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Inhibition of perilipin 2 expression reduces pro-inflammatory gene expression and increases lipid droplet size

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Abstract

Our lab previously demonstrated that triglyceride-rich lipoprotein (TGRL) lipolysis products induce lipid droplet formation and pro-inflammatory gene expression in monocytes. We hypothesized that the inhibition of perilipin 2 expression in THP-1 monocytes would reduce lipid droplet formation and suppress pro-inflammatory gene expression induced by TGRL lipolysis products. In the current study, we use microarray analysis to identify gene expression altered by TGRL lipolysis products in THP-1 monocytes. We confirmed the expression of selected genes by quantitative reverse transcription PCR and characterized lipid droplet formation in these cells after exposure to TGRL lipolysis products. Using siRNA inhibition of perilipin 2 expression, we examined the role of perilipin 2 in the response of THP-1 monocytes to TGRL lipolysis products. We found that perilipin 2 siRNA increased the intracellular triglyceride content, increased the size of lipid droplets, and reduced pro-atherogenic and pro-inflammatory gene expression. We saw a reduction of serum/glucocorticoid kinase 1, v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian), chemokine (C-C motif) ligand 3, and interleukin 8 gene expression induced by TGRL lipolysis products. This study supports previous findings that reduction of perilipin 2 expression is protective against atherogenesis, while finding an unexpected increase in lipid droplet size with reduced perilipin 2 expression.

Graphical Abstract

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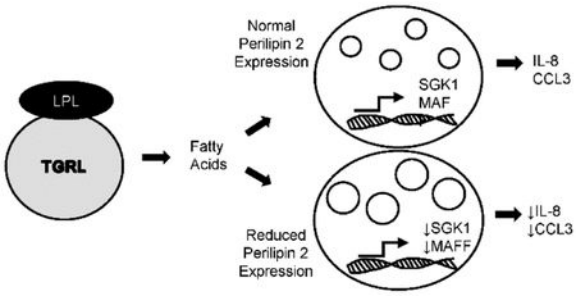
Conflicts of Interest

There are no conflicts to declare.



Reduced perilipin 2 expression modifies the response of THP-1 monocytes to TGRL lipolysis product exposure.

Introduction

Hypertriglyceridemia has been shown to be a risk factor for atherosclerosis, which can be modified by diet[1]. Triglycerides are contained within triglyceride-rich lipoproteins (TGRL) in the blood[2], which consist of chylomicrons produced by the intestine, and VLDL produced by the liver[2]. The enzyme lipoprotein lipase (LPL) hydrolyzes triglycerides within TGRL, releasing primarily free fatty acids (hereafter referred to as lipolysis products)[3]. Monocytes, the precursors to the lipid filled foam cell macrophages in the core of atherosclerotic plaques[4], form lipid droplets and exhibit an inflammatory phenotype when exposed to lipolysis products[5, 6].

Lipid droplet formation is a key event in the development of foam cells[4]. Atherosclerotic plaques have been shown to have increased gene expression of perilipin 2 (also referred to as adipophilin or adipose differentiation-related protein), a lipid droplet associated protein[7, 8]. Perilipin 2 has been shown to be increased in monocytes and macrophages after incubation with modified LDL, VLDL, TGRL, and fatty acids[9–13]. In addition, studies have indicated that perilipin 2 enhances uptake of long chain fatty acids, incorporation of fatty acids into triglycerides, and inhibition of β-oxidation[12, 14]. Inhibition of perilipin 2 expression by siRNA has been shown to reduce lipid droplet formation in THP-1 derived macrophages in response to acetylated LDL and VLDL[7, 12]. Additionally, reduction of perilipin 2 expression in THP-1 derived macrophages was shown to reduce the expression of pro-inflammatory cytokines after exposure to acetylated LDL[15]. Further, lack of perilipin 2 in germline or in bone marrow alone has been shown to reduce atherosclerotic plaque formation in a mouse model of atherosclerosis[16]. Although there is ample literature on the role of perilipin 2 in THP-1 derived macrophages differentiated with phorbol 12-myristate 13-acetate (PMA), little work has been done on its role in undifferentiated THP-1 monocytes. Further, to our knowledge, although studies have examined the role of perilipin 2 in macrophage response to acetylated LDL, whole VLDL, and whole TGRL, no studies have looked at the role of perilipin 2 in monocyte or macrophage response to TGRL lipolysis products.

Our laboratory has previously shown that lipolysis products induce lipid droplet formation and activate inflammatory pathways in monocytes[5, 6]. We wanted to further address the relationship between lipolysis product-induced lipid droplets and inflammatory pathways in

monocytes. In this study, we first used a microarray analysis to explore the effects of TGRL lipolysis products on undifferentiated THP-1 cells using an unbiased approach. Next, we examined the role of perilipin 2 in the response to TGRL lipolysis products using perilipin 2 siRNA. We hypothesized that the inhibition of perilipin 2 expression would reduce lipid droplet formation and suppress pro-inflammatory gene expression induced by TGRL lipolysis products.

Experimental Materials and Methods

TGRL isolation and generation of lipolysis products

This study adheres to all federal and state regulations related to the protection of human research subjects. The protocol was approved by the University of California, Davis Institutional Review Board (IRB ID: 447043). The University of California, Davis Federalwide Assurance Number with the Department of Health & Human Services is 00004557. Informed consent was obtained from all participants.

Healthy human volunteers, ages 18 and older, were recruited on the University of Davis, California campus. They were asked to eat a moderately high fat meal of their choosing. Blood was drawn by venipuncture into K_2EDTA containing Vacutainer tubes (Becton Dickinson) approximately 3.5 hours after consumption of the meal. Plasma was isolated from whole blood by centrifugation. TGRL were isolated from the plasma (pooled from multiple participants) by centrifugation at 40,000 rpm for 18 h at 14°C using a SW41 Ti swinging bucket rotor (Beckman Coulter) in a Beckman L8–70 M ultracentrifuge. Lipoproteins with a density less than 1.0063 g/mL were collected. The TGRL was collected and dialyzed in Spectrapor membrane tubing (molecular weight cut off 3,500; Spectrum Medical Industries) at 4°C overnight against a saline solution containing 0.01% EDTA. Bovine milk LPL (Sigma) was used for generation of lipolysis products. Lipolysis products were generated by incubating TGRL containing 150 mg/dL triglyceride with 2 U/mL LPL in normal culture media at 37°C for 30 minutes prior to addition to the cells. This procedure has been used by our lab to study the effects of TGRL lipolysis products on various cell types[3, 17–23].

Cell culture and treatment

THP-1 cells (ATCC) were maintained in RPMI 1640 containing 2 mM L-glutamine, 10 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, and 1500 mg/L sodium bicarbonate (ATCC), supplemented with 10% fetal bovine serum (Hyclone Characterized Fetal Bovine Serum, US Origin SH3007103), 10 μg/mL Gentamicin, 0.25 μg/mL Amphotericin B and 0.05 mM 2-mercaptoethanol. During growth and treatment cells were maintained as suspensions incubated in 5% CO_2 and 95% air at 37°C. Cells were maintained at a density of 200,000 – 800,000 cells/mL for growth. All experiments were performed on cells less than 50 passages from the original vial provided by ATCC. All treatments without siRNA modification were performed at a concentration of 1,000,000 cells/mL for 3 hours. Treatments were prepared in normal culture media as follows: media control (normal culture media only), LPL (2 U/mL LPL in normal culture media), TGRL (150 mg/dL TGRL in normal culture media), and lipolysis products (150 mg/dL TGRL and 2 U/mL LPL in

normal culture media). These treatments were incubated at 37°C for 30 minutes prior to addition to cells.

siRNA inhibition of perilipin 2 expression

THP-1 cells were transfected with dicer substrate siRNA to perilipin 2 (Integrated DNA Technologies) or a non-targeting sequence, Negative Control DS ScrambledNeg (Integrated DNA Technologies), hereafter referred to as scramble siRNA, using HiPerFect transfection reagent (Qiagen) for 48 hours prior to treatment. The sequences of the dicer substrate siRNA are shown in Table 1. The first 6 hours of the transfection was carried out without serum in a concentrated state (2.5 million cells/mL, 125 nM siRNA, and 1:20 HiPerFect). After 6 hours, normal culture media was added bringing the concentration to 500,000 cells/mL, 25 nM siRNA, and 1:100 dilution of HiPerFect and the cells were transfected for an additional 42 hours. The scramble siRNA or perilipin 2 siRNA transfected cells were treated in fresh culture media with or without TGRL lipolysis products for 3 hours.

Lipid droplet staining and imaging

After treatment, cells were washed once with 37°C PBS, fixed in 4% paraformaldehyde for 15 minutes, washed once again with PBS, then stained with nile red (Sigma). The nile red was kept in a saturated solution in acetone at −20°C until use, this stock solution was then diluted 1:1000 in PBS for staining. Cells were incubated in the nile red staining solution for 45 minutes. All wash steps were completed by bringing up volume with PBS to 50 mL, then pelleting cells. After staining, cells were pelleted and resuspended in 7 μL prolong gold containing DAPI (Molecular Probes) and mounted on slides with a coverslip. Lipid droplets were imaged using a 63x objective on a Leica DMIL microscope (Leica) with a X-cite series 120q fluorescent light source (Lumen Dynamics). A FitC filter was used to obtain images of nile red stained lipid droplets and a DAPI filter was used to obtain images of DAPI stained nuclei. Images were taken using a Leica DFC400 camera (Leica) and the Leica Application Suite Version 3 software (Leica). Lipid droplets were analyzed using the Image J software. The number of FitC positive objects was counted per image field and divided by the number of DAPI positive nuclei in the image field to determine the number of lipid droplets per cell. The area of FitC positive staining per image field was divided by the number of DAPI positive nuclei in the image field to determine the area of nile red staining per cell. Image fields were adjusted to exclude any partial cells to ensure accuracy.

Total cellular lipid content analysis

Cells were lysed after treatment with lysis buffer (50 mM Tris, 150 mM Na Cl, 1% Nonidet P-40 Substitute, 0.25% sodium deoxycholate, 0.1% sodium dodecyl sulfate, pH 7.4). Total triglycerides were quantified using a colorimetric triglyceride quantification assay (Sigma). Total cholesterol was quantified using a colorimetric Total Cholesterol Assay Kit (Cell Biolabs Inc.). The cell lysate was also assayed for protein content by a BCA protein assay kit (Pierce). Triglyceride and cholesterol content were determined relative to protein content to control for any differences in cell numbers.

Microarray analysis

RNA was isolated from cells using the RNeasy mini kit (Qiagen), including the on column DNase digest step, according to manufacturer instructions. Microarray analyses of the pooled total RNA samples (n = 3 separate samples per group for media, LPL, TGRL, and lipolysis products treatments) were performed. A 200 ng aliquot of total RNA from each pooled sample was aRNA amplified, reverse transcribed to synthesize first-strand cDNA and second-strand cDNA, transcribed in vitro to synthesize labeled aRNA, purified, and fragmented as described in the Affymetrix 3′ IVT Express Kit protocol. The fragments of labeled aRNA samples were hybridized to the Human Genome U133A 2.0 Array (Affymetrix). The hybridization, washing, labeling, and scanning of the microarray were performed as described in the Affymetrix protocols by the Microarray Core Facility in the UC Davis Genome and Biomedical Sciences Facility. Differentially expressed genes were defined as genes which had a detection p-value less than 0.05 and a fold change of ± 2.0 or when compared to the media treatment. Venn diagrams were generated using the online software at: http://bioinformatics.psb.ugent.be/webtools/Venn/.

Quantitative reverse transcription PCR (qRT-PCR)

After treatment, RNA was isolated using the RNeasy mini kit (Qiagen) according to the manufacturer's instructions, including the on column DNase digest step. RNA from each sample was reverse transcribed using Superscript III First Strand Synthesis Kit (Life Technologies) according to manufacturer's instructions. qRT-PCR was performed with FastStart Universal SYBR Green Master (Roche) to quantify the gene expression. Reactions were carried out in 384-well optical plates containing approximately 25 ng RNA in each well. The quantity of applied RNA was normalized by simultaneously amplifying cDNA samples with gene of interest specific primers and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) specific primers. The primer sequences used can be found in Table 2. Transcript levels were measured by qRT-PCR using the ABI Vii7 Sequence detection system (PE Applied Biosystems). The PCR amplification parameters were: initial denaturation step at 95°C for 10 min followed by 40 cycles, each at 95°C for 15 s (melting) and 60°C for 1 min (annealing and extension). A comparative threshold cycle (Ct) method[24] was used to calculate relative changes in gene expression determined from qRT-PCR experiments. The Ct, which correlates inversely with the target mRNA levels, was measured as the cycle number at which the SYBR Green emission increases above a preset threshold level. Fold change determinations were made using the media treatment or scramble siRNA media treatment for comparison.

Western blot

Cells were lysed after treatment with lysis buffer (50 mM Tris, 150 mM Na Cl, 1% Nonidet P-40 substitute, 0.25% sodium deoxycholate, 0.1% sodium dodecyl sulfate, pH 7.4). Total protein content was quantified by a BCA protein assay kit (Pierce). Equal amounts of denatured protein in a reducing buffer was loaded into each well of a precast polyacrylamide gel (4–15%, Bio-Rad). Proteins were separated by electrophoresis then electro-transferred to a polyvinylidene difluoride membrane with 0.2 μM pore size (Bio-Rad). The membrane was blocked with 5% nonfat dry milk in TBS with Tween 20. For each protein of interest, the

membrane was incubated in 5% nonfat dry milk in TBS with Tween 20 containing the primary antibody overnight at 4°C. Following the primary incubation, the membrane was washed three times with TBS with Tween 20 for at least 10 minutes, each wash. The membrane was then incubated with the secondary antibody in 5% nonfat dry milk in TBS with Tween 20 for one hour at room temperature and washed in the same manner as the primary antibody. The primary antibodies used were as follows: chicken anti-Perilipin 2 (Abcam), rabbit anti-MAFF(Aviva Systems Biology), rabbit anti-SGK1 (Cell Signaling) and mouse anti-β-actin (Sigma). Horseradish peroxidase linked secondary antibodies were used and protein was detected using Amersham ECL or ECL Prime (GE Healthcare) and autoradiography film. The Multi Gauge V2.3 software (Fujifilm) was used for densitometry analysis, relative expression in arbitrary units was determined relative to β actin.

ELISA

After treatment, the supernatant was collected and kept at −20°C until analyzed. Cytokine content was measured by commercially available ELISA kits for IL-8 (BD Biosciences) and CCL3 (R&D Systems), according to manufacturer's instructions. Cytokine concentration was the normalized to the total protein content of the corresponding cell pellet, to control for any differences in cell concentration.

Statistical analysis

Data was analyzed using the Sigma Stat 3.5 statistical software. All data was analyzed by one way or two way ANOVA using and a Tukey's post hoc test was used where applicable.

Results

TGRL lipolysis products induce differential gene expression in THP-1 monocytes.

We performed microarray analysis of 3 pooled samples from THP-1 monocytes treated with media control, LPL alone, TGRL alone or TGRL lipolysis products. More genes were differentially expressed when cells were exposed to TGRL lipolysis products than when exposed to LPL or TGRL alone. Figure 1 summarizes the number of differentially expressed genes for each treatment in Venn diagram format, when compared to the media control. Supplemental Tables S1, S2, and S3 show the list of genes differentially expressed by LPL, TGRL and Lipolysis Product treatment, respectively, when compared to the media control.

qRT-PCR confirms the results of the pooled microarray.

We confirmed the top 10 differentially expressed genes resulting from the microarray analysis by qRT-PCR. The top 10 differentially expressed genes discovered by the microarray analysis were: heat shock 70 kDa protein 6 (HSPA6, 38.1 fold), growth differentiation factor (GDF)15 (21.8 fold), serum/glucocorticoid regulated kinase (SGK)1 (13.7 fold), activating transcription factor (ATF)3 (13.1 fold), pleckstrin homology-like domain, family A, member 1 (PHLDA1, 10.6 fold), DNA-damage-inducible transcript (DDIT)4 (8.5 fold), heat shock 70 kDa protein 1A (HSPA1A,7.8 fold), DDIT3 (7.8 fold), v-maf musculoaponeurotic fibrosarcoma oncogene homolog F (avian) (MAFF, 7.6 fold), and v-maf musculoaponeurotic fibrosarcoma oncogene homolog B (avian) (MAFB, 7.2 fold). For each of the top 10 genes, qRT-PCR confirmed a differential expression for cells exposed

to TGRL lipolysis products when compared to the media control. The results of the qRT-PCR confirmation of the top 10 differentially expressed genes can be seen in Figure 2A.

After confirming the differential expression of the top ten genes, we chose to further investigate genes identified by the microarray which were directly related to our hypothesis. We focused on the TGRL lipolysis product-induced differential gene expression of pro-inflammatory cytokines identified by the pooled microarray analysis. The microarray analysis indicated that TGRL lipolysis products increased the expression of the pro-inflammatory cytokine genes: chemokine (C-C motif) ligand (CCL)2 (2.1 fold), CCL3 (5.8 fold), CCL20 (6.5 fold), and interleukin(IL)-8 (2.5 fold). We confirmed these findings using qRT-PCR (Figure 2B).

Exposure to TGRL lipolysis products increases perilipin 2 expression, lipid droplet formation, and cellular triglyceride content.

The microarray analysis identified an increase in perilipin 2 gene expression with exposure to TGRL lipolysis products. We confirmed this finding by qRT-PCR (Figure 3A) and further confirmed an increase in perilipin 2 protein expression by western blot (Figure 3B).

Exposure to TGRL lipolysis products produced an increase in lipid droplet formation as demonstrated by an increase in area of nile red staining and number of lipid droplets per cell (Figure 4A-C). Whole cell triglyceride content increased, while whole cell cholesterol content was not changed by exposure to TGRL lipolysis products (Figure 4D-E).

Knockdown of perilipin 2 increases cellular lipid staining area and triglyceride content.

We wanted to determine if TGRL lipolysis product-induced lipid droplet formation was an upstream process to the increase in inflammation related gene expression found in the microarray. We reduced perilipin 2 expression using siRNA prior to TGRL lipolysis product exposure, then characterized lipid droplet formation and measured the selected genes we confirmed from the microarray analysis. We found a significant reduction of both RNA (Figure 5A) and protein (Figure 5B) expression of perilipin 2. We found that perilipin 2 knockdown by siRNA appears to increase lipid droplet size (Figure 6A). While perilipin 2 knockdown did not change lipid droplet number, the area of lipid staining per cell and cellular triglyceride content increased (Figure 6B-D).

Perilipin 2 siRNA alters TGRL lipolysis product-induced SGK1, MAFF, IL-8, and CCL3 expression.

Inhibition of perilipin 2 expression reduced the gene expression of SGK1, MAFF, IL-8, and CCL3 after exposure to TGRL lipolysis products (Figure 7). We found no change in the gene expression response of the remaining top ten differentially expressed genes and pro-inflammatory cytokines identified in the microarray (data not shown).

We used western blot to determine protein expression of SGK1 and MAFF and measured secretion of CCL3 and IL-8 by ELISA. We found that perilipin 2 siRNA suppressed TGRL lipolysis product-induced SGK1 protein expression, while MAFF protein expression was not

altered (Figure 8). Perilipin 2 siRNA reduced TGRL lipolysis product-induced secretion of CCL3 and IL-8 (Figure 9).

Discussion

Elevated serum triglycerides are known to be a risk factor for atherosclerosis[1]. Our lab has previously shown that exposure to lipolysis products increases monocyte adhesion, a key step in atherosclerosis[5]. In the current study, of the top ten differentially expressed genes induced by TGRL lipolysis products two genes, SGK1 and DDIT3, have been demonstrated to be directly pro-atherogenic in the literature[25, 26]. Microarray analysis also demonstrated that TGRL lipolysis products increased the gene expression of pro-inflammatory cytokines IL-8, CCL2, CCL3, and CCL20, all of which have been implicated in the progression of atherosclerosis[27–31].

Previous studies in our lab have demonstrated an increase in lipid droplets, a key factor in the development of atherosclerosis, in monocytes with exposure to TGRL lipolysis products[5, 6]. In the current study, we replicated this result and further analyzed cellular lipid content. Lipid droplets are composed of a neutral lipid core, made up primarily of triglycerides and cholesterol esters surrounded by a phospholipid monolayer[32]. We therefore examined total cellular triglyceride and cholesterol content and found that the increase in lipid droplets occurs alongside an increase in cellular triglyceride content, but no change in cholesterol content. This would seem to indicate that the lipid droplets that form after exposure to TGRL lipolysis products are composed primarily of triglycerides. Given that the action of LPL on TGRL releases fatty acids and monoglycerides[2], this would be the expected result if the cells were taking up these components and esterifying them into triglycerides.

We have shown that the lipid droplet protein, perilipin 2 RNA and protein expression increases alongside lipid droplet formation, as previously reported[9, 13, 33]. Further, perilipin 2 has been implicated as a pro-atherogenic gene. Perilipin 2 has been found in human atherosclerotic plaques, and was shown to correlate with plaque instability[34]. Deficiency of perilipin 2 or transplant of perilipin 2 knockout bone marrow in apoE knockout mice (a common model of atherosclerosis) has been shown to be protective against atherosclerosis[16].

Previous literature indicates that a reduction in perilipin 2 expression in THP-1 derived macrophages leads to reduced lipid droplet formation when cells are exposed to lipoproteins[7, 12]. We expected to see a similar response in undifferentiated THP-1 cells exposed to TGRL lipolysis products. However, our study found that the reduced expression of perilipin 2 contributed to increased intracellular triglyceride levels and apparently larger lipid droplets, given that the image analysis showed no change in the number of lipid droplets alongside an increase in area of lipid staining. This unexpected finding indicates that perhaps the role of perilipin 2 in lipid droplet formation is different in monocytes exposed to lipolysis products than it is in differentiated macrophages exposed to whole lipoproteins. We believe that this may be due to one or both of two key features of our study that differ from those previously reported in the literature. The first of these differences is that we are studying undifferentiated THP-1 cells in a monocyte-like state, while previous

studies were conducted on THP-1 cells differentiated with PMA into a macrophage-like state. The other key difference in our study is the lipid stimulus to which the cells are exposed. Perhaps perilipin 2 has a different function when cells are exposed to lipolysis products when compared to whole lipoproteins. Future studies will be needed to clarify which of these factors produced the larger lipid droplets when perilipin 2 expression is suppressed.

We found a reduction in TGRL lipolysis product-induced MAFF gene expression when perilipin 2 expression was inhibited prior to TGRL lipolysis product exposure. Although we did not observe an increase in MAFF protein expression, it is possible that there is a change in protein levels at a later time point. MAFF is a transcription factor, which has been implicated in stress response and inflammation[35]. Further, MAFF has previously been shown to be upregulated in classically activated macrophages[36]. Therefore the reduction in lipolysis product-induced MAFF expression appears to be an anti-inflammatory effect of perilipin 2 knockdown.

We found that knockdown of perilipin 2 reduced TGRL lipolysis product-induced SGK1, CCL3, and IL-8 gene expression. This reduction was also seen in TGRL lipolysis product-induced SGK1 protein expression, and secretion of the cytokines CCL3 and IL-8. SGK1, CCL3, and IL-8 have each been directly implicated in the development of atherosclerosis. SGK1 has been shown to be pro-atherogenic in a mouse model of atherosclerosis, with high cholesterol diet fed apo E, SGK1 double knockout mice having smaller atherosclerotic lesions than apo E knockout mice with normal SGK1 expression[25]. Further, monocytes lacking SGK1 exhibit reduced migration and recruitment to sites of inflammation[25]. Leukocyte specific CCL3 has been shown to contribute to atherosclerotic plaque formation, with bone marrow transplants from CCL3 knockout mice into the LDL receptor knockout mouse model of atherosclerosis reducing plaque formation when compared to LDL receptor knockout mice with CCL3 expression in the bone marrow[31]. IL-8 has been shown to play a significant role in atherosclerotic plaque development throughout the progression of the disease[27]. At this point it is unclear if the reduction in expression of these genes is due to a common signaling pathway. Future studies are needed to determine which pathways lead to these reductions in pro-atherogenic gene expression.

Conclusions

It has previously been described that a lack of perilipin 2 reduces atherosclerotic plaque formation in vivo[16]. Our data support this finding and further inform some of the mechanisms by which this may occur. The unexpected result of our study was an increase in cellular triglyceride content and presence of larger lipid droplets in cells with a knockdown of perilipin 2 expression. Even with this increased cellular lipid content, the THP-1 cells with reduced perilipin 2 expression seem to have a less atherogenic phenotype, with reduced pro-inflammatory and pro-atherogenic gene expression induced by TGRL lipolysis products. One possibility is that the larger lipid droplets are protective against inflammatory responses, sequestering fatty acids that induce inflammation. Alternatively, this could be due to an active signaling role of perilipin 2. Future studies will be needed to further elucidate the signaling pathways which facilitate the reductions in pro-inflammatory and pro-atherogenic

gene expression and whether this is due to an active signaling role of perilipin 2 or simply due to the changes in lipid metabolism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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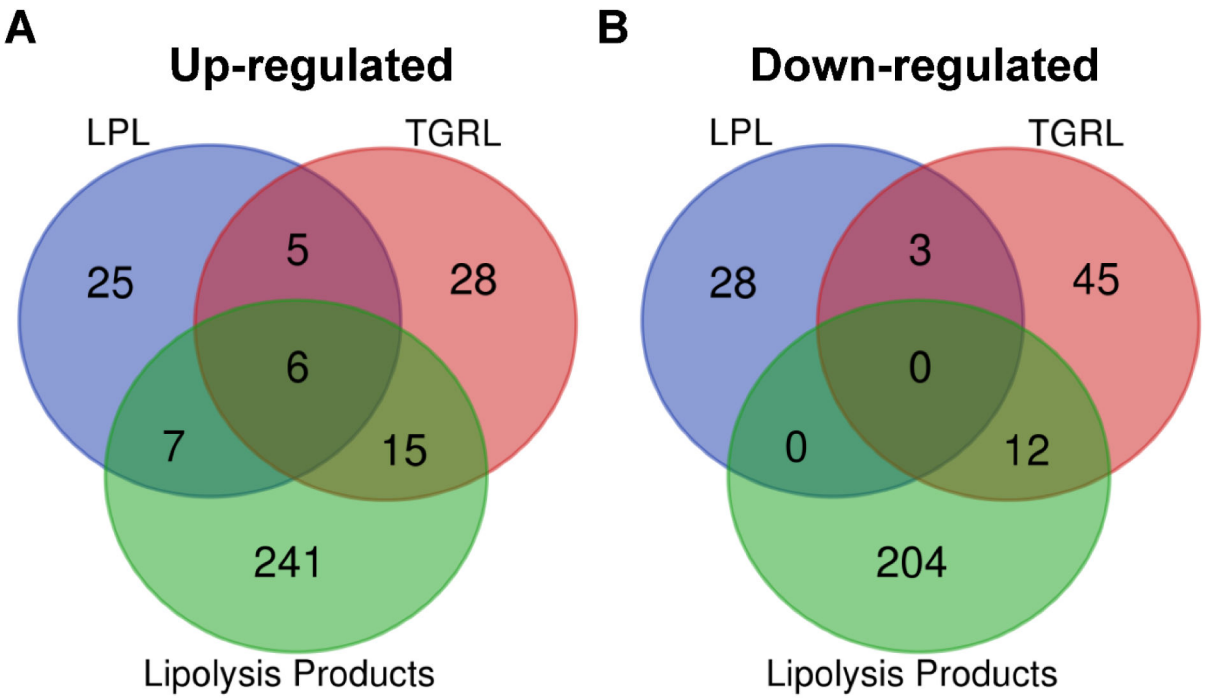

Figure 1. Numbers of genes differentially expressed by treatment.

THP-1 monocytes were treated with media (normal culture media only), LPL (2 U/mL LPL in normal culture media), TGRL (150 mg/dL TGRL in normal culture media), and lipolysis products (150 mg/dL TGRL and 2 U/mL LPL in normal culture media) for 3 hours. Microarray analysis was conduced on 3 pooled samples for each treatment, these samples were obtained from 3 separate experiments with one culture from each treatment group. **A.** Venn diagram of up-regulated genes for LPL, TGRL, and lipolysis products treated cells when compared to media control group. **B.** Venn diagram of down-regulated genes for LPL, TGRL, and lipolysis products treated cells when compared to media control group.

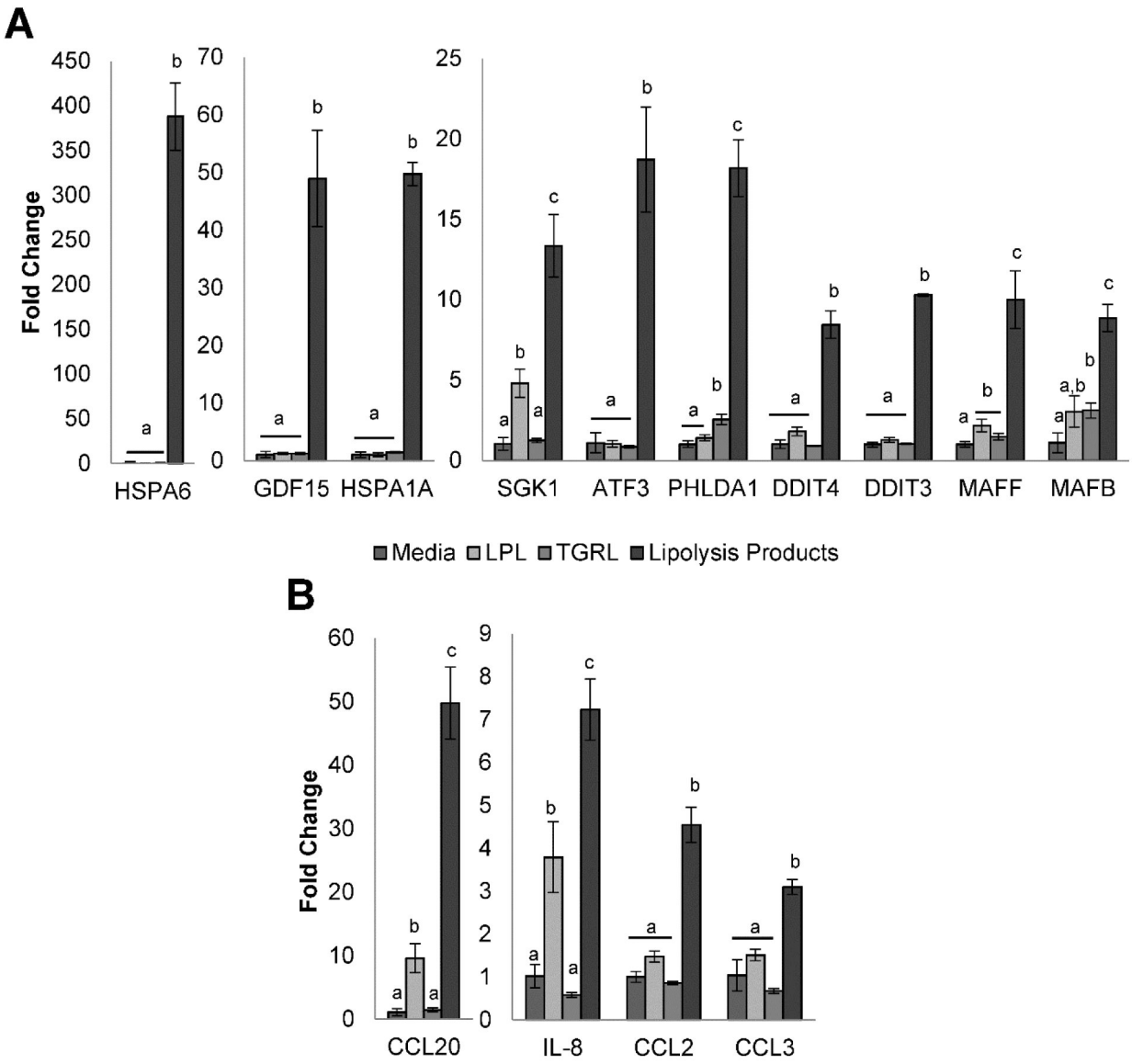

Figure 2. Confirmation of differential gene expression induced by TGRL lipolysis products in THP-1 cells.

THP-1 monocytes were treated with media (normal culture media only), LPL (2 U/mL LPL in normal culture media), TGRL (150 mg/dL TGRL in normal culture media), and lipolysis products (150 mg/dL TGRL and 2 U/mL LPL in normal culture media) for 3 hours. The **A.** top ten differentially expressed genes as determined by microarray analysis and **B.** differentially expressed pro-inflammatory cytokines were analyzed by qRT-PCR, normalized to GAPDH expression. Fold change was determined relative to the media treated group. Data shown is mean +/− standard deviation, n=3 separate samples per treatment group obtained from one experimental run with triplicate cultures. Different letter superscripts represent groups which are significantly different from each other as determined by ANOVA with Tukey's post hoc test ($p<0.05$).

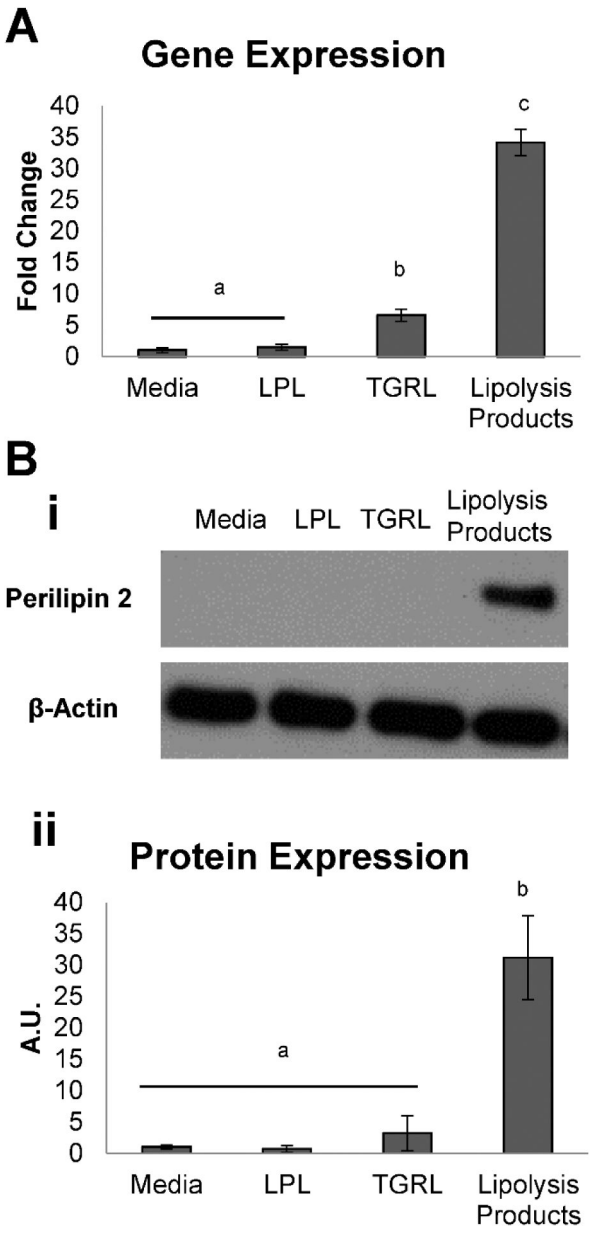

Figure 3. TGRL lipolysis products increase expression of perilipin 2.

THP-1 monocytes were treated with media, LPL, TGRL or lipolysis products for 3 hours. **A.** Lipolysis products increase perilipin 2 gene expression as measured by qRT-PCR, normalized to GAPDH expression. Fold change was determined relative to the media treated group. **B.** Lipolysis products increase perilipin 2 protein expression. **i.** Representative western blot image. **ii.** Densitometry analysis of western blot, protein expression determined relative to β-actin. All data shown is mean +/− standard deviation, n=3 separate samples per treatment group obtained from one experimental run with triplicate cultures. Different letter superscripts represent groups which are significantly different from each other as determined by ANOVA with Tukey's post hoc test ($p<0.05$).

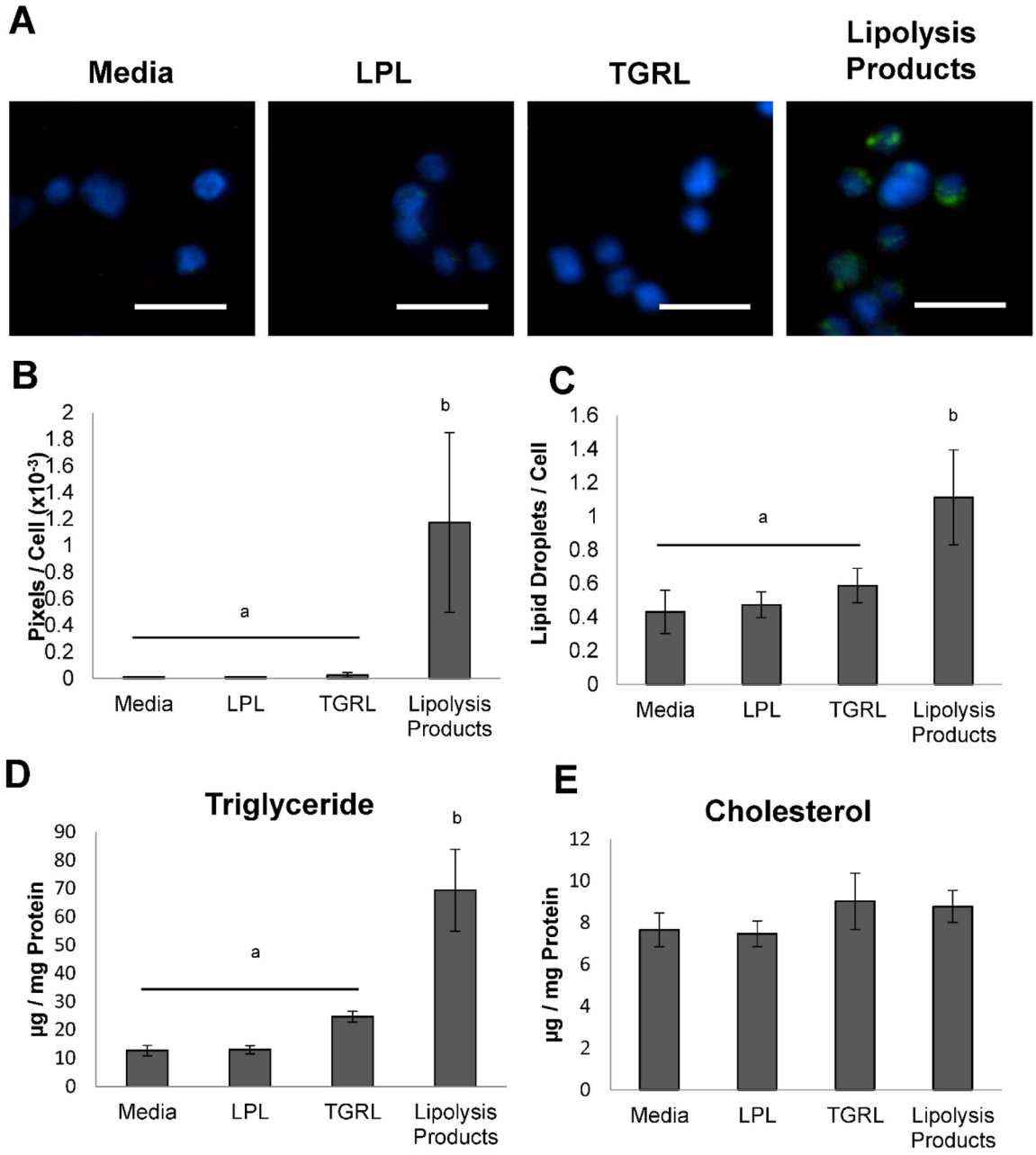

Figure 4. Characterization of TGRL lipolysis product-induced lipid droplet formation.

THP-1 monocytes were treated with media, LPL, TGRL or lipolysis products for 3 hours. **A.** Lipolysis products induced a significant increase in lipid droplet formation when compared to media, LPL, or TGRL treated cells. Representative images of THP-1 cells after each treatment stained with nile red (green) and DAPI nuclear stain (blue), scale bar represents 25 µm. **B.** Lipolysis products significantly increased area of lipid droplet staining when compared to media, LPL, or TGRL treated cells. Image analysis of area of nile red staining was averaged per number of nuclei in image field, n=3 separate samples per treatment group obtained from one experimental run with triplicate cultures. **C.** Lipolysis products significantly increased the number of lipid droplets per cell when compared to media, LPL,

or TGRL treated cells. Image analysis of nile red staining as number of stained structures averaged per number of nuclei in image field, n=3 separate samples per treatment group obtained from one experimental run with triplicate cultures. **D.** Lipolysis products significantly increased whole cell triglyceride content when compared to media, LPL or TGRL treated cells. Cellular triglyceride content is expressed relative to cellular protein, n=6 separate samples per treatment group obtained from two experimental runs with triplicate cultures. **E.** Whole cell cholesterol content did not differ between treatment groups. Cellular cholesterol content is expressed relative to cellular protein, n=6 separate samples per treatment group obtained from two experimental runs with triplicate cultures. All data shown is mean +/− standard deviation. Different letter superscripts indicate groups which are significantly different from each other as determined by ANOVA with Tukey's post hoc test ($p<0.05$).

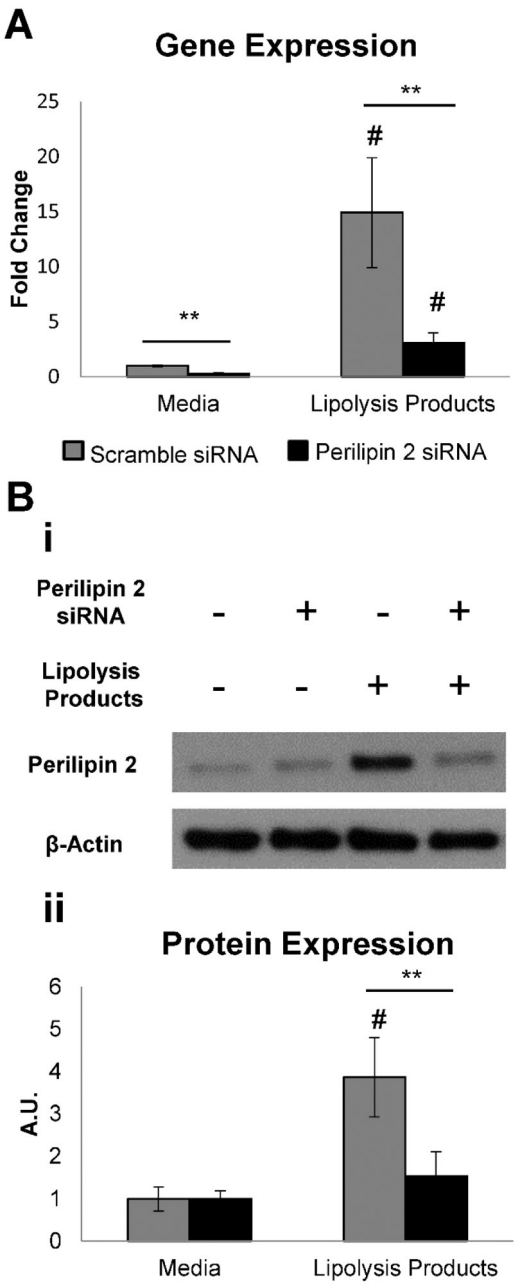

Figure 5. Effect of perilipin 2 siRNA on lipolysis product-induced perilipin 2 expression.

THP-1 monocytes were pre-incubated with perilipin 2 siRNA or scramble siRNA prior to incubation with media control or lipolysis products for 3 hours. **A.** Perilipin 2 siRNA reduced perilipin 2 gene expression in THP-1 cells treated with media and lipolysis products. Gene expression was measured by qRT-PCR, and normalized to GAPDH expression. Fold change was determined relative to the scramble siRNA media control group, n=9 separate samples per treatment group obtained from three experimental runs with triplicate cultures. Analysis by two-way ANOVA indicated an interaction of the two factors. **B.** Perilipin 2 siRNA reduced lipolysis product-induced perilipin 2 protein expression. **i.** Representative image of western blot. **ii.** Densitometry analysis of western blot, protein

expression determined relative to β-actin, n=3 separate samples per treatment group obtained from one experimental run with triplicate cultures. Analysis by two-way ANOVA indicated an interaction of the two factors. All data shown is mean +/− standard deviation. Statistical differences are indicated by **($p<0.001$, for indicated comparison) and # ($p<0.001$, for comparison to corresponding media control).

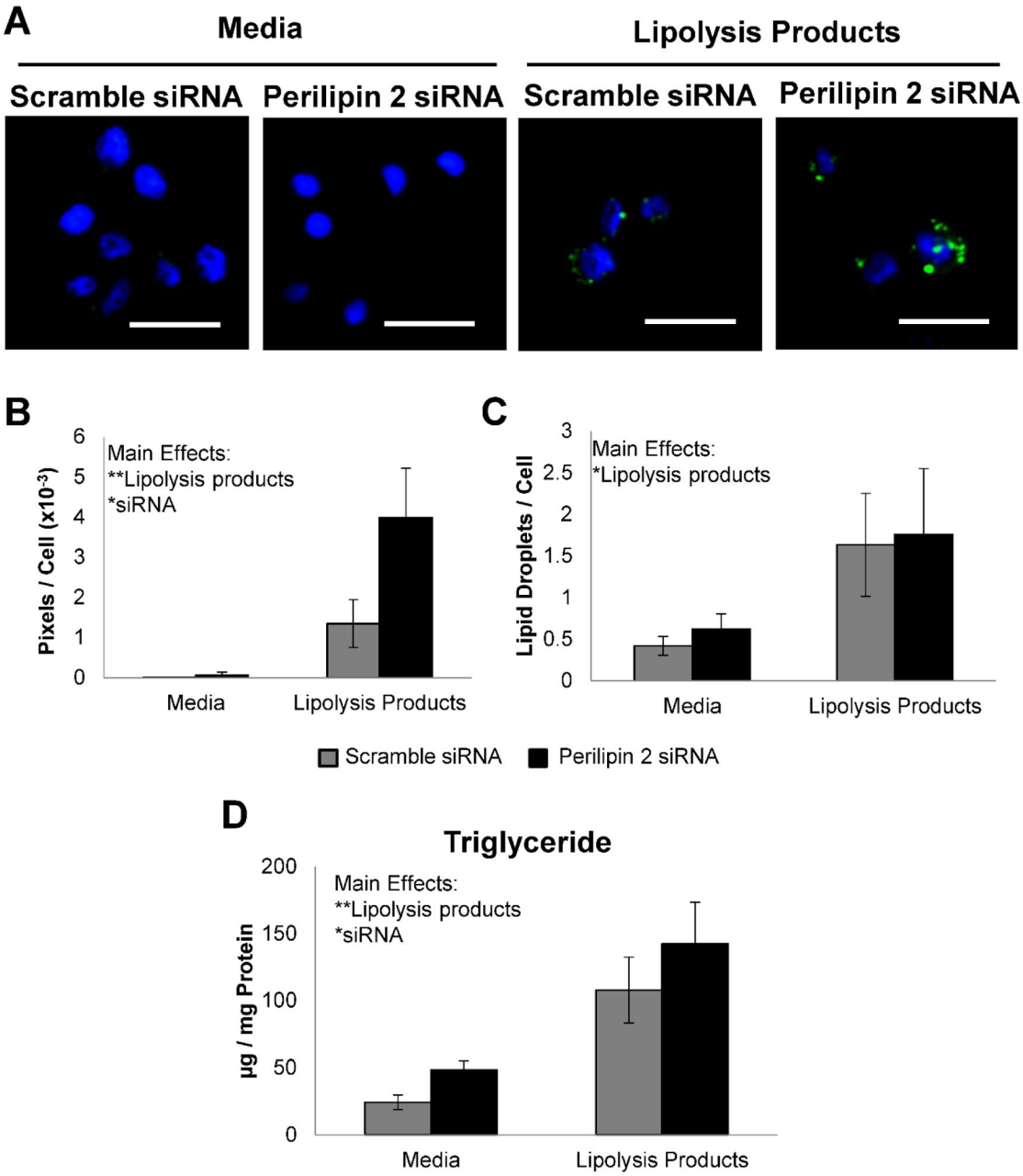

Figure 6. Characterization of lipolysis product-induced lipid droplet formation with perilipin 2 siRNA knockdown.

THP-1 monocytes were pre-incubated with perilipin 2 siRNA or scramble siRNA prior to incubation with media or lipolysis products for 3 hours. **A.** Larger lipid droplets are visible in cells pre-incubated with perilipin 2 siRNA prior to lipolysis product treatment. Representative images of THP-1 cells after each treatment stained with nile red (green) and DAPI nuclear stain (blue), scale bar represents 25 µm. **B.** Perilipin 2 siRNA increases the lipolysis product-induced area of lipid staining per cell. Image analysis of area of nile red staining was averaged per number of nuclei in image field, n=3 separate samples per treatment group obtained from one experimental run with triplicate cultures. Analysis by

two-way ANOVA indicated main effects of both factors, lipolysis products and siRNA. **C.** Perilipin 2 siRNA does not have a significant effect on the number of lipolysis product-induced lipid droplets per cell. Image analysis of nile red staining as number of stained structures averaged per number of nuclei in image field, n=3 separate samples per treatment group obtained from one experimental run with triplicate cultures. Analysis by two-way ANOVA indicated a main effect of lipolysis products only. **D.** Perilipin 2 siRNA increases whole cell triglyceride content induced by lipolysis products. Cellular triglyceride content is expressed relative to cellular protein, n=6 separate samples per treatment group obtained from two experimental runs with triplicate cultures. Analysis by two-way ANOVA indicated main effects of both factors, lipolysis products and siRNA. All data shown is mean +/− standard deviation. Statistical differences are indicated by *($p<0.05$, for indicated main effect) and **($p<0.001$, for indicated main effect).

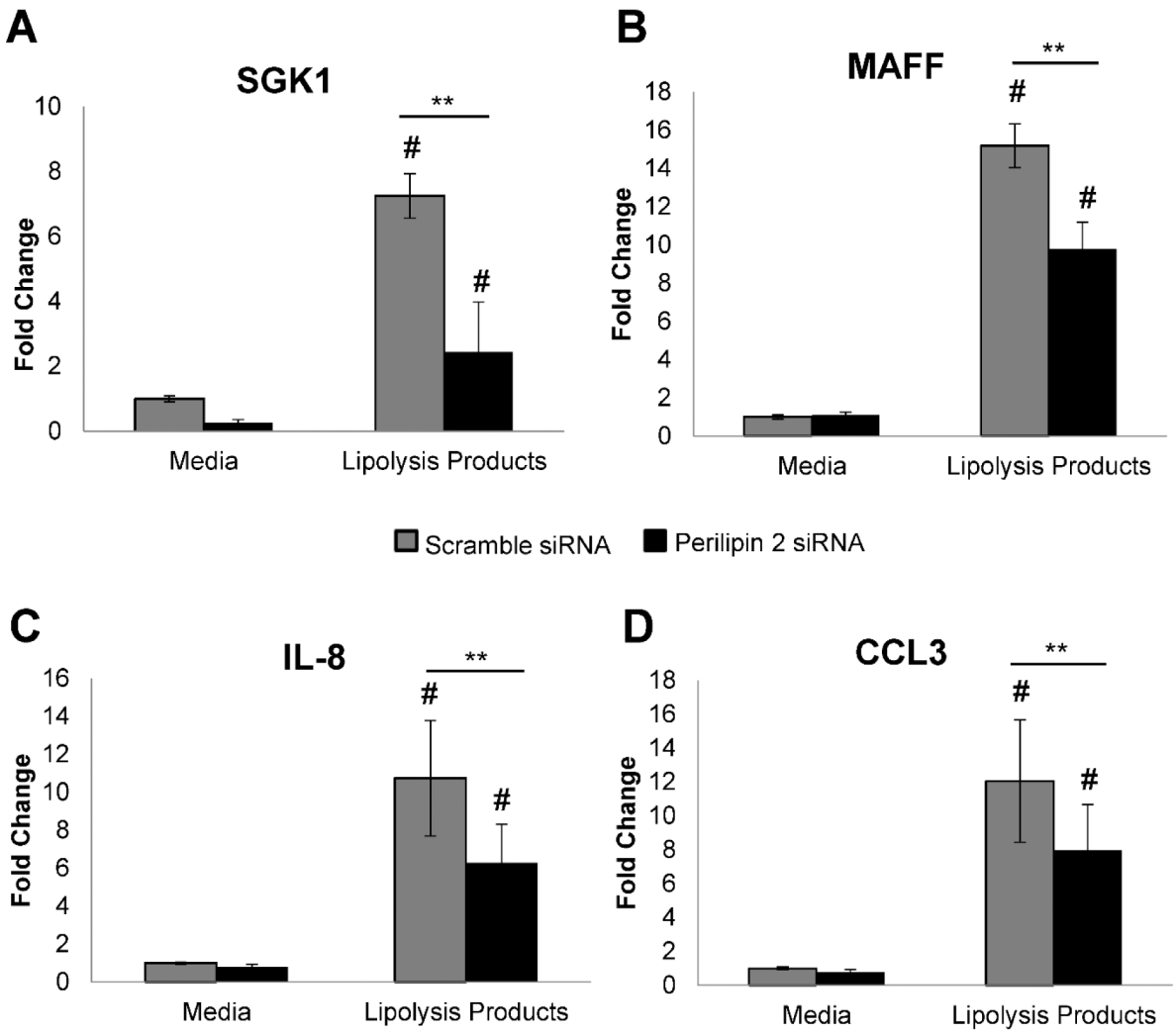

Figure 7. Effects of perilipin 2 siRNA on TGRL lipolysis product-induced gene expression.

THP-1 monocytes were pre-incubated with perilipin 2 siRNA or scramble siRNA prior to incubation with media or TGRL lipolysis products for 3 hours. Gene expression for **A.** SGK1, **B.** MAFF, **C.** IL-8, and **D.** CCL3. Gene expression was analyzed by qRT-PCR, normalized to GAPDH. Fold change is relative to scramble siRNA media control group. All data shown is mean +/− standard deviation, n=9 separate samples per treatment group obtained from three experimental runs with triplicate cultures. For each gene, analysis by two-way ANOVA indicated an interaction of the two factors. Tukey's post hoc test was used to determine differences between groups. Statistical differences are indicated by **($p<0.001$, for indicated comparison) and #($p<0.001$, for comparison to corresponding media control).

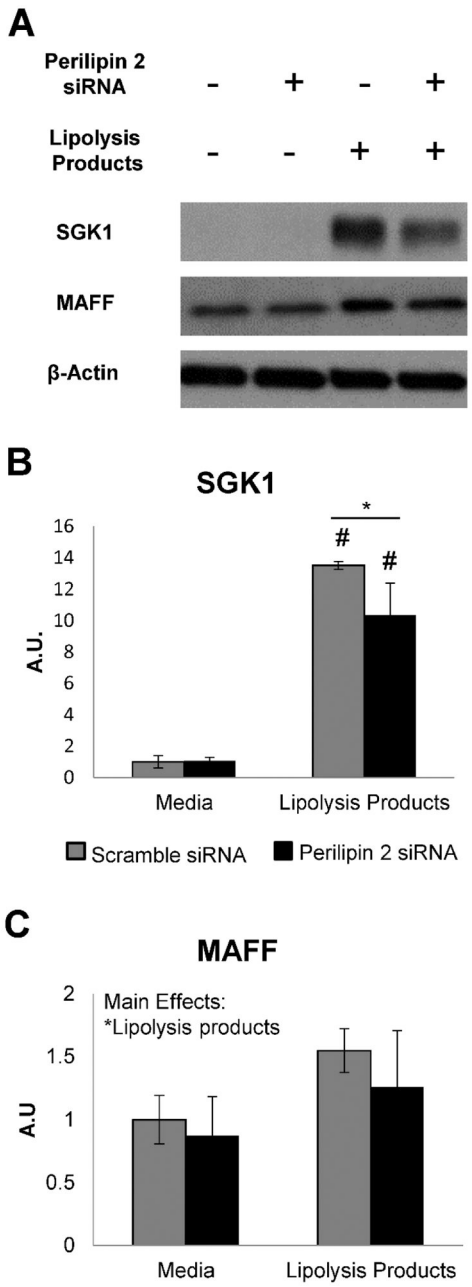

Figure 8. Effects of perilipin 2 siRNA on lipolysis product-induced protein expression of SGK1 and MAFF.

THP-1 monocytes were pre-treated with scramble siRNA or perilipin 2 siRNA prior to treatment with media or lipolysis products. Perilipin 2 siRNA significantly reduced TGRL lipolysis product-induced SGK1 protein expression, but did not have a significant effect on MAFF protein expression. **A.** Representative image of western blot. Densitometry analysis of **B.** SGK1 and **C.** MAFF western blots, protein expression was determined relative to β-actin. All data shown is mean +/− standard deviation, n=3 separate samples per treatment group obtained from one experimental run with triplicate cultures. Data was analyzed by two-way ANOVA with Tukey's post hoc test (where applicable). Analysis indicated an

interaction of the two factors for SGK1 expression and a main effect of lipolysis products for MAFF expression. Statistical differences are indicated by *($p<0.05$, for indicated main effect or comparison) and #($p<0.001$, for comparison to corresponding media control).

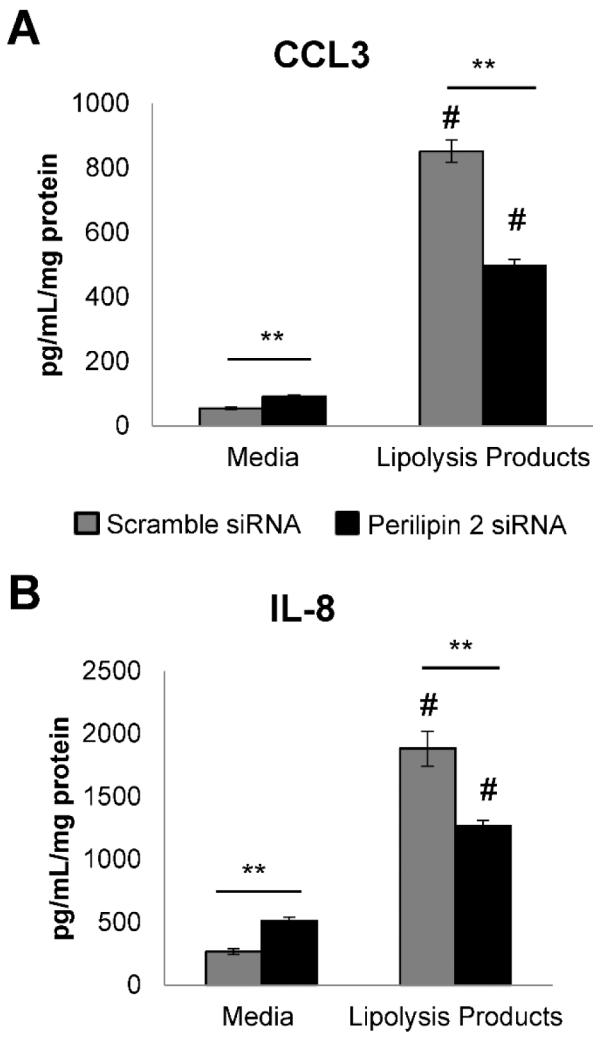

Figure 9. Effects of perilipin 2 siRNA on lipolysis product-induced cytokine secretion.

THP-1 monocytes were pre-treated with scramble siRNA or perilipin 2 siRNA prior to treatment with media or lipolysis products. Perilipin 2 siRNA reduced TGRL lipolysis product-induced secretion of CCL3 and IL-8. Secretion of **A.** CCL3 and **B.** IL-8 was determined by ELISA of cell supernatants collected after cells were treated with media or lipolysis products. Cytokine concentrations are expressed relative to the amount of protein in the cell pellet. All data shown is mean +/− standard deviation, n=3 separate samples per treatment group obtained from one experimental run with triplicate cultures. Analysis by two-way ANOVA indicated an interaction of the two factors for both cytokines. Tukey's post hoc test was used to determine differences between groups. Statistical differences are indicated by **($P<0.001$, for indicated comparison) and #($p<0.001$, for comparison to corresponding media control).

Table 1.

Dicer substrate siRNA sequences.

siRNA	Sense	Anti-Sense
Perilipin 2	GGAUUAUCUUGUUAACAACACGCCC	GGGCGUGUUGUUAACAAGAUAAUCCAU
Scramble	/5Phos/CUUCCUCUCUUUCUCUCCCUUGUGA	UCACAAGGGAGAGAAAGAGAGGAAGGA

Table 2.

Primer sequences used for qRT-PCR.

Gene	Forward	Reverse
ATF3	TTCTCCCAGCGTTAACACAAAA	AGAGGACCTGCCATCATGCT
CCL2	CAGCAGCAAGTGTCCCAAAG	TTGGCCACAATGGTCTTGAA
CCL3	GGTGACAACCGAGTGGCTGT	TTGGTGCCATGACTGCCTAC
CCL20	TGGAATGGAATTGGACATAGCC	CAACCCCAGCAAGGTTCTTTC
DDIT3	AGAGTGGTCATTCCCCAGCC	CTTTCTCCTTCATGCGCTGC
DDIT4	GCAGCTGCGTTTAAGCCTTC	TGCCAGCTCAACTCTGCAGT
GAPDH	CACCAACTGCTTAGCACCCC	TGGTCATGAGTCCTTCCACG
GDF15	TGGTGCTCATTCAAAAGACCG	CATCATAGGTCTGGAGCGACAC
HSPA1A	GAAGGAGCTGGAGCAGGTGT	CCCTGGTACAGTCCGCTGAT
HSPA6	GTGAGAGGGCCATGACCAAG	TGAGTTCAAAACGCCCCAG
IL-8	CCTTTCCACCCCAAATTTATCA	TGGTCCACTCTCAATCACTCTCAG
MAFB	CGCATGGTGCTTGCAGTTT	TTCTGATGCAGGACAAATATCCAC
MAFF	TGCCCAGGTCCCATTTCTC	GGCCCACGAAGGGAATGT
PERILIPIN 2	CCAAGGTGCAGAGATGGACA	TGCTCAGATCGCTGGGTCT
PHLDA1	TTGGATCAAAAGCAAAACAGTCTC	AAACTACTTGATCTGGTGCGGG
SGK1	TTATGGACCAATGCCCCAGT	CAATGAAAAACACCAACGGCT