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Research Article

Tributyltin Enhances Macrophage Inflammation and Lipolysis, Contributing to Adipose Tissue Dysfunction

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Abstract

Tributyltin (TBT) is a synthetic chemical widely used in industrial and commercial applications. TBT exposure has been proven to elicit obesogenic effects. Gestational exposure led to increased white adipose tissue depot size in exposed (F1, F2) animals and in unexposed generations (F3, F4), an example of transgenerational inheritance. TBT exerts these effects in part by increasing the number and size of white adipocytes, altering the fate of multipotent mesenchymal stromal stem cells to favor the adipocyte lineage, altering adipokine secretion, and modulating chromatin structure. Adipose tissue resident macrophages are critical regulators in adipose tissue; however, the effects of TBT on adipose tissue macrophages remained unclear. Here we investigated the effects of TBT on macrophages and consequent impacts on adipocyte function. TBT significantly enhanced palmitate-induced inflammatory gene expression in mouse bone marrow derived macrophages and this effect was attenuated by the antagonizing action of the nuclear receptor peroxisome proliferator activated receptor gamma. TBT-treated macrophages decreased lipid accumulation in white adipocytes differentiated from mesenchymal stromal stem cells accompanied by increased expression of lipolysis genes. Lastly, ancestral TBT exposure increased *Tnf* expression in adipose tissue resident macrophages in both exposed (F2) and unexposed (F3) generations, suggesting that TBT exposure led to an inherited predisposition toward inflammatory adipose tissue macrophages that can manipulate adipose tissue function. These findings provide new insights into the interplay between adipocytes and adipose tissue macrophages in obesity, further establishing a role for obesogens such as TBT in the development of obesity-related metabolic disorders.

Key Words: obesity, tributyltin, TBT, adipocyte, macrophage, peroxisome proliferator activated receptor gamma, PPARγ, lipolysis

Obesity is a worldwide public health issue resulting from a variety of factors, but energy imbalance is generally considered to be a major driving force [1]. Compelling evidence suggests that obesity is a critical contributor to the development of adipose tissue inflammation and insulin resistance [2, 3], 2 major causal factors in the pathogenesis of obesity-associated type 2 diabetes (T2D) [4]. Adipose tissue plays an important role in regulating obesity-associated health issues and is intricately connected with various metabolic factors including insulin sensitivity, lipid metabolism, and inflammation. Therefore, it is important to delve further into the complexity of adipose tissue biology.

Several lines of evidence suggest that disrupted energy balance alone cannot fully account for the obesity pandemic. A growing number of studies have established links between exposure to endocrine-disrupting chemicals and the obesity pandemic [5-7]. One subset of endocrine-disrupting chemicals, obesogens, increased white adipocyte number and/or size and consequently promoted adiposity [8]. The obesogens tributyltin (TBT) and triphenyltin activate the so-called master regulator of adipogenesis, peroxisome proliferator activated receptor gamma (PPARγ), and its heterodimeric partner, the retinoid X receptor (RXR) [9-12] to promote adipogenesis at low nanomolar levels in human and mouse multipotent mesenchymal stromal stem cells (MSCs) in vitro [12, 13]. We also showed that exposure of pregnant F0 mouse dams to TBT biased MSCs toward the adipose lineage in F1 offspring [13] and increased lipid accumulation in liver, testis, and adipose depots in F1, F2, and F3 generations [8]. Interestingly, male F4 descendants of TBT-treated pregnant F0 dams exhibited a large increase in fat storage compared with controls when fed with a diet containing modestly increased fat content whereas females did not [14].

Chronic inflammation can lead to or exacerbate life-threatening conditions such as T2D and cardiovascular disease (CVD). The adipose tissue niche, containing preadipocytes, adipocytes, and immune cells, is crucial in maintaining systemic metabolic balance and immune response in the white adipose tissue (WAT) [15-17]. Adipose tissue macrophages (ATMs) interact with neighboring adipocytes via cell-cell communication, thus impacting adipogenesis in either a positive or negative way. It is generally agreed that classically activated (M1) ATMs inhibit adipogenesis by secreting proinflammatory factors [15, 18].

Compelling evidence suggests that obesity is a critical contributor to the development of adipose tissue inflammation and insulin resistance, 2 major causal factors to the

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pathogenesis of obesity-associated T2D and CVDs [19-21]. Therefore, adipose tissue is believed to play a crucial role in regulating obesity-associated health issues. ATMs are an major population of resident immune cells in WAT, accounting for more than 50% of the total stromal cell population in individuals with obesity, and are crucial regulators of adipose tissue function [17]. Increased numbers of ATMs in obese adipose tissues are important contributors to obesity-related metabolic syndromes. Furthermore, ATMs display a wide range of activation states in responding to local microenvironmental cues. These include primarily anti-inflammatory states (alternative activation, M2) in the lean adipose tissue and a dominant proinflammatory state (classic activation, M1) in the obese tissue [20, 22]. Proinflammatory factors such as TNF-α and excess fatty acids such as palmitate induce M1 response by activating TLR4/NF-κB, JNK, and other pathways in ATMs [23]. In contrast, Th2 cytokines, such as IL-4 and IL-13, stimulate the alternative activation of ATMs [24].

ATMs are also critical regulators of adipocyte function. For example, classically activated M1 macrophages cause inflammation in adjacent adipocytes by secreting proinflammatory cytokines that impair insulin signaling in nearby adipocytes, leading to insulin resistance, whereas alternatively activated M2 macrophages exert anti-inflammatory functions and contribute to tissue remodeling/repairing [25, 26]. This interaction forms a feedback loop, perpetuating inflammation and contributing to conditions such as T2D and CVD. Understanding this crosstalk is crucial for fully understanding the links between obesity, inflammation, and metabolic dysfunction. Exploring these interactions is critical to identify potential.

Here we showed that exposure to the obesogen TBT induced palmitic acid-activated macrophage inflammation through the PPARγ pathway, leading to and enhancing lipolysis in surrounding adipocytes. Furthermore, we found that treatment of pregnant F0 mouse dams with TBT led to elevated levels of inflamed macrophages in the WAT of male descendants through the F3 generation. This demonstrated an important role for TBT and PPARγ signaling in the transgenerational inheritance of predisposition to inflammation in WAT.

Materials and Methods

Chemicals and Reagents

TBT, LG100268, HX531, UVI3003, dexamethasone, isobutylmethylxanthine, insulin, Nile Red, and Hoechst 33342 were purchased from Sigma-Aldrich (St. Louis, MO). T0070907 was purchased from Enzo Life Sciences (Farmingdale, NY). Rosiglitazone was purchased from Cayman Chemical (Ann Arbor, MI). Mouse Leptin ELISA Kit (#90080, RRID: AB_2783626) was purchased from Crystal Chem (Elk Grove Village, IL).

Cell Culture

Bone marrow derived hematopoietic stem cells from C57BL/6J mice were seeded in presence of L-929 conditioned medium continuously for 7 days to induce differentiation into macrophages [27]. Bone marrow derived multipotent MSCs from the long bones of C57BL/6J mice (MSCs) were purchased at passage 6 (OriCell; Cyagen Biosciences, Santa Clara, CA) and stored at passage 8 or 9 in liquid N_2. Cells were maintained, as previously described [8], in DMEM containing 10% calf bovine serum, 10 mM HEPES, 1 mM sodium pyruvate, 100 IU/mL penicillin, and 100 µg/mL streptomycin [12]. MSCs were plated at 60,000 cells/cm^2 in 12-well cell culture plates for adipogenesis assays. Cells were allowed to attach and acclimate for 24 hours prior to 48 hours of chemical treatment or for conditioned media collection of adipogenesis experiment. Forty-eight-hour chemical treated macrophage was replenished with fresh media and supernatants were collected and saved for adipogenesis assay. The PPARγ antagonist T0070907 (50 nM), the RXR antagonists HX531 (100 nM) or UVI3003 (500 nM), or dimethyl sulfoxide (DMSO) vehicle control were added every 3 days. The amount of DMSO vehicle was kept at <0.1% in all assays.

Adipogenesis Assay

Once seeded cells reached 100% confluency in culture plates, cells were induced to differentiate with an adipose induction cocktail (500 µM isobutylmethylxanthine, 1 µM dexamethasone, and 5 µg/mL insulin) in macrophage conditioned media as previously described. Wells were replenished with fresh conditioned media, differentiation factors, and chemical ligands every 3 days. Cells were differentiated over the course of 14 days and then fixed in buffered 3.7% formaldehyde, followed by 1 wash with PBS for 1 minute, and then maintained at 4 °C in PBS overnight to remove residual phenol red from the culture medium. To quantify lipid accumulation, Nile Red (1 µg/mL) was used to stain neutral lipids and Hoechst 33342 (1 µg/mL) to stain DNA [28]. For each biological replicate, Nile Red relative fluorescence units (excitation/emission: 485/590 nm) were measured relative to Hoechst relative fluorescence units (excitation/emission: 355/460 nm) using a SpectraMax Gemini XS spectrofluorometer (Molecular Devices, Sunnyvale, CA) using SoftMax Pro (Molecular Devices) [12].

Quantitative PCR

Differentiated adipocytes were lysed with Trizol following the manufacturer's recommended protocol (ThermoFisher Scientific, MA) and total RNA recovered after isopropanol precipitation (Fisher Chemical, PA). Gene expression was assessed with real-time quantitative PCR (RT-qPCR) using SYBR™ Green PCR Master Mix (Thermo Fisher Scientific, Waltham, MA) on a Roche LightCycler 480 II (Roche). Cycle threshold values were quantified as the second derivative maximum using LightCycler software (Roche). The $2^{-\Delta\Delta Ct}$ method [12] was used to analyze RT-qPCR data and determine relative quantification. Standard propagation of error was used throughout for each treatment group [29]. Error bars represent the SEM from 3 to 4 biological replicates, calculated using standard propagation of error [29].

Statistics

GraphPad Prism 7.0 (GraphPad Software, Inc.) was used to perform statistical analysis for all datasets. A one-way ANOVA followed by Dunnett's post hoc test was performed to compare the treatment group, palmitate (PA), TBT, or PA plus TBT, to DMSO control in gene expression qPCR analysis. In antagonist assays, treatment groups without antagonists were compared to a corresponding treatment group

treated with T0070907 or HX531 using Student's *t*-test. $P \leq .05$ was considered statistically significant.

Results

TBT Enhanced PA-induced Murine Macrophage Inflammatory Gene Expression

To study the effects of TBT on macrophage activation, we treated male mouse bone marrow derived macrophages with DMSO vehicle, 200 µM PA, 50 nM TBT, or PA plus TBT. The expression of mRNA encoding 4 inflammatory markers, *Tnf*, *Nos2*, *Stat1*, and *Scos3*, was measured in 24-hour-exposed macrophages. TBT showed a significant enhancement of PA-induced macrophage inflammatory marker gene expression (Fig. 1A-1D). We repeated this assay using the RAW293 mouse macrophage cell line and observed a similar effect of TBT on inducing PA-induced macrophage inflammation (Fig. 1E-1H).

TBT Enhanced PA-induced Macrophage Inflammatory Gene Expression Through PPARγ Activation

TBT acts through the nuclear receptor PPARγ and its heterodimeric partner RXR to promote adipogenesis in vitro and in vivo [8, 10, 12]. To elucidate the role of TBT-induced macrophage activation under PA stress and its pathway of action, cells were treated for 24 hours in the presence of the strong PPARγ antagonist T0070907 or the RXR antagonist HX531 in all groups. Treatment with T0070907 significantly suppressed TBT-enhanced PA-induced inflammatory gene expression whereas HX531 did not, revealing that the effects in PA-induced inflammation was strongly attenuated by antagonizing PPARγ (Fig. 2).

TBT Treated PA-activated Macrophages Exhibited Decreased Lipid Accumulation and Increased Lipolysis Gene Expression in MSCs

The interplay between macrophages and adipocytes in the adipose tissue niche has been an important model to study adipose tissue inflammation in obesity. To study the effect of TBT-affected inflammatory macrophages during adipogenesis, we performed adipogenesis assays on mouse MSCs in macrophage conditioned medium for 14 days. Conditioned media from PA-treated macrophage significantly suppressed lipid accumulation during differentiation of mouse adipocytes, and conditioned media from TBT exposed macrophage cultures further decreased the lipid content (Fig. 3A). We measured representative marker genes for adipogenesis (*Fabp4* and *Fsp27*) and lipolysis (*Pnpla2* and *Lipe*). No changes were observed in the expression of adipogenesis genes *Fabp4* (Fig. 3B) and *Fsp27* (Fig. 3C). We found that expression of PA-induced lipolysis genes *Pnpla2* (Fig. 3D) and *Lipe* (Fig. 4E) was significantly induced by conditioned media from TBT-exposed macrophage cultures. We infer that exposure to TBT-treated macrophage conditioned medium led to increased adipose tissue lipolysis by targeting ATMs.

Ancestral TBT Exposure Increased TNF Expression in Adipose Macrophages

The effects of prenatal TBT treatment on fat depot size have been reported as transgenerational and detectable in the F1, F2, F3, and F4 descendants of F0 mouse dams exposed during pregnancy [8] or during pregnancy and lactation [14], whereas rosiglitazone was unable to elicit transgenerational effects on fat depot size [8]. The role of ATMs in mediating some effects of ancestral TBT exposure was unclear. We sought to examine the phenotypes of ATMs in the offspring of F0 dams treated with TBT or DMSO vehicle control. After purifying macrophages from WAT stromal cells using the adhesion selection method, we measured TNF-α gene expression and cytokine secretion to determine the inflammatory status of ATMs. We found that WAT-derived macrophages from F2 and F3 male offspring of TBT group dams showed increased levels of *Tnf* mRNA (Fig. 4A) as well as TNF cytokine (Fig. 4C). In addition to TNF expression, *Il1b* mRNA (Fig. 4B) expression and levels of IL1B cytokine (Fig. 4D) were measured; these echoed the TNF results (Fig. 4B and D). In addition to proinflammatory cytokine profiles, we measured anti-inflammatory makers *Tgfb* and *Il10* and detected no significant difference between DMSO or TBT ancestral exposed ATMs (Fig. 4E-H). These results are consistent with the possibility that ancestral TBT exposure produced a chronic inflammatory state in WAT macrophages that, in turn, manipulated WAT function.

Discussion

This study aimed to investigate the effects of TBT on macrophage activation and the subsequent impact on white adipocyte function. We first treated male mouse bone marrow derived macrophages with PA, TBT, or PA plus TBT and measured the expression of 4 inflammatory genes. The results showed that TBT significantly enhanced PA-induced expression of proinflammatory genes in macrophages. The same effect was observed in the RAW293 mouse macrophage cell line. We also found that TBT-induced macrophage inflammation was attenuated by antagonizing PPARγ, suggesting that TBT acts through this nuclear receptor to promote macrophage activation under PA stress.

To further study the interplay between macrophages and adipocytes, we performed adipogenesis assays on mouse MSCs using macrophage-conditioned medium. Strikingly, TBT exposure significantly heightened the expression of proinflammatory genes in macrophages already under the influence of PA. This effect was not confined to bone marrow-derived macrophages alone; we replicated it in the RAW293 mouse macrophage cell line. Intriguingly, our findings indicated that TBT-induced macrophage inflammation could be curbed by interfering with the nuclear receptor PPARγ, suggesting that TBT acted at least in part via this receptor, thereby promoting macrophage activation under PA-induced stress.

To gain a deeper understanding of the interplay between macrophages and white adipocytes—the chief constituents of WAT—we conducted adipogenesis assays using mouse MSCs exposed to macrophage-conditioned medium. We found that PA-induced macrophage-conditioned medium exerted a substantial inhibitory effect on lipid accumulation during the differentiation of mouse adipocytes. Adding a layer of complexity, TBT treatment further decreased lipid content within these differentiating adipocytes. However, this effect was not associated with alterations in lipogenesis gene expression. Instead, TBT appeared to bolster the breakdown of lipids, primarily via upregulating expression of lipolysis genes. We infer that TBT can augment adipose tissue lipolysis by targeting and inducing an inflammatory state within macrophages residing in WAT.

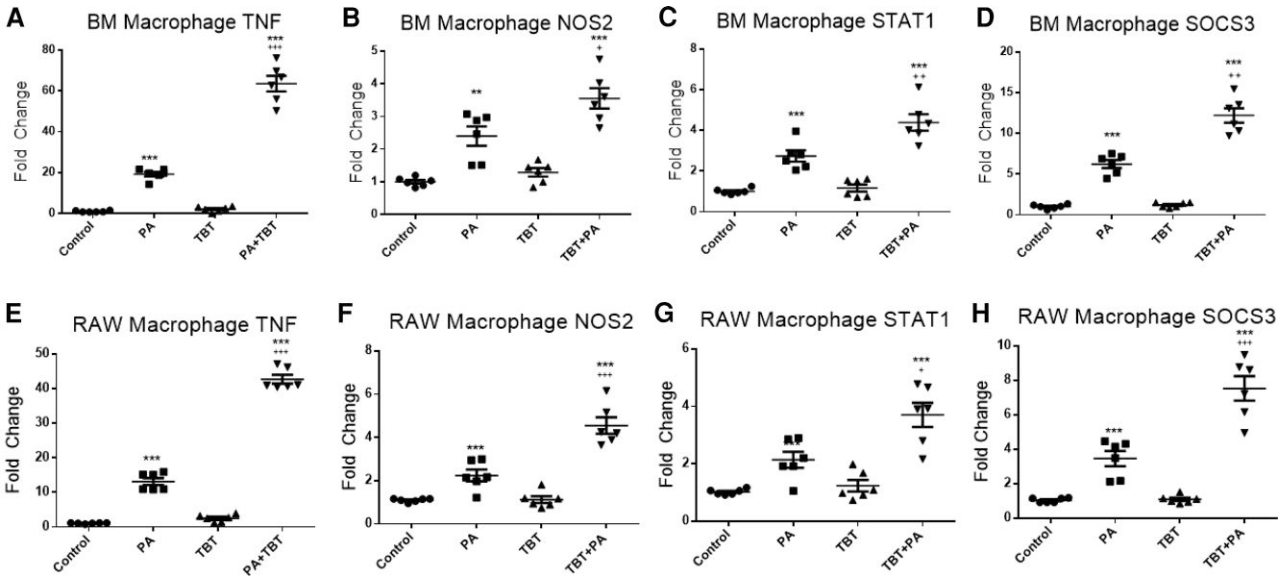

Figure 1. TBT enhanced PA-induced murine macrophage inflammatory gene expression. Mouse bone marrow derived macrophage was treated with DMSO vehicle, 200 μM PA, 50 nM TBT, or PA plus TBT. The expression of mRNA encoding 4 inflammatory markers, (A) *Tnf*, (B) *Nos2*, (C) *Stat1*, and (D) *Scos3,* were measured in 24-hour-exposed macrophages. RAW264.7 macrophage was treated with DMSO vehicle, 200 μM PA, 50 nM TBT, or PA plus TBT. The expression of mRNA encoding 4 inflammatory markers, (E) *Tnf*, (F) *Nos2*, (G) *Stat1,* and (H) *Scos3,* were measured in 24-hour-exposed macrophages. For the quantitative RT-PCR analyses, measured Ct values were normalized to *36b4* and graphed relative to the control treatment. Error bars represent SEM $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ comparing to control group and $^{+}P < .05$, $^{++}P < .01$, and $^{+++}P < .001$ comparing to PA group. Data analyzed using either an unpaired *t*-test or a one-way ANOVA followed by Dunnett's post hoc analysis.

Abbreviations: DMSO, dimethyl sulfoxide; PA, palmitate; TBT, tributyltin.

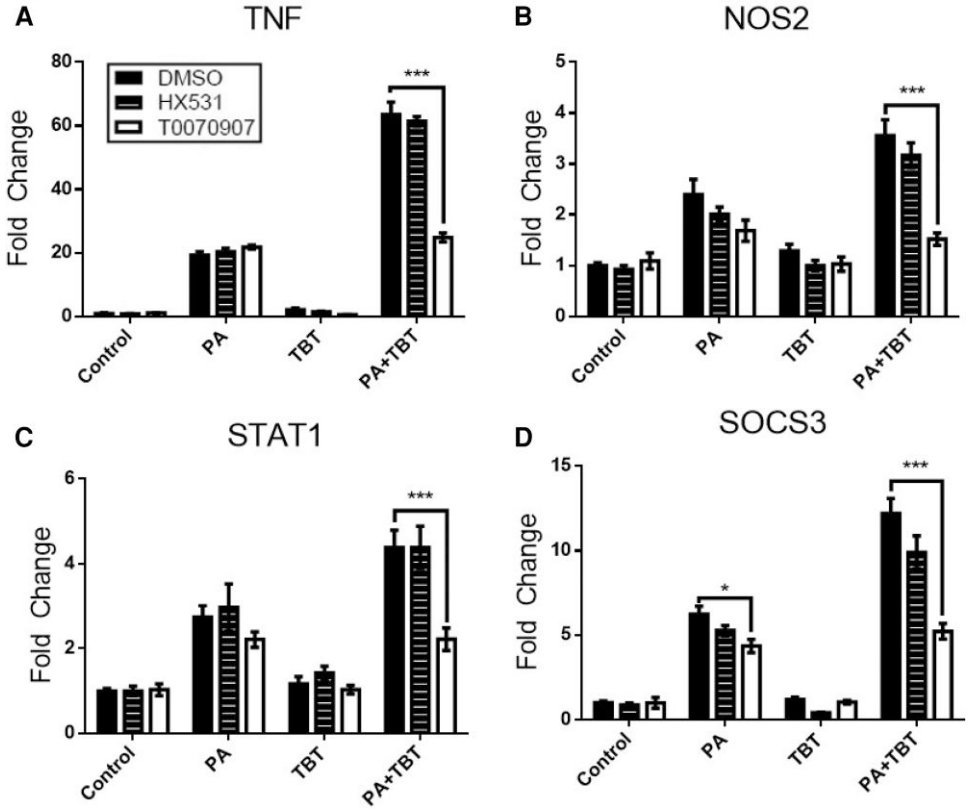

Figure 2. TBT enhanced PA-induced macrophage inflammatory gene expression through PPARγ activation. Mouse bone marrow derived macrophage was treated for 24 hours in the presence of PPARγ antagonist T0070907 or the RXR antagonist HX531 in all groups and the expression inflammatory genes (A) *Tnf*, (B) *Nos2,* (C) *Stat1,* and (D) *Scos3* were measured by quantitative RT-PCR analyses. Measured Ct values were normalized to *36b4* and graphed relative to the control treatment. Error bars represent SEM $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$. Data analyzed using either an unpaired *t*-test or a one-way ANOVA followed by Dunnett's post hoc analysis.

Abbreviations: PA, palmitate; PPARγ, peroxisome proliferator activated receptor gamma; RXR, retinoid X receptor; TBT, tributyltin.

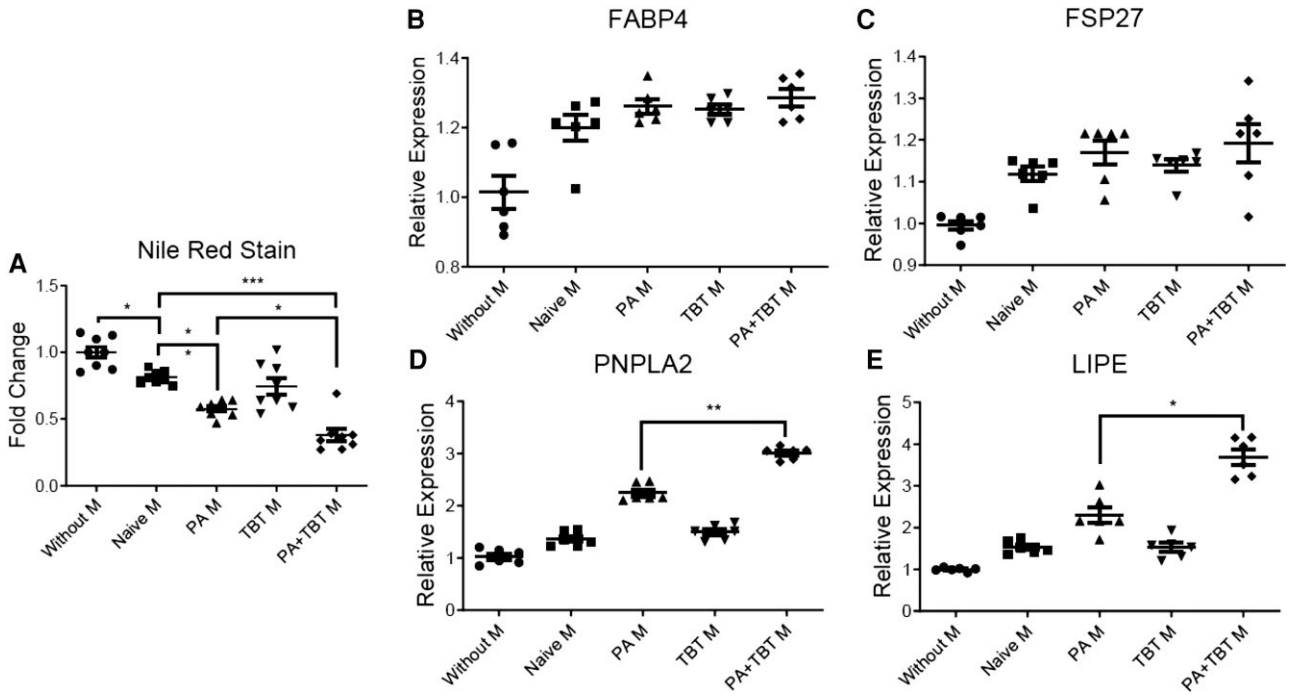

Figure 3. TBT treated PA-activated macrophage decreased MSC lipid accumulation and increased lipolysis genes. A standard adipogenesis assay was performed in MSCs using macrophage conditioned media. All treatment groups were compared to control, the media without prior macrophage exposure. (A) Lipid accumulation is shown as the ratio between fluorescence units of Nile Red and Hoechst, which were used to quantify lipid content and the number of cells per well, respectively. Quantitative RT-PCR validation of lipogenesis genes (B) *Fabp4*, (C) *Fsp27*, (D) *Pnpla2*, and (E) *Lipe*. Error bars represent SEM $*P < .05$, $**P < .01$, and $***P < .001$. Data analyzed using either an unpaired *t*-test or a one-way ANOVA followed by Dunnett's post hoc analysis.

Abbreviations: MSC, mesenchymal stromal stem cell; PA, palmitate; TBT, tributyltin.

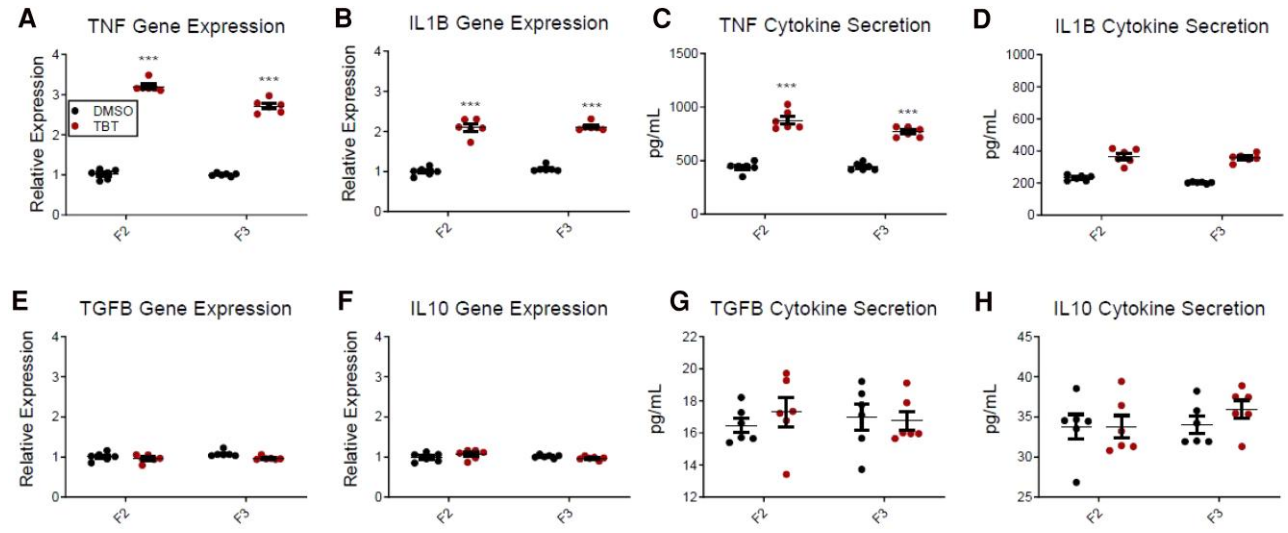

Figure 4. Ancestral TBT exposure induced inflammatory marker but not anti-inflammatory marker expression and secretion by primary adipose macrophages. Adipose tissue resident macrophage was purified from gonad white adipose tissue and inflammatory gene (A) *Tnf* and (B) *Il1b,* and anti-inflammatory gene (E) *Tgfb* and (F) *Il10* were measured by quantitative RT-PCR assay. In addition, inflammatory cytokine (C) *Tnf* and (D) *Il1b* and anti-inflammatory cytokine (G) TGF-beta and (H) IL10 secretion was measured by ELISA. Error bars represent SEM $*P < .05$, $**P < .01$, and $***P < .001$. Data analyzed using either an unpaired *t*-test or a one-way ANOVA followed by Dunnett's post hoc analysis.

Abbreviations: TBT, tributyltin.

While PPARγ activation is traditionally associated with anti-inflammatory effects in macrophages, our findings revealed that TBT enhanced PA-induced inflammation, highlighting a context-dependent effect. This discrepancy may stem from the interaction between TBT, PPARγ, and additional signaling pathways activated by inflammatory stimuli such as PA. Previous studies have shown that PPARγ activation can have varying effects depending on the presence of different ligands and coregulatory proteins [30]. It is possible that TBT modifies the typical anti-inflammatory role of PPARγ under certain conditions, such as in the presence of fatty acids like PA, driving a proinflammatory response. TBT is also only a partial agonist of

PPARγ that has been shown to produce dysfunctional adipocytes from MSCs, so this may also be a contributing factor to the discrepancy [12]. Further studies are needed to dissect the precise molecular mechanisms governing this dual role of PPARγ in macrophages exposed to TBT and PA. Our findings that the PPARγ antagonist T0070907 effectively reduced TBT-induced macrophage inflammation, while the RXR antagonist HX531 did not, are consistent with the possibility that TBT may induce PPARγ activity through mechanisms that are independent of RXR activation. Although PPARγ typically forms a heterodimer with RXR to exert its effects, certain gene transrepression functions or alternative pathways may allow PPARγ to act independently [31].

We also investigated the effects of ancestral TBT exposure on ATMs using our established diet-induced obesity mouse model. We found that the descendants of TBT-treated dams showed increased *Tnf* gene expression and cytokine secretion in purified ATMs. This indicated that ancestral TBT exposure led to a proinflammatory state in WAT-resident macrophages in both exposed (F2) and unexposed (F3) generations.

The in vivo and in vitro effects of TBT exposure might seem contradictory since gestational TBT exposure increased WAT depot size in F1, F2, and transgenerationally in F3 and F4 animals, whereas our in vitro results showed decreased lipid accumulation and increased lipolysis gene expression in MSC-derived adipocytes. However, these observations likely represent different aspects of TBT action at various levels of adipose tissue regulation. In the in vivo context, TBT exposure leads to systemic metabolic changes, including alterations in the endocrine environment and possibly epigenetic modifications across generations. These long-term effects could predispose offspring to increased adiposity and changes in energy balance, particularly under specific dietary conditions, as seen in the F1-F4 generations.

In contrast, the in vitro effects observed in MSC-derived adipocytes exposed to TBT-treated macrophage-conditioned medium reflect a more immediate response, where TBT-induced macrophage inflammation promotes lipolysis. This proinflammatory environment could be driving lipolysis in vitro, whereas in vivo, the chronic low-grade inflammation and systemic metabolic effects of TBT exposure may contribute to overall adiposity. These findings suggest that TBT has multifaceted effects on adipose tissue, influencing both adipogenesis and lipolysis depending on the context, timing, and cellular interactions, which underscores the complexity of obesogen action.

Overall, our study supports a model in which TBT can enhance PA-induced macrophage inflammation and increase adipose tissue lipolysis, potentially contributing to obesity-induced inflammation. Further research is needed to fully understand the mechanisms underlying these effects and to explore potential interventions to mitigate the negative impact of exposure to TBT or other obesogens on WAT function. Our study adds to the growing body of literature highlighting potential negative impacts of environmental pollutants on obesity and other aspects of human health and underscores the need for greater awareness and regulation of environmental toxicants to protect public health and prevent long-term negative consequences.

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Disclosures

B.B. is a named inventor on US patents 5,861,274, 6,200,802, 6,815,168, and 7,250,273 related to PPARγ. All other authors declare they have nothing to disclose.

Data Availability

Original data generated and analyzed during this study are included in this published article or in the data repositories listed in References.

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