygen species and activates the NLRP3 inflammasome causing the secretion of IL1β (15,26). The role for the non-oxidative lipid metabolism pathway in regulating NLRP3 inflammasome is not clear; given the association of mitochondrial oxidative stress with cellular lipid accumulation, we have hypothesized that de novo synthesis of ceramide via Sptlc2, is required for inflammasome-induced inflammation in diet-induced obesity. We found that ceramide-induced IL1β requires Nlrp3 and the accumulation of reactive oxygen species (ROS); however, surprisingly, we found that palmitate-induced IL1β does not require Sptlc2, indicating that Sptlc2 is not necessary for Nlrp3 inflammasome activation. Furthermore, we show that saturated fat diet-induced adipose tissue inflammation is unaffected in isolated adipose tissue from mice with myeloid cell-deletion of Sptlc2. Our findings reveal that in vitro and in vivo, myeloid cell-specific Sptlc2 is dispensable for fatty acid-mediated inflammation and insulin resistance.

**Methods:**

**Animals/mice:** Sptlc2-flox mice have been previously described (27). To ablate de novo synthesis in myeloid cells, Sptlc2-flox (Sptlc2^fl/fl; Dr. Xian-Cheng Jiang, SUNY) mice were crossed to LysM-Cre (B6.129P2-Lyz2^2m1(cre)Jfo/J Jackson Laboratory) mice to generate Sptlc2^fl/fl; LysM^cre- mice. Sptlc2^fl/fl; LysM^cre- mice were backcrossed to Sptlc2^fl/fl; LysM^cre- mice generating the littermates Sptlc2^fl/fl; LysM^cre- (CRE') experimental animals. MCAT transgenic mice and wild-type littermate controls were obtained from Dr. Gerald Shadel, (Yale University) and have been previously described (28). For diet studies: mice were placed on standard chow diet (LFD; 13.4% fat; LabDiet, Purina 5001) or high fat (HFD; 60% fat; Research Diets) at 6-7 weeks of age for 13 weeks of feeding. All experiments and animal use were conducted in compliance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at Yale University.

**Bone marrow derived macrophage (BMDMs) and cell culture:** The BMDMs were prepared and inflammasome activation assays were performed as described by us previously (13, 32). All steps were performed using sterile technique. Femurs were collected in RPMI (Life Technologies, Inc.). Using a needle and syringe, marrow was flushed into RPMI containing 10% FBS (Omega Scientific, Inc.) and 5% antibacterial/antimycotics (Life Technologies, Inc.). Red blood cells were lysed using ACK lyses buffer (Quality Biological) and lysis was neutralized with RPMI. Bone marrow cells were differentiated into macrophages using MCSF (10ng/ml; R&D) and L929 conditioned media. Non-adherent cells were collected on day 7, counted and replated at 1x10^6 cells/ml. BMDMs were treated on day 8. Cells were primed by four hour treatment with ultrapure LPS (1ug/ml;Sigma) alone; inflammasome stimulation was provided by treatment with ATP (5mM; 1hr), sodium palmitate conjugated to BSA (200 or 400uM; 24hr treatment; Sigma) or ceramide (40-120mg; 6 hour; Cayman Chemical). Myricetin (Caymen Chemical) was added to some treatments, in combination with LPS priming, at 1 or 5uM. Supernatants were collected and stored at -80°. BMDMs were washed with PBS and collected in RIPA supplemented with protease inhibitors for protein analysis. BMDMs were polarized to M1 or M2 by treatment with LPS (1ug/ml) and IFNγ (20ng/ml; eBioscience) or IL4 (10ng/ml; eBioscience). After 24 hours, cells were washed with PBS and collected in trizol for RNA extraction.

**Western blot:** Samples were left on ice for one hour with vortexing every 10min to disrupt
membranes. Samples were centrifuged at 14,000g for 15 minutes, supernatant was collected and protein concentration was quantified using the DC protein assay (Bio-Rad). IL1β (GeneTex), SPTLC2 (Proteintech), catalase (Sigma), caspase-1 (Genentech) and Actin (Cell Signaling) were probed for.

**RNA extraction and gene expression analysis:** RNA extraction and purification was performed using RNeasy kits (Qiagen) according to manufacturer’s instructions. Total RNA was measured using a nanodrop and 500ng used to reverse transcribe cDNA. Quantitative PCR was performed as described (29). Primer sequences for Gapdh, Arg1, Tnfa, Il1b, iNos, Sptlc1, Sptlc2, Sptlc3, CerS5, CerS6, Nsmaf, and Smpd1 are listed in table 1.

**Adipose digestion and stromavascular staining:** Visceral adipose was harvested at sacrifice and weighed. Tissue was enzymatically digested in 0.1% collagenase I (Worthington Biochemicals) in Hanks Buffered Salt Solution (Life Technologies, Inc.) for 45min at 37°. The stromavascular fraction (SVF) was pelleted by centrifugation at 1500rpm for 10min, then washed and filtered. Red blood cells are lysed using ACK lysing buffer. Cells were resuspended in 1ml for counting prior to staining or positive selection of F4/80+ macrophages (Biotinylated; eBioscience) using Dynabeads Biotine Binder (LifeTechnologies). Positively selected cells were stored in trizol for RNA isolation. For staining, the SVF was incubated with FcBlock, surface antibodies for 30min on ice, in the dark, then washed and stained with Fixable Viability Dye (eBioscience) and intracellular antibodies using cytoste (BD Bioscience). Analysis was performed on a BD LSRII and using FlowJo vX.

**Antibodies:** Fixable Viability Dye Aqua; F4/80-eFlour450; CD11b-PerCPCy5.5; CD11c-APC; CD206-PECCy7; CD3-FITC; B220-PECy7; MHCII-Alexa Fluor 700 (eBioscience)

**Hyperinsulinemic–euglycemic clamps:** Experiments were performed according to recent recommendations of the Mouse Metabolic Phenotyping Center Consortium (30) and as previously published (31).

**Glucose and Insulin tolerance test:** Mice were fasted for 12 (GTT) or 4 (ITT) hours and blood glucose measured from the tail vein using a glucometer (Breeze) at baseline, 10, 20,30,45,60 and 90min. Insulin (Sigma-Aldrich) was injected intraperitoneal at 0.8U/kg. Glucose (Sigma-Aldrich) was injected intraperitoneal at 0.4g/kg.

**Statistical analysis:** We used a two-tailed Student’s T test to determine significance between genotype. The differences between means and the effects of treatments were analyzed by one-way analysis of variance with Tukey’s test which corrects for multiple hypotheses.

**Results:**

Ceramides activate the Nlrp3 inflammasome via mitochondrial oxidative stress- Our prior studies have demonstrated that ceramides activate caspase-1 induced IL1β secretion in a Nlrp3 inflammasome dependent manner(13,32); however the exact mechanism by which ceramides activate the Nlrp3 inflammasome is not yet understood. Consistent with our previous studies, ceramide activates Nlrp3 inflammasome causing IL1β secretion from bone marrow derived macrophages (BMDMs) in a dose dependent manner (Fig. 1A&B). Given that ceramides and lipids increase the production of ROS causing oxidative stress and that mitochondrial damage and ROS generation has been linked to the activation of the Nlrp3 inflammasome (33,34), we tested the potential role of ROS in ceramide-induced inflammasome activation. We investigated this question using BMDMs from transgenic mice with targeted overexpression of the human catalase gene to mitochondria (MCAT mice), an enzyme which reduces mitochondrial oxidative damage and improves mitochondrial function, by degrading hydrogen peroxide and preventing ROS accumulation (35,36). Consistent with recent studies (37) that ROS is not critical for ATP-mediated inflammasome activation, catalase overexpression did not attenuate extracellular ATP-induced caspase-1 activation (Fig. 1C). In contrast, ceramide-induced caspase-1 activation was decreased in BMDMs from MCAT transgenic mice, indicating that inhibition of ROS accumulation prevents ceramide-induced Nlrp3 activation (Fig. 1C) and, similar to saturated fatty acids, ceramide induction of mitochondrial oxidative stress drives Nlrp3 activation.

Mitochondrial oxidative activity is associated with intracellular lipid accumulation (38,39); we wanted to ask whether prevention of lipid accumulation could reduce ROS-induced Nlrp3
activation. SPT is the rate-limiting enzyme in the de novo synthesis of ceramide (Fig. 1D), and is an important intersection for regulating cellular levels of saturated fatty acids and sphingolipids by generating ceramide from palmitoyl-CoA precursors (20). SPT-specific inhibition, using myriocin, prevents palmitate-induced accumulation of ceramide and downstream cytokine production in macrophages (26). Palmitate induces IL1β secretion in a Nlrp3-dependent manner (Fig. 1B & (15)); however, myriocin-inhibition of SPT failed to abrogate IL1β secretion (Fig. 1E). These data suggest that chemical inhibition of de novo synthesis may not be sufficient to reduce saturated fat-induced IL1β.

Myeloid cell-specific deletion of Serinepalmitoyltransferase 2- To further address the role of SPT in saturated fat-induced Nlrp3 activation, we wanted to genetically delete SPT; therefore we first measured the expression level of Sptlc subunits in BMDMs. Sptlc2 has the highest expression level, being nearly 5-fold higher than Sptlc1 in pro-inflammatory-polarized M1 macrophages and in anti-inflammatory-polarized M2 macrophages (Fig. 2A). Sptlc3 was not detected in BMDMs under any condition (Fig. 2A).

We sought to generate mice with myeloid cell-specific knockouts of Sptlc2. To test for cre deletion efficiency, we compared gene expression between littermates Sptlc2<sup>Bfl/fl</sup> LysM<sup>Cre</sup>- (CRE<sup>-</sup>) controls and Sptlc2<sup>Bfl/fl</sup> LysM<sup>Cre<sup>Cre</sup>+</sup> (CRE<sup>+</sup>) experimental BMDMs. In both M1 and M2 polarized BMDMs, Sptlc2 gene was significantly reduced in CRE<sup>+</sup> mice as compared to their CRE<sup>-</sup> littermate control (Fig. 2B). Sptlc2 gene level was not affected by polarization towards M1 or M2 phenotype or by having a single allele floxed (fl<sup>-</sup>/; CRE<sup>+</sup>). The expression level of Sptlc1 was unaltered by genotype or macrophage polarization (Fig. 2B). The immunoblot analysis confirmed that compared to littermate control cells, Sptlc2 protein was not expressed in CRE<sup>+</sup> BMDMs (Fig. 2C). These data indicate that in BMDMs from CRE<sup>+</sup>, Sptlc2 is efficiently deleted, without altering the expression of Sptlc1.

Sptlc2-deficient BMDM activation in vitro- Sptlc2 heterozygous macrophages have reduced palmitate-induced inflammatory gene expression and myeloid cell-specific deletion of Sptlc2 improves atherosclerotic lesions (40). To examine in vitro whether CRE<sup>+</sup> macrophages are appropriately activated by traditional pro- or anti-inflammatory cytokines, we analyzed gene expression of traditional M1 or M2 markers following polarization of BMDMs from CRE or CRE<sup>+</sup> mice. M1 macrophages from CRE<sup>+</sup> mice had comparable expression levels of the M1 markers, Tnfa and iNos, but failed to express the M2 marker, Arg1 (Fig. 3A). Similarly, M2 polarized macrophages from CRE<sup>+</sup> and CRE<sup>+</sup> mice had comparable expression levels of Arg1 (Fig. 3A), but failed to express Tnfa and iNos. These data indicate that Sptlc2-deficiency in macrophages does not alter macrophage polarization towards M1 or M2.

To examine whether the de novo synthesis pathway is required for saturated fatty acid activation of the inflammasome, BMDMs from CRE<sup>-</sup> or CRE<sup>+</sup> mice were primed with LPS prior to overnight culture in palmitate to activate the inflammasome. Secretion of active IL1β into the supernatants was comparable between CRE<sup>-</sup> and CRE<sup>+</sup> BMDMs that were cultured with LPS plus palmitate (Fig. 3B). Surprisingly, secretion of active IL1β in the presence of ATP or ceramide was higher in BMDMs from CRE<sup>+</sup> mice, as compared to the CRE<sup>-</sup> BMDMs. These data indicate that Sptlc2 is not required for palmitate-induced Nlrp3 activation, but may have role in inhibiting Nlrp3 activation, in the presence of other activators.

Macrophage SPTLC2-deficiency does not regulate adipose tissue mass or leukocytosis in response to high-fat diet- Saturated-fat diet-induced obesity is characterized by increased adipose tissue mass and inflammation that involves increased numbers of macrophages surrounding hypertrophied adipocytes with increased ER stress and release fatty acids upon cell death (3). Adipose tissue macrophage fatty acid-uptake during obesity is a contributing factor of their inflammatory status (9); however the fate of lipids in macrophages is still unclear. To identify whether myeloid cell-specific de novo synthesis of ceramides is required for adipose tissue inflammation driven by a high saturated fat diet, CRE<sup>-</sup> or CRE<sup>+</sup> mice were fed a 60% saturated fat diet for 13 weeks prior to analysis of the visceral adipose tissue. CRE<sup>-</sup> or CRE<sup>+</sup> mice on the high-fat diet (HFD) showed increased weight gain (data not shown) and total body-weight over
control mice on low fat diet (LFD), but there was no difference between CRE^−^ and CRE^+^ mice on HFD (Fig. 4A). Similarly, HFD-fed mice showed increased visceral adipose tissue mass and increased cellularity in visceral adipose tissue, but there were no differences between CRE^−^ and CRE^+^ mice (Fig. 4B&C).

Adipose tissue macrophages play a major role in regulating the homeostasis of the adipose tissue (41). Pro-inflammatory macrophages are recruited to the adipose tissue during HFD, where they promote the inflammatory response by secreting inflammatory cytokines such as IL1β and TNFα (41). The gating strategy for quantifying adipose tissue macrophages and lymphocytes are shown in figure 4D with representative dot plots in figure 4E. Adipose tissue macrophages were characterized into M1 (CD11c^+^), M2 (CD206^+^) and HFD-induced CD11c^+^CD206^+^ macrophages. We found that the percentages of total F4/80^+^CD11b^+^ macrophages are increased with HFD (Fig. 4F). When examining the subpopulation of macrophages, there was a small increase in the percentage of F4/80^+^CD11b^+^CD11c^+^CD206^+^ macrophages that are increased in both CRE^−^ and CRE^+^ mice on HFD, as compared to control mice on LFD (Fig. 4F). As lymphocyte populations are altered with HFD (5,42), we quantified T and B cells in the visceral adipose tissue in HFD-fed mice. CD3^+^ T cells and B220^+^ B cells were comparable between CRE^−^ and CRE^+^ on HFD (Fig. 4G).

When normalized to the adipose tissue weight, the number of total F4/80^+^CD11b^+^ macrophages and F4/80^+^CD11b^+^CD11c^+^CD206^+^ macrophages were significantly increased in both CRE^−^ and CRE^+^ on HFD as compared to control mice on LFD (Fig. 5A). To examine the gene expression of adipose tissue macrophages; F4/80^+^ cells were positively selected from CRE^−^ and CRE^+^ mice on HFD. Sptlc2 gene expression was significantly reduced in macrophages from CRE^+^ mice, whereas Sptlc1 gene expression was comparable between CRE^−^ mice, whereas Sptlc1 gene expression was comparable between CRE^−^ and CRE^+^ mice on HFD (Fig. 5B). There was no difference in anti-inflammatory gene, Arg1, or in pro-inflammatory genes, iNos, Il1β or Tnfa (Fig. 5C). Taken together these data indicate that myeloid cell-specific Sptlc2 is not required for HFD-induced adipose tissue inflammation. To ask whether other pathways to generate ceramide are compensating for the loss of Sptlc2, we examined gene expression of ceramide synthases (CerS) and sphingomyelinases in isolated adipose tissue macrophages. The expression level of CerS6, CerS5, Nsmaf and Smad1 were not altered by myeloid-deficiency of Sptlc2 (Fig. 5D).

Macrophage Sptlc2-deficiency does not impact HFD-induced insulin resistance - Myriocin-treatment, to block systemic de novo synthesis of ceramides, decreases systemic sphingolipid and ceramide levels, improves insulin sensitivity and reduces adipose tissue mass in mice on HFD (24). On LFD, CRE^−^ and CRE^+^ mice have comparable baseline glucose and ability to clear glucose following intraperitoneal injection of glucose (Fig. 6A). We examined whether myeloid-specific de novo synthesis of ceramides is responsible for HFD-induced decreases in insulin sensitivity using hyperinsulinemic-euglycemic clamp studies. There was no difference in glucose infusion rate (GIR) between CRE^−^ and CRE^+^ mice on HFD (Fig. 6B). Furthermore, whole-body glucose uptake and endogenous glucose production (EGP) at basal or following clamp was comparable between HFD-mice (Fig. 4C&D). These data suggest that myeloid cell-specific Sptlc2 is not required for systemic insulin sensitivity. In addition, clamp was equally able to decrease non-esterified fatty acids (NEFA) in CRE^−^ and CRE^+^ HFD-mice (Fig. 6E). In agreement with this data, an insulin tolerance test in CRE^−^ and CRE^+^ on HFD, revealed similar baseline glucose and similar ability to restore glucose levels following insulin challenge (Fig. 6F&G).

**Discussion:**

Diet-induced obesity (DIO) is characterized as a state of chronic, low grade inflammation with lipid and glucose alterations mediated in part by macrophage infiltration of adipose tissue (41). Palmitate induces Nlrp3 inflammasome activation and subsequent IL1β secretion during DIO; however the mechanism for activation has not been fully elucidated (15). We have hypothesized that Nlrp3 inflammasome activation requires saturated fatty acid entry into the non-oxidative pathway and de novo generation of ceramide via Sptlc2. In these experiments we have shown that myeloid cell-specific deletion of Sptlc2 is not required for inflammasome-induced adipose tissue inflammation and insulin resistance. In *vivo,*
Sptlc2-deficiency does not alter macrophage polarization or palmitate-induced IL1β secretion by the Nlrp3 inflammasome. In a model of saturated fat-induced inflammation, adipose tissue macrophage numbers, polarization and gene expression are comparable, in control mice and mice lacking myeloid cell expression of Sptlc2. Taken together, these data indicate that myeloid cell expression of Sptlc2 is dispensable for inflammasome-induced adipose tissue inflammation and insulin resistance.

In vitro work using Sptlc2-/+ BMDMs have shown that Sptlc2 is required for LPS or palmitate induced inflammatory cytokine production (40). Furthermore, in vivo investigations have shown that macrophage-specific Sptlc2 promote atherosclerotic lesions (40), highlighting the importance of ceramide synthesis in macrophages in metabolic diseases. A number of sphingolipids, downstream of ceramide synthesis, including plasminogen activator inhibitor (PA)-1, sphingosine-1-phosphate and ceramide-1-phosphate, have been identified as possible mediators in driving metabolic-induced inflammation (17,43,44). These data suggested that macrophage de novo synthesis of ceramide was critical in regulating macrophage-driven inflammation in metabolic diseases. Here, we show that myeloid cell-specific deletion of Sptlc2, as shown by 75% knockdown of gene expression and complete deletion of the protein, has no alteration on macrophage response in vitro to a single stimulus or in a mouse model of DIO. Our data, while unexpected, help to portray a more complete picture of fatty acid fate in macrophages and eliminates mechanisms that are responsible for ceramide generation as therapeutic targets of certain metabolic complications.

Furthermore, a number of publications have identified that myriocin, an SPT-specific inhibitor, promotes remarkable reductions in DIO-induced symptoms, including, reduced ceramide accumulation, reduced adipose tissue, smaller adipocytes, improved insulin signaling through Akt, and improved metabolic function (21,24,25). The differences between these and our data are likely due to the ability of myriocin to inhibit whole-body ceramide synthesis. SPT is a constitutive enzyme with activities in regulating cellular sphingolipids in all cell types; its inhibition alters total cellular sphingolipids and these alterations are likely to be beneficial to cells with lipid dysregulation, but damaging to cells that lack exposure to excess palmitate and ceramide synthesis. Tissue-specific or cell-specific inhibition of ceramide synthesis during lipid dysregulation, for example in the liver or muscle, is an attractive prospect for reducing inflammation and improving insulin sensitivity. In agreement with this concept, overexpression of acid ceramidase in liver or adipocytes improves systemic insulin sensitivity, hepatic lipid accumulation and adipose tissue inflammation (23). It remains to be studied if elevation of ceramide degradation enzymes in macrophages will lower the ‘lipotoxic DAMP load’ that causes inflammasome activation in obesity.

Sptlc2 is the rate-limiting subunit of the SPT enzyme and is required for the de novo synthesis of ceramide; however other mechanisms for generating ceramide include the hydrolysis of sphingomyelin (salvage pathway) or synthesis from sphingosine and more complex sphingolipids (recycling pathway) (20,45). Degradation of sphingomyelin requires sphingomyelinases (SMase), whereas ceramide synthases catalyze the recycling of sphingolipids, both as part of a carefully regulated process for meeting the cellular demands of lipids (20). Our data shows that there is no change in gene expression of these ceramide synthases in adipose tissue macrophages due to deletion of Sptlc2. This suggests that other pathways for generating ceramides (salvage, recycling pathways) are not upregulated in compensation for loss of the de novo pathway. Recent publications are in agreement with our data, suggesting that ceramide synthesis in myeloid cells is not critical for diet induced inflammation. Deletion of CerS6, which is upregulated in the white adipose tissue of high fat-fed mice, in macrophages failed to prevent diet-induced adipose tissue inflammation or insulin resistance (46). In our experiments, adipose tissue macrophages highly express both CerS5 and CerS6. Taken together, these data indicate that ceramide synthesis in macrophages is dispensable when targeting DIO-induced inflammation and metabolic disorders. Given that ceramides are also present with the cell membranes, macrophages may accumulate ceramide via cellular membrane degradation following phagocytosis of dead or dying cells.
Macrophages are present in the lean state and are critical in promoting diet-induced adipose tissue inflammation. CD11c+ macrophages infiltrate the adipose tissue, surround necrotic adipocytes and release inflammatory cytokines (10). Not only are macrophages directly exposed to fatty acids released from dying adipocytes, but systemically, diet-induced increased serum fatty acids cause chronic exposure to macrophages. Macrophages express fatty acid receptors, including CD36, which when deleted, prevents diet-induced adipose tissue inflammation (47), indicating that macrophage uptake of fatty acids mediates HFD-induced inflammation. Upon lipid uptake, fatty acids can be stored as triglyceride in lipid droplets, enter into an oxidative pathway for metabolism to ATP, or a non-oxidative pathway for conversion into cell-required sphingolipids or signaling molecules (20). The fate of fatty acids following release from dying adipocytes is unclear, although a recent study has shown the importance of lysosomal biogenesis and metabolism of lipids in adipose tissue macrophages following DIO (9), suggesting that a portion may be metabolized. Other investigations have underscored the importance of the type of fatty acids in eliciting inflammation, as omega-3 supplementation is sufficient to reduce HFD-induced adipose tissue inflammation (48). In this publication, we have eliminated the possibility that palmitate entry into the non-oxidative pathway causes Nlrp3 inflammasome-driven inflammation. These data suggest that the metabolic fate of palmitate could be at least partly independent from its ability to induce inflammation; alternatively storage as triglycerides is a potential mechanism for inflammation.

Macrophages are tissue resident cells which are critical for maintaining homeostasis through immunometabolic interactions. Saturated fatty acid is a metabolite capable of eliciting Nlrp3 inflammasome activation and promoting dysregulated glucose metabolism (13). Its mechanism of action is known to involve AMPK inhibition and ROS, but whether its metabolism is required for activation is still incompletely understood. Therapeutic attempts at improving metabolic dysfunction have been mostly unsuccessful; narrowing the number of viable translatable approaches to improve metabolic syndrome is critical in type 2 diabetes and human obesity. We have used in vitro and in vivo mouse models to eliminate the de novo ceramide synthesis as a potential mechanism and allow future research to focus on other significant pathways of ceramide homeostasis or degradation in macrophages.
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Author Contributions: C.D.C. participated in the design of the study, coordinated and carried out experiments, performed the analysis and wrote the manuscript. K.Y.N participated in study design, assisted with experiments and manuscript edits. M.J.J. performed experiments and analysis shown in figure 6. G.I.S. participated in experimental design and edits of the manuscript. G.S.S. assisted with experimental design and data analysis. V.D.D. conceived and coordinated the study and participated in the writing of the manuscript. All authors reviewed the results and approved the final version of the manuscript.

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Sptlc2 is dispensable for Nlrp3 inflammasome activation

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**Figure Legends**

**Table 1. Sequences of primers used in Syber green quantitative PCR.** Forward and reverse sequences are listed (5’ to 3’) for *Gapdh, Arg1, Tnfa, Il1β, iNos, Sptlc1, Sptlc2, Sptlc3, CerS5, CerS6, Nsmaf* and *Smpd1*.

**Figure 1. Ceramides activate the Nlrp3 inflammasome via mitochondrial oxidative stress.** (A) Representative western blot analysis of IL1β (active p17) protein in supernatants of wild-type (WT) BMDMs that have been primed with LPS for 4hrs and stimulated with increasing doses of ceramide (C6) for 6 hrs. (B) Western blot analysis of IL1β protein in BMDMs from WT or *Nlrp3*−/− mice that have been stimulated with LPS, LPS plus C6 (80ug/ml) for 6 hours or LPS plus palmitate (400uM) for 24hrs. (C) Western blot analysis of caspase-1 (active p20) and catalase protein in BMDMs from WT or MCAT transgenic mice that have been treated with LPS alone, LPS plus C6 or LPS plus ATP (5mM) for one hour. Two representative blots from individual experiments are shown. (D) Schematic depicting palmitate entry into oxidative pathway to generate ATP or the non-oxidative pathway causing the de novo synthesis of ceramide. Western blot analysis of IL1β protein in the supernatants of WT BMDMs, treated with LPS or LPS plus palmitate, in which some received pretreatment with serine palmitoyltransferase-inhibitor, myriocin. Data are representative of 2-3 individual experiments.

**Figure 2. Myeloid cell-specific deletion of Sptlc2.** (A) Gene expression of *Sptlc1, Sptlc2* or *Sptlc3*, normalized to *gapdh*, in WT BMDMs that have been polarized to M1 (LPS (1ug/ml) plus IFNγ
(20ng/ml)) or M2 (IL4 (10ng/ml)). (B) Gene expression of Sptlc2 or Sptlc1 normalized to gapdh in M1 (filled) or M2 (open) polarized BMDMs from CRE−, CRE+, or fl/−; CRE+. (C) Western blot analysis of SPTLC2 protein expression in treated BMDMs from CRE− or CRE+ mice. Actin is shown as a loading control. (N=2-3 individual experiments; T-test; *P<0.05; error bars represent mean±SEM)

**Figure 3. Sptlc2 is not required for macrophage polarization or inflammasome activation in vitro.** (A) iNos, Tnfa or arg1 gene expression, normalized to gapdh, from CRE− or CRE+ BMDMs that have been polarized to M1 (filled) or M2 (open). (B) Two western blots from individual experiments of IL1β protein in supernatants of CRE− or CRE+ BMDMs following no treatment, treatment with LPS alone, LPS plus palmitate, LPS plus ATP or LPS plus C6. (N=2-3 individual experiments; error bars represent mean±SEM)

**Figure 4. Myeloid cell-specific Sptlc2 is not required for diet-induced inflammation.** (A) Body-weight (BW), (B) visceral adipose tissue (AT) weight and (C) cells per gram of adipose tissue after 13 weeks of LFD or HFD in CRE− or CRE+ mice. (D) Gating strategy to analyze stromavascular fraction of adipose tissue. (E) Representative dot plots of F4/80CD11b+ cells from adipose tissue of CRE− mice on LFD, CRE− mice on HFD or CRE+ mice on HFD. F4/80CD11b+ cells were gated on to analyze CD206 and CD11c expression. (F) Quantification of the percentage of macrophage and the macrophage subpopulations. (G) Quantification of the percentage of lymphocytes, CD3+ T cells and B220+ B cells, in adipose tissue from HFD-mice. (N=9-10 biological replicates; 1-way ANOVA or t-test as appropriate; *P<0.05; error bars represent mean±SEM)

**Figure 5. Sptlc2-deficient adipose tissue macrophages maintain HFD-induced inflammation.** (A) Quantification of the cells/g AT for macrophages and the macrophage subpopulations from the visceral adipose tissue of CRE− or CRE+ mice on HFD. (B) Expression level of Sptlc2 or Sptlc1 in isolated F4/80+ adipose tissue macrophages from CRE− or CRE+ mice on HFD. Gene expression of (C) arg1, iNos, Il1β Tnfa, (D) CerS6, CerS5, Nsmaf or Smpd1 (normalized to Gapdh) in isolated adipose tissue macrophages. (N=9-10 biological replicates; 1-way ANOVA or t-test as appropriate; *P<0.05; error bars represent mean±SEM)

**Figure 6. Macrophage-specific Sptlc2 is not required for diet-induced insulin resistance.** (A) Glucose tolerance test (GTT) on LFD-fed CRE− or CRE+ mice. (B) Glucose infusion rates (GIR) during hyperinsulinemic-euglycemic clamps in CRE− or CRE+ mice on HFD for 12 weeks. Average GIR inset. (C) Whole body glucose uptake. (D) Endogenous glucose production (EGP) at basal and after clamp. (E) Non-esterified fatty acid level in the blood at basal and after clamp. (N=4 CRE−; N=7 CRE+) (F) Baseline glucose levels after four hour fast, and (G) at time points after intraperitoneal injection of insulin (insulin tolerance test) in CRE− or CRE+ mice on HFD. (N=8) (T-test; *P<0.05; error bars represent mean±SEM)
| Gene | Forward  | Reverse                                  |
|------|----------|------------------------------------------|
| Gapdh | TCAACGGCACAGTCAAG | CATGGACTGTGGTGTGATGAG |
| Arg1  | ATTATCGGAGCCTTTTCTC | TTTTCCAGCAGACCAGCTT |
| Tnfa  | CGAGTGACAAGCCTGAC | CTTTCTGCTGGTATGAGATGAGCA |
| Il1β  | GGTCAAAGGTTTGGGAAGCAG | TGTGAAATGCCACCTTTTGA |
| iNos  | CCCTCCTGATCTTTGTGTGG | GGCTGAGCATACCATTCCTCA |
| Sptlc1 | AGTCACCAGCACTATGGGA | GAGAGCCGCTGATGGTCA |
| Sptlc2 | CCAAAATTGGGCGCTTTTGA | GGCTGACAGGAAATCCACC |
| Sptlc3 | GACTAGTAAAAGGCTCTGCTTGA | ATGGTGAGGATGTGCTGGAG |
| CerS5 | ATCAAGGACACGCTCAACG | ACGCAGGCACTCGGCAGAG |
| CerS6 | AGGGTTGAAGCTGCTTTGTGGTC | GTCACTCTGGATACCTTGCCT |
| Nsmaf | TGAACATGATGTCAGCCTCAAA | ACCATGCTTCTTTGGTGC |
| Smpd1 | GACCAGCAGCTGATGACCTCC | AACTCGGTAGCCAGGTTAAG |
Figure 1

A.)

|                | LPS | LPS+C6 | LPS+C6 | LPS+C6 |
|----------------|-----|--------|--------|--------|
| Supernatants (kDa) | 110 | 50    | 30    | 20    |
| LPS + EtOH     |     |        |        |        |
| LPS            | 40 μg|        |        |        |
| LPS            | 80 μg|        |        |        |
| LPS            | 120 μg|       |        |        |

B.)

|                | LPS | LPS+C6 | LPS+PA |
|----------------|-----|--------|--------|
| Supernatants (kDa) | 30 | 20    | 15    |
| IL1β            |     |        | p17   |
| p17            |     |        |       |

C.)

|                | LPS | LPS+C6 | LPS+ATP | LPS | LPS+C6 | LPS+ATP |
|----------------|-----|--------|---------|-----|--------|---------|
| Exp.1          |     |        |         |     |        |         |
| Exp.2          |     |        |         |     |        |         |
| Supernatants (kDa) | 50 | 30    | 20     | 15 |       |         |
| Cell Extracts (kDa) | 50 | 30    | 20     | 15 |       |         |
| Caspase-1      |     |        | p20    |     |        |         |
| Catalase       |     |        |        |     |        |         |
| Actin          |     |        |        |     |        |         |

D.)

Oxidative (Mitochondria)

- Palmitate
- mj C → A
- β-oxidation
- ATP

Non-oxidative (ER)

- Palmitoyl-CoA + L-Serine
- Serine palmitoyltransferase
- 3-ketosphinganine
- 3-Keto. Reductase
- Sphinganine
- Ceramide Synthase
- Dihydro-ceramide
- Dihydroceramide desaturase
- Ceramide

E.)

|                | LPS | LPS+C6 | LPS+PA |
|----------------|-----|--------|--------|
| Supernatants (kDa) | 30 | 20    | 15    |
| IL1β            |     |        | p17   |
| p17            |     |        |       |
A.)

Figure 2

B.)

C.)
Figure 3

A.)

![Graph showing gene expression](image)

B.)

![Western blot analysis](image)

**Exp. 1**

- UNT
- LPS
- LPS+PA
- LPS+ATP
- LPS+C6

**Exp. 2**

- UNT
- LPS
- LPS+PA
- LPS+ATP
- LPS+C6

**Supernatants (kDa):**

- 50
- 30
- 20
- 15

**IL-1β p17**

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Macrophage-specific de novo synthesis of ceramide is dispensable for inflammasome-driven inflammation and insulin-resistance in obesity
Christina D. Camell, Kim Y. Nguyen, Michael J. Jurczak, Gerald I. Shulman, Gerald S. Shadel and Vishwa Deep Dixit

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