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Gamma-tocotrienol attenuates the aberrant lipid mediator production in NLRP3 inflammasome-stimulated macrophages

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Abstract

The activation of NLRP3 inflammasome in innate immune cells is associated with enhanced production of pro-inflammatory lipid mediator eicosanoids that play a crucial role in propagating inflammation. Gamma-tocotrienol (γ T3) is an unsaturated vitamin E that has been demonstrated to attenuate NLRP3-inflammasome. However, the role of γ T3 in regulating eicosanoid formation is unknown. We hypothesized that γ T3 abolishes the eicosanoid production by modulating the macrophage lipidome. LPS-primed bone marrow-derived macrophages (BMDM) were stimulated with saturated fatty acids (SFA) along with γ T3, and the effects of γ T3 in modulating macrophage lipidome were quantified by using mass spectrometry based-shotgun lipidomic approaches. The SFA-mediated inflammasome activation induced robust changes in lipid species of glycerolipids (GL), glycerophospholipids (GPL), and sphingolipids in BMDM, which were distinctly different in the γ T3-treated BMDM. The γ T3 treatment caused substantial decreases of lysophospholipids (LysoPL), diacylglycerol (DAG), and free arachidonic acid (AA, C20:4), indicating that γ T3 limits the availability of AA, the precursor for eicosanoids. This was confirmed by the pulse-chase experiment using [³H]-AA, and by diminished prostaglandin E₂ (PGE₂) secretion by ELISA. Concurrently, γ T3 inhibited LPS-induced cyclooxygenases 2 (COX2) induction, further suppressing prostaglandin synthesis. In addition, γ T3 attenuated ceramide synthesis by transcriptional downregulation of key enzymes for *de novo* synthesis. The altered lipid metabolism during inflammation is linked to reduced ATP production, which was partly rescued by γ T3. Taken together, our work revealed that γ T3 induces distinct modification of the macrophage lipidome to reduce AA release and corresponding lipid mediator synthesis, leading to attenuated cellular lipotoxicity.

Keywords

gamma tocotrienol; NLRP3 inflammasome; lipidomics; arachidonic acid; eicosanoids; PGE₂

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1. Introduction

Obesity and insulin resistance are regarded as inflammatory diseases in which NF κ B activation orchestrates the innate immune responses [1, 2]. Aberrant NF κ B activation and proinflammatory cytokine secretion are important regulators for pathogenic progression of obesity. Nod-like receptor protein 3 (NLRP3) inflammasome is an innate immune effector that recognizes molecular patterns of dangerous signals to mature and secrete cytokines IL-1 β and IL-18 [3]. In addition to pathogen-associated molecular patterns, NLRP3 inflammasome assembly is activated by damage-associated molecular patterns (DAMPs) such as free fatty acids (FFA) [4]. The elevated levels of FFA, arising from either the lipolysis of inflamed adipose tissue or excessive dietary intake, serve as a key DAMP signal to instigate NLRP3 inflammasome in adipose tissue macrophages (ATM), thereby perpetuating lipotoxicity [5].

In addition to the release of cytotoxic cytokines such as IL-1 β , activation of the NLRP3 inflammasome in ATM is accompanied by large quantities of lipid mediator production including eicosanoids and ceramides, which propagates the metabolic damages to systemic level via autocrine and paracrine signaling [6]. It is proposed that NF κ B activation alters lipid profiles in macrophages [7, 8], albeit it has not been completely understood regarding the role of FFA in modulating the proinflammatory lipid mediators in macrophages. Understanding the interplay between lipotoxicity-mediated NLRP3 inflammasome activation and synthesis of lipid mediators may provide insights into the pathogenic progression of obesity to type 2 diabetes.

Gamma-tocotrienol (γ T3) is an unsaturated form of vitamin E that has been demonstrated to attenuate adipose inflammation and obesity-induced insulin resistance [9]. Accumulating lines of evidence showed that γ T3 inhibits NF κ B activation in innate immune cells such as macrophages as well as metabolic cells such as adipocytes and hepatocytes [10, 11]. Recently, our group reported the immunomodulatory role of γ T3 demonstrating that γ T3 suppresses NLRP3 inflammasome in cultured macrophages as well as ATM in diabetic leptin receptor knockout mice (DB) [12]. Given the fat-soluble nature of γ T3 and its potential to modify signaling in cell membranes, it is speculative that γ T3 may exert direct roles in altering lipid metabolism in macrophages in favor of attenuated synthesis of pro-inflammatory lipid mediators. Hitherto, γ T3 has been shown to decrease ceramide production by inhibiting the dihydroceramide desaturase activity in cancerous cells [13]. However, it is largely unknown whether γ T3 has a potential to alter macrophage lipidome, especially in conditions of SFA-induced NLRP3 inflammasome activation.

The aim of this study is to investigate the role of γ T3 in regulating the production of lipid mediators in macrophages under the NLRP3 inflammasome-stimulated conditions by endotoxin LPS in combination with palmitate, C16:0 saturated fatty acid (SFA). By using lipidomic approaches, we identified that γ T3 significantly alters arachidonic acid (AA) and sphingolipid metabolism in macrophages, resulting in reduced eicosanoid production and protection of energy metabolism from aberrant immune response-mediated metabolic damages.

2. Materials and Methods

2.1. Chemicals and Reagents

Gamma-tocotrienol (γ T3; with 90% purity) was kindly provided by Carotech (Edison, NJ, USA). γ T3 was prepared as previously described [12]. Pam3CSK4, a TLR2 agonist, was purchased from R&D Systems. All other chemicals and reagents were purchased from Sigma Chemical unless otherwise stated.

2.2. Preparation of bone marrow-derived macrophages (BMDM)

Primary bone marrows were isolated from 6-week-old C57BL/6 mice (Jackson Laboratory) as we conducted previously [10]. All animal experimental procedures were approved by the Institutional Animal Care and Use Committee at the University of Nebraska-Lincoln. Briefly, bone marrows were obtained by flushing the femur, and the cells were suspended and cultured in DMEM containing 20% FBS, penicillin (100 U/ml), and streptomycin (100 μ g/ml), in the presence of 30 % L929-cell conditioned medium containing monocyte stimulating factor. Differentiated macrophages (BMDM, unstimulated, M0) were allowed to attach and form a monolayer for 7–10 days [14].

2.3. Activation of NLRP3 inflammasome

The differentiated bone marrow-derived macrophages (BMDMs) were pretreated with either 1 μ M of γ T3 or vehicle (DMSO) for 24 hours. BMDM were first primed with lipopolysaccharide (LPS) (100 ng/ml) for 1 hour, and with palmitic acid (400 μ M) for 12 hours to activate NLRP3 inflammasome (LPS/pal). For separate activation of toll like receptor 2 (TLR2) and 4 (TLR4), BMDM were stimulated with pharmacological agonist of Pam3CSK4 (200 ng/mL, R&D systems) or KLA (100 ng/mL, Sigma), respectively.

2.4. Shotgun lipidomics study using LC-MS/MS

After stimulation of NLRP3 inflammasome, BMDMs (10×10^6) were harvested with ice-cold HBSS and pelleted for keeping in -80°C until lipid profile analysis. Two hundred μ L of cell suspension were used for protein quantification, and 800 μ L of suspension were analyzed with an LC-MS/MS (AB Sciex TripleTOF 5600 system). The data was processed using the software Peakview & lipidview. All data were normalized to mg protein. A detailed description of this method has been previously described [15].

2.5. qPCR

Gene expression levels were measured using real-time qPCR [12]. Gene-specific primers for real-time quantitative PCR (qPCR) were synthesized from Integrated DNA Technologies (Chicago, IL, USA). Total RNA were isolated with Trizol reagent (Invitrogen). To remove the potential genomic DNA contamination, mRNA will be treated with DNase (DNAase free kit, Invitrogen), and 2 μ g of mRNA were converted into cDNA in a total volume of 20 μ L (iScript cDNA synthesis kit, Bio-Rad). Gene expression was determined by real-time qPCR (QuantStudio 6 Flex Real-Time PCR, Applied Biosystem) using SYBR green (Fisher scientific), and relative gene expression were normalized to the average of reference gene, hypoxanthine guanine phosphoribosyltransferase (*Hprt*).

2.6. Measurement of prostaglandin E₂ (PGE₂)

To measure PGE₂ levels, BMDMs were stimulated with LPS/palmitate for 12 hours and medium was collected, centrifuged to remove cell debris, and used for measurement of PGE₂ by ELISA kit (Cayman chemical, #514010) according to the manufacturer's instructions.

2.7. [³H]-radiolabeled Arachidonic acid (AA) release

To measure the Arachidonic acid (AA) release, we used the pulse-chase metabolic chasing method according to the published protocol [16]. The AA [5,6,8,9,11,12,14,15-³H(N)] was purchased from PerkinElmer. Briefly, BMDMs were plated at a density of 0.5×10^6 million/per well in 6-well plate and incubated overnight at 37 °C in a humidified incubator. The cells were pretreated with or without 1 μM of γT3 for 12 hours, then incubated [³H]-AA (0.5 μCi/1mL/well) for 18 h at 37 °C for loading. The cells were washed three times with 2 mg/mL of bovine serum albumin in phosphate-buffered saline to remove unincorporated [³H]-AA, followed by NLRP3 inflammasome stimulation using LPS/palmitate as described in 2.3. After 6 hours of NLRP3 inflammasome activation, released [³H]-AA to the culture medium was collected, centrifuged to remove cell debris, and used for measurement of [³H] radioactivity by liquid scintillation counter (Tri-carb 2900TR liquid scintillation analyzer, Perkin Elmer).

2.8. Measurement of mitochondrial respiration and glycolysis

The Mito Stress tests were conducted to measure basal respiration, maximal respiration, and ATP production in BMDM by using XFe24 extracellular flux analyzer (Seahorse) as we described previously [17]. Briefly, macrophages were seeded in gelatin-coated seahorse microplates, and pretreated with or without 1 μM of γT3 for 24 hrs. The Oxygen consumption rate (OCR) was calculated by plotting the O₂ tension of the medium in the microenvironment above the cells as a function of time (pmol of O₂/min). Glycolysis Stress tests were conducted to measure glycolysis, glycolytic capacity, and glycolytic reserve under the same conditions for mitochondrial respiration using the BMDM. The Extracellular acidification rate (ECAR) was calculated by measuring the acidity of the medium above the cells as a function of time (mpH/min). Once the assay was finished, total the protein was collected from each well and used to normalize the data.

2.9. Western Blot Analysis

For Western blot analysis, total cell extract of BMDM were prepared [12]. The antibodies recognizing phospho(p)-cPLA(Ser505), and Iκ-Bα were purchased from Cell Signaling Technology.

2.10. Statistical Analysis

The heat map production and principal component analysis (PCA) were performed with JMP software (SAS Institute Inc. Cary, NC, USA). The heatmap was depicted with every row for each lipid species and the relative abundance data were auto-scaled for multivariate analyses. From the green to red, it represents low to high concentration of each lipid profile. In PCA analysis, the data was transformed from the original variables to principal

components. For the statistical analyses of two-group comparisons, two-tailed Student's t-test was used. P values less than 0.05 ($P < 0.05$) were considered significant. The values were expressed as mean $\pm$ SEM. Analysis for multi-group comparisons were performed by a one-way ANOVA followed by Tukey's multiple comparison test. All statistical analyses were performed using GraphPad Prism 6 (version 6.02).

3. Results

3.1. Shotgun lipidomic analysis revealed that γ T3 distinctively modifies macrophage lipidome against SFA-induced NLRP3 inflammasome

To gain an insight into whether SFA-driven inflammasome activation alters macrophage lipid metabolism and to determine the potential role of γ T3, LPS-primed BMDM were stimulated with palmitate (LPS/pal) in the presence or absence of γ T3 (1 μ M). Before the lipidomics assay, the suppression of NLRP3 inflammasome activation by γ T3 was confirmed by IL-1 β gene expression and secretion to the medium (Fig 1A). Lipidomic analyses were performed in both unstimulated conditions (Cont vs. γ T3) and NLRP3 inflammasome-stimulated conditions (LPS/pal vs. LPS/pal+ γ T3). By using 14 internal standard lipids, shotgun lipidomic analyses resulted in the identification of total 209 lipid species from the major 20 (sub) classes. The heatmap visualization of the distribution of the different lipid classes (by summing the individual lipid species) demonstrated that LPS/pal stimulation robustly promoted the synthesis of the majority of lipid classes in inflammasome-stimulated BMDM (LPS/pal) compared to unstimulated control (Cont) (Fig 1B). To gain an initial overview, multivalent principal component analysis (PCA) relative to the lipid composition was performed. As we expected, PCA with compositional data resulted in a pronounced separation of unstimulated and LPS/pal-stimulated BMDM. More importantly, there was a distinct γ T3-dependent difference in the lipid profiles of BMDM in both unstimulated as well as LPS/pal-stimulated conditions (Fig 1C). Collectively, these results suggest that γ T3 significantly modulates cellular lipid profiles and the magnitude of lipid profile differences was more evident in NLRP3 inflammasome-stimulated conditions in the primary murine BMDM.

3.2. γ T3 suppressed AA formation in BMDM against palmitate-induced inflammasome

Regardless of γ T3 treatment, the increase of total phospholipid (PL) content was phenomenal in response to LPS/pal stimulation, suggesting that palmitate is incorporated into PL. However, the total lysophospholipids (LysoPL) were significantly lower in γ T3-received BMDM than LPS/pal alone (Fig 2A). To identify the individual lipid distinguishing the impact of γ T3 in response to SFA-mediated NLRP3 inflammasome, we compared lipid composition between LPS/pal and LPS/pal+ γ T3. Interestingly, monoacylglycerol (MAG) content was higher in the presence of γ T3, and the magnitude was further increased by LPS/pal especially in 14:1, 20:2, 22:1, and 22:2 MAG (Fig 2B). In contrast, there was an opposite trend in diacylglycerol (DAG) regulation. LPS/pal stimulation dramatically induced the 32:0, 36:4 and 38:4 DAG in BMDM, which were dampened by γ T3 treatment (Fig 2C). Although there was no difference in total FFA level, arachidonic acid (AA, 20:4) content was significantly reduced with γ T3 treatment (Fig 2D). Among the PL classes, phosphatidylethanolamine (PE) levels, but not phosphatidylcholine (PC), were significantly

lower in the presence of γ T3 especially in 36:4, 40:5, 36:1, and 38:4 PE (Fig 2E). Consequently, PC/PE ratio was significantly lowered in LPS/pal than LPS/pal+ γ T3 (data not shown). It is also noteworthy that increased levels of LysoPL during NLRP3 inflammasome activation were significantly reduced in γ T3-treated BMDM in the major species of lysophosphatidyl choline (16:0, 14:1, 16:1 LysoPC), lysophosphatidyl ethanolamine (16:0, 18:0, 20:4, 20:0 LysoPE), and lysophosphatidyl serine (18:1, 22:4 LysoPS) (Fig 2F).

Given that AA release is a rate-limiting step for proinflammatory eicosanoid synthesis, we further examined the direct effects of γ T3 on AA release by using ^3H -AA as a molecular probe. BMDM were first incubated with ^3H -AA in the absence or presence of γ T3 for membrane loading. After removing unincorporated AA, BMDM were stimulated by LPS/pal for 6 hours, and the medium was collected to measure the released radiolabeled-AA and its metabolites. There was no significant difference in ^3H -AA loading to the BMDM between two groups (data not shown). However, the release of ^3H -AA and its metabolites were markedly lower in the presence of γ T3 (Fig 3A). In addition, the LPS/pal-induced phosphorylation of cytosolic phospholipase A2 (cPLA2) (Ser505), a sign for cPLA2 activation, was significantly reduced by γ T3 (Fig 3B). This result implicate that γ T3 considerably represses inflammation-mediated AA release by diminishing the membrane PL hydrolysis via cytosolic phospholipase A2 (cPLA2) resulting in reduced lysophosphatidic acid (lysoPA) and AA release. In combination with lipidomic data (Fig 1 and 2), these results suggest that γ T3 reduces AA release from membrane PL via mechanisms in which γ T3 1) diminishes cPLA2-mediated membrane PL hydrolysis resulting in reduced lysophosphatidic acid (lysoPA) and AA levels, and 2) inhibits DAG formation from the dephosphorylation of phosphatidic acid (PA), presumably due to reduced phospholipase C activation (Fig 3C).

3.3. γ T3 reduced prostaglandin E₂ production by blocking cyclooxygenase 2 induction in BMDMs

Eicosanoids include prostaglandins, thromboxanes (TXs), and leukotrienes (LTs). Prostaglandins and TXs, which are generated by the two isoforms of cyclooxygenases (COX), COX-1 and COX-2, whereas LTs are generated by 5-lipoxygenase (5-LOX). We quantified prostaglandin E₂ (PGE₂) levels from the BMDM medium that was used for lipidomic analysis in Fig 1. The activation of inflammasome led to a dramatic secretion of prostaglandin E₂ (PGE₂), which was abolished by γ T3 comparable to unstimulated levels (Fig 4A). Consistent with PGE₂ secretion, there was over 40-fold induction of *Cox2* gene expression upon inflammasome activation, while it was reduced to approximately to half in the presence of γ T3. However, *Cox1* and *5-Lox* were not increased by LPS/pal-induced inflammasome conditions (Fig 4B), suggesting that production of prostaglandins by COX2 is the major constituent for eicosanoid storms in response to LPS/pal stimulation.

It is known that COX2 is induced by toll-like receptor (TLR)/NF κ B pathways [18]. To tease out the separate role of γ T3 on TLR2 and TLR4-mediated COX2 activation, BMDM were either stimulated with Kdo2 lipid A (KLA), a TLR4-specific agonist, or Pam3CSK4 (Pam3), a TLR2-specific agonist (Fig 4C). The increase of *Cox2* and *Il-1 β* by both KLA and Pam3 was significantly suppressed by γ T3 comparable to the unstimulated levels (Fig 4D, E). As

we expected, γ T3 treatment significantly reduced KLA and Pam3-induced I κ B α degradation (Fig 4D, E). These results suggest that γ T3 inhibits TLR2/NF κ B signaling axis as well as TLR4/NF κ B, thereby synergistically reducing COX2 induction and corresponding PGE₂ formation.

3.4. γ T3 suppressed the NLRP3 inflammasome-mediated ceramide synthesis

Sphingolipids (SL) are crucial lipid mediators of inflammatory diseases [19, 20]. The lipidomic analysis showed that total SL [sums of Ceramide (Cer), hexosyl-ceramide (HexCer), sulfatagalatosyl-ceramide (SGalCer), and sphingomyelin (SM)], as well as ceramide alone, were significantly increased in NLRP3 inflammasome-stimulated BMDM. This effects were reversed by γ T3 pretreatment (Fig 5A, B). The gene expression levels of key enzymes for *de novo* synthesis of ceramide were measured using qPCR. The inflammasome-induced mRNA transcripts of serine-palmitoyltransferase (*Spt*) and dihydroceramide desaturase (*Des*) were significantly lower in γ T3-treated BMDM than Control (Fig 5C). Combining the results from lipidomic analysis and qPCR, it is suggestive that palmitate-induced inflammasome promotes *de novo* ceramide synthesis, which is attenuated by γ T3 partly through the transcriptional suppression of key enzymes for ceramide biosynthesis (Fig 5D).

3.5. γ T3 protected from inflammation-mediated impairment of energy metabolism

Emerging evidence suggests that energy-sensing mechanisms are linked with immunity. The reduced ATP production in innate immune cells triggers innate immune responses including NLRP3 inflammasome activation in macrophages [21, 22]. Next, we hypothesized that reduced inflammation and lipid remodeling by γ T3 help maintain the energy production capacity of macrophages, contributing to suppression of excessive immune responses. The LPS-stimulation impaired respiration rate and dampened mitochondrial ATP production. In contrast, γ T3-treated BMDM were able to maintain mitochondrial function and rescue the mitochondrial ATP production ~50 % (Fig 6A). Similarly, LPS-stimulation substantially decreased glycolytic functions, while γ T3 treatment help maintains glycolytic function comparable to unstimulated macrophages (Fig 6B). These results led us to speculate that the unique lipidomic signature modulated by γ T3 (e.g., decreased AA release) may contribute to maintaining energy homeostasis by protecting glycolytic and mitochondrial function.

4. Discussion

Inflamed adipose tissue is a reservoir of FFA that serves as a potent DAMP signal to instigate NLRP3 inflammasome in ATM. The activation of inflammasome accompanies a massive production of lipid mediators, so-called eicosanoid storm [23, 24]. This study is designed to define the changes of macrophage lipidome by γ T3 in the SFA-stimulated NLRP3 inflammasome. Here, we demonstrated that γ T3 substantially modulates inflammasome-mediated lipid profiles in BMDM (Fig 1, 2), inhibiting AA release (Fig 3) and suppressing COX2 induction and subsequent PGE₂ production (Fig 4). Our work also showed that γ T3 was effective in decreasing ceramide formation against excess SFA-stimulated inflammasome (Fig 5). Moreover, γ T3 at least partly reversed the inflammation-mediated impairment of mitochondrial and glycolytic function (Fig 6). This work

demonstrated that γ T3 suppresses the aberrant production of pro-inflammatory lipid mediators and protects the energy metabolism of macrophages against SFA-driven NLRP3 inflammasome.

In this study, we focused on lipidomic changes caused by γ T3 in the unstimulated and NLRP3 inflammasome-stimulated BMDM. In the unstimulated conditions, changes of lipidome by γ T3 were negligible except MAG content. The overall content of MAG was fairly low in both basal and stimulated BMDM. In contrast, γ T3 treatment promoted MAG levels even in unstimulated conditions, which was further augmented by inflammasome-stimulated conditions (Fig 1B, 2B). There is increasing knowledge that monoacylglycerol lipase (MAGL) is an important regulator of simultaneous prostaglandins synthesis [25]. Additionally, MAGL can hydrolyze endocannabinoid 2-arachidonoyl glycerol (2-AG) yielding glycerol and AA, which further metabolizes into prostaglandins [26, 27]. Based on this, we assumed γ T3 might play a role as MAGL inhibitor, thereby accumulating MAG but decreasing AA production. Unfortunately, it is unlikely because we found *Magl* message levels were very low, and they were not increased by γ T3 treatment (data not shown). Currently, it is uncertain whether the increased content of MAG is a part of the anti-inflammatory mechanism of γ T3 or metabolic side effects unrelated with inflammation. Further research is warranted to determine the exact role of increased MAG by γ T3 in macrophages regarding metabolic and immunological consequences.

In our experimental setting, γ T3 treatment has minimal effects on FFA activation of acyl CoA or its conversion into PL, as we did not see any differences in the total content of GPL. Importantly, the most conspicuous change by γ T3 was the modulation of AA metabolism. Despite no difference of total FFA content, free AA content was significantly lower in γ T3-treated BMDM (Fig 2D). It is well known that availability of free AA is a rate-limiting step for eicosanoids production in innate immune cells [28]. The AA levels are modulated by two opposing reactions, phospholipid de-acylation by cytosolic phospholipase A2 (cPLA2) and re-acylation back into phospholipid by lysophospholipid acyltransferase (LPLAT) [29]. In the stimulated conditions, cPLA2-mediated de-acylation dominates over re-acylation, resulting in elevated levels of free AA, while the availability of AA is limited in unstimulated conditions due to dominant re-acylation reaction [29]. In the presence of γ T3, there were marked reductions in LysoPL as well as free AA (Fig 1, 2F), suggesting that γ T3 suppressed the LPS/pal-induced cPLA2 activation and its enzymatic action to hydrolyze PL into LysoPL and AA. It is reported that cPLA2 levels are decreased by α -tocotrienol in neuronal cells [30]. However, it was unknown whether γ T3 inhibits cPLA2 activation in innate immune cells. Here, we provided the first evidence that γ T3 decreases cPLA2 activation against inflammation in macrophages (Fig 3B). Moreover, γ T3 treatment dampened the increase of DAG, another source of AA, suggesting the possibility that γ T3 may inhibit the phospholipase C (PLC) action to dephosphorylate PL into DAG. Although further verification is required to determine whether γ T3 inhibits cPLA2 and/or PLC, we demonstrated that γ T3 decreased the substrate availability for eicosanoids by limiting AA release (Fig 3, 4).

The released AA from membrane PL is subsequently metabolized to form a wide variety of oxygenated metabolites (eicosanoids) including prostaglandins and TXs by cyclooxygenase

(COX), and LTs by lipoxygenase (LOX). The uncontrolled formation of eicosanoids upon stimulation with FFA-mediated inflammation can be self-destructive and lead to irreversible damage [31]. In addition to reduced AA, we examined that γ T3 inhibits induction of *Cox2* (Fig 4B). Consistent with our results, there are lines of evidence that γ T3 suppresses *Cox2* expression in Raw macrophages [32, 33] likely through inhibition of NF κ B activation (reviewed in [34]). In fact, NF κ B signaling is the most critical and common denominator for both priming of the NLRP3 inflammasome and proinflammatory eicosanoid production. By using the agonists specific to TLR2 and 4 (Fig 4D and E), our results reinforce the notion that γ T3 attenuates the TLR2/TLR4-NF κ B signaling axis activation, resulting in reduced NLRP3 inflammasome activation as well as proinflammatory eicosanoid production.

It is notable that emerging research indicates the positive feedback loop between biosynthesis of PGE₂ and innate immune responses; 1) the inhibition of COX2 activity by Celecoxib abolished NLRP3 inflammasome activation [35], and 2) the enhanced COX2 activity and PGE₂ production are required for priming of pro-IL-1 β [36]. Considering this intimate relationship, the ability of γ T3 to decrease PGE₂ production may be a fundamental mechanism that γ T3 inhibits NLRP3 inflammasome activation. In addition to the reduced NF κ B activation, AA release, and PGE₂ production, other lipidomic changes induced by γ T3 may contribute to attenuation of NLRP3 inflammasome activation. In support of our notion, Scholz et al. proposed that an increase of LysoPC triggers caspase-1 activation and NLRP3 inflammasome activation in microglia [37]. Based on this study, decreased LysoPC by γ T3 could be an additional mechanism to downregulate NLRP3 inflammasome activation.

It has been demonstrated that TLR4 signaling in combination with palmitate upregulates ceramide synthesis [38]. As we expected, ceramide content was considerably increased by combined stimuli of LPS/pal in our experimental setting, while γ T3 significantly decreased ceramide content in both basal and stimulated conditions (Fig 1B, 5A). It is consistent with the reports that γ T3 inhibits *de novo* synthesis of ceramide by inhibiting the dihydroceramide desaturase activities in various cancer cells without altering mRNA levels [13, 39, 40]. Unlike the previous cancer cell studies, γ T3 significantly downregulated the LPS/pal-induced transcriptional augmentation of serine-palmitoyl transferase and dihydroceramide desaturase in BMDM (Fig 5B). Although the crosstalk of sphingolipids and inflammasome has been well-known [41], the role of ceramide in regulating obesity-mediated inflammasome is controversial; Camell et al. showed that *de novo* synthesis of ceramide is not necessary for inflammasome-driven inflammation and insulin resistance [42]. By using the primary BMDM, we demonstrated that γ T3 can decrease SFA-mediated ceramide formation by transcriptional activation, but its impact on macrophage metabolism and immunity may require further verification.

The energy sensing mechanism is intertwined with immune responses in innate immune cells such as macrophages [21, 22]. In our studies, γ T3 treatment was effective in sustaining ATP production capacity against inflammation (Fig 6). We propose that protection of energy metabolism in macrophage would be another important mechanism by which γ T3 suppresses aberrant innate immune responses. The PC and PE are the two major PL, and PC/PE ratio is critical index for membrane integrity. The decreased PC/PE ratio, due to an

ineffective conversion of PE to PC and the corresponding accumulation of PE, promotes membrane permeability and influences energy metabolism [43, 44]. In this regard, we had the very interesting observation that PC/PE molar ratio was significantly different in the presence of γ T3; PC/PE ratio was decreased upon LPS/pal stimulation from 10.4 ± 0.5 (unstimulated) to 8.3 ± 1.9 (LPS/pal). In contrast, γ T3-treatment decreased PE content (PE) recovering the PC/PE ratio similar to the unstimulated range, 11.0 ± 0.6 (Fig 1, 2E). We speculate that reduced PC/PE ratio in the inflamed macrophage is responsible for reduced glycolytic flux and mitochondrial ATP production, which was partly rescued by γ T3. Although more research is warranted to identify the molecular targets to explain the specific reduction of PE by γ T3, our study provides insights into γ T3-mediated lipidomic changes, energy metabolism, and innate immunity.

In summary, shotgun lipidomic analysis revealed that γ T3 not only suppresses cytokine IL-1 β secretion but also interferes with lipid remodeling for eicosanoid production in BMDM. Moreover, γ T3 protects energy metabolism against inflammation, maintaining the ATP production capacity. Our results implicate that γ T3 possesses the translational potential to prevent and/or delay the pathogenic progression from obesity to type 2 diabetes by preventing pyroptotic cell death and disconnecting the propagation of lipotoxic damages. Based on our study, the inclusion of γ T3 as personalized medicine may be useful for those who are obese and susceptible to obesity-driven type 2 diabetes.

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Abbreviations

AA	arachidonic acid
ATM	adipose tissue macrophages
BMDM	bone marrow-derived macrophages
Cer	ceramide
COX2	cyclooxygenase 2
DAG	diacylglycerol
DAMP	damage-associated molecular pattern
Des	dihydroceramide desaturase
FFA	free fatty acid
γT3	gamma tocotrienol

GPL	glycerophospholipids
NLRP3	NOD-like receptor protein 3
LPS	lipopolysaccharide
LPS/pal	palmitate stimulation in LPS-primed conditions
LysoPA	lysophosphatidic acid
LysoPC	lysophosphatidyl choline
LysoPE	Lysophosphatidyl ethanolamine
LysoPL	lysophospholipids
LysoPS	lysophosphatidyl serine
PA	phosphatidic acid
PC	phosphatidyl choline
PE	phosphatidyl ethanolamine
PL	phospholipids
cPLA2	cytosolic phospholipase A2
PLC	phospholipase C
PGE₂	prostaglandin E ₂
Spt	serine-palmitoyl transferase
SM	sphingomyelin

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Figure 1. γ T3 distinctively modulated macrophage lipidome against SFA-induced NLRP3 inflammasome

Bone marrow derived macrophages (BMDM) were stimulated for NLRP3 inflammasome by LPS/pal with or without γ T3 pretreatment. Then, the total cellular lipids were extracted and subjected to LC-MS/MS for shotgun lipidomic analysis. (A) IL-1 β gene expression and its secretion to medium were measured in the same samples for lipidomic analysis by qPCR and ELISA, respectively (n=6 per group). (B) Heatmap representation of the lipid profile changes in response to LPS/pal stimulation with or without γ T3 treatment (n=4 each group). The content of each lipid (nmol/mg protein) was color-coded from green (low) to red (high). Ranges of lipid classes were placed to the right. (C) Principal component analysis (PCA) scatterplot obtained from shotgun LC-MS/MS spectra of BMDM lipid extracts. Values are presented as mean $\pm$ SEM in (A). Bars with different letters are significantly different (a>b>c, P<0.05) by one-way ANOVA. **Abbreviation used:** FFA: free fatty acids, MAG: monoacylglyceride, DAG: diacylglyceride, TAG: triacylglyceride, PA: phosphatidic acid, PC: phosphatidyl choline, PE: phosphatidyl ethanolamine, PG: phosphatidyl glycerol, PI: phosphatidyl inositol, PS: phosphatidyl serine, LPA: lysophosphatidic acid, LPC: lysophosphatidyl choline, LPE: lysophosphatidyl ethanolamine, LPG: lysophosphatidyl glycerol, LPI: lysophosphatidyl inositol, LysoPS: lysophosphatidyl serine, Cer: Ceramide, HexCer: hexosyl ceramide, SM: sphingomyelin, SGalCer: sulfategalactosylceramide

Figure 2. γ T3 altered the specific lipid profiles of DAG, LysoPL, PE, and arachidonic acid in NLRP3-inflammasome stimulated BMDM

BMDMs were stimulated with LPS/palmitic acid, and lipidomic analyses were performed using LC-MS/MS. (A) Concentrations of total lipid, total phospholipids (PL), and total Lysophospholipids (LysoPL). Changes in lipid species in monoacylglycerol (MAG) (B), Diacylglycerol (DAG) (C), Free fatty acids (FFA) (D), Phosphatidyl ethanolamine (E), Lysophospholipids (LysoPL) (F) in NLRP3 inflammasome-stimulated BMDM without (white) or with γ T3 (black) treatment. Data are shown as mean $\pm$ SEM (n=4). Bars with different letters are significantly different (P<0.05) by one-way ANOVA in A. *P<0.05 (LPS/pal vs. LPS/pal+ γ T3) by student's *t*-test in B-F.

Figure 3. γ T3 attenuated the release of free AA and its metabolites in the inflammasome-stimulated BMDM

(A) BMDMs were loaded with [³H]-AA overnight before stimulation with LPS/pal for 6 hours, then the release of AA and its metabolites to the medium was quantified by [³H]-radioactivity. Data are shown as mean $\pm$ SEM. Bars with different letters are significantly different ($P < 0.05$) by one-way ANOVA. (B) Western blot analysis of phosphor-cPLA2 as a marker for cPLA2 activation. (C) Schematic diagram to explain the arachidonic acid (AA) generation in FFA-stimulated inflammatory conditions. The exogenous FFA will be activated to acyl CoA and incorporated into PL. Subsequently, PL are hydrolyzed to LysoPL and AA by cPLA2 (de-acylation), or de-phosphorylated into DAG by PLC. Dotted line is showing the regeneration of PL from AA (re-acylation) in the unstimulated conditions. The increased levels of AA are used for production of eicosanoids.

Figure 4. γ T3 attenuated *Cox2* induction in BMDM against toll-like receptor agonist 2 and 4
 Bone marrow derived macrophages (BMDM) were stimulated for NLRP3 inflammasome by LPS/pal with or without pretreatment of γ T3. (A) PGE₂ secretion to the medium by ELISA. (B) mRNA expression levels of *Cox1*, *Cox2* and *5Lox* upon LPS/pal stimulation. (C) Schematic representation to explain the inhibition of TLR2/4-NF κ B axis by γ T3 resulting in decreased *Cox2* induction and corresponding PGE₂ production. (D) mRNA expressions of *Cox2*, *Tlr4* and *Il-1 β* upon stimulation with KLA (100ng/mL), TLR4 agonist, for 3hr. (E) mRNA expressions of *Cox2*, *Tlr2*, and *Il-1 β* upon stimulation with Pam3Csk4 (200ng/mL), TLR2 agonist. (Data are shown as mean $\pm$ SEM (n=3). Bars with different letters are significantly different (P<0.05) by one-way ANOVA.

Figure 5. γ T3 modulated sphingolipid metabolism in the inflammasome-stimulated BMDM
 (A) The cellular content of total SPL and Cer. (B) The mRNA levels of key enzymes for Ceramide biosynthesis, serine palmitoyl transferase (*Spt*) and dihydroceramide desaturase (*Des*). (C) The schematic presentation to show γ T3 decreases the *de novo* ceramide formation by transcriptional repression of key *Spt* and *Des*. Data are shown as mean $\pm$ SEM. Bars with different letters are significantly different ($P < 0.05$) by one-way ANOVA. **CerS**, ceramide synthase; **DES**, dihydroceramide desaturase; **dhCer**, dihydroceramide; **dhSph**, dihydrophingosine; **sph**, sphingosine; **SPT**, serine palmitoyltransferase; **SM**, sphingomyelin.

Figure 6. γ T3 attenuated LPS-induced metabolic dysfunction in BMDM

BMDM treated with either vehicle, LPS, or LPS plus γ T3. (A) Changes in basal respiration rate and ATP production measured by Seahorse XF analyzer (average of three separate experiments, $n=5$ /experiment). (B) Glycolytic flux measured by Seahorse analyzer. Arrow indicates the addition of glucose, oligomycin, 2-deoxyglucose (2DG) (upper). The basal and oligomycin-stimulated glycolysis, and reserved glycolysis (%) were calculated (lower). Data are shown as mean $\pm$ SEM ($n=6$ per group). Bars with different letters are significantly different ($P<0.05$) by one-way ANOVA.