Malays. Appl. Biol. (2022) 51(4): 153-167
https://doi.org/10.55230/mabjournal.v51i4.24

HDL AND ITS SUBPOPULATION (HDL2 AND HDL3) PROMOTE CHOLESTEROL TRANSPORTERS EXPRESSION AND ATTENUATE INFLAMMATION IN 3T3-L1 MATURE ADIPOCYTES INDUCED BY TUMOR NECROSIS FACTOR-ALPHA

SUHAILA ABD MUID[1,2,*], REMEE AWANG JALIL[2], NOOR HANISA HARUN[2], HAPIZAH MOHD NAWAWI[1,2] and GABRIELE RUTH ANISAH FROEMMING[3]

[1]*Institute of Pathology, Laboratory and Forensic Medicine (I-PPerForM), Universiti Teknologi MARA Sungai Buloh, Selangor, Malaysia*
[2]*Faculty of Medicine, Universiti Teknologi MARA Sungai Buloh, Selangor, Malaysia*
[3]*Faculty of Medicine and Health Sciences, Universiti Malaysia Sarawak, Sarawak, Malaysia*
[*]*E-mail: suhaila_muid@uitm.edu.my*

Accepted 3 October 2022, Published online 31 October 2022

ABSTRACT

Obesity activates inflammation causing dysfunction of adipocytes. Increasing high-density lipoprotein (HDL) levels in obesity may be beneficial in overcoming this effect. However, not much data is available on the effects of HDL and its subpopulations in inflamed adipocytes. The objective of this study was to investigate the effects of total HDL (tHDL) and the comparison between its subpopulations (HDL2 & HDL3) on protein and gene expression of cholesterol transporters, inflammation, and adipokines in TNF-α stimulated 3T3-L1 mature adipocytes. TNFα alone had lower adiponectin and higher protein and gene expression of IL-6 and NF-κβ (p65) compared to unstimulated adipocytes and these effects were attenuated by HDLs especially HDL3 (in most of the biomarkers). HDL and its subpopulation had higher cholesterol transporters expression in 3T3-L1 mature adipocytes induced by TNF-α compared to unstimulated cells. Increment of cholesterol transporters expression by HDL leads to reduce secretion of inflammatory markers [IL-6 & NF-kB (p65)] and visfatin and increases adiponectin secretion in the inflamed mature adipocytes. HDL exhibits beyond its reverse cholesterol transporter property by exhibiting anti-inflammatory effects thru the deactivation of NF-κβ (p65). This may contribute to reducing the progression of obesity-related complications.

Key words: Adipokines, cholesterol transporters (ABCA1 & SR-B1), HDL, HDL2, HDL3, inflamed adipocytes

INTRODUCTION

The imbalance of lipid homeostasis and adiponectin secretion by dysfunctional adipose tissue are some of the main factors that promote the increase of circulating free fatty acids (FFAs) and lipolysis in an individual (Guilherme *et al*., 2008). It is known that chronic low-grade inflammation and increment of macrophage infiltration observed in obese individuals are some of the major characteristics of hypertrophic adipose tissues [dysfunctional adipose tissue due to increase of adipocyte cell size] (Wellen & Hotamisligil, 2003).

Obesity is well known to be associated with chronic low-grade inflammation and causes the release of pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) in adipose tissue (Das, 2001). Increased expression of TNF-α leads to insulin resistance and dyslipidemias, affecting apolipoprotein (apo) B metabolism and inhibiting reverse cholesterol transport which may increase the risk of getting diabetes and cardiovascular diseases (Gutierrez *et al*., 2009). It is alarming that the prevalence of obesity in Malaysia is the highest among Asian countries and the number is increasing by the year. According to the press media during "World Health Day 2019", Malaysia has the highest rate of obesity and overweight among Asian countries. About 64% of men and 65% of women in Malaysia are either obese or overweight (Ruiz *et al*., 2019).

Adipocytes are the main cells in the adipose tissue. It is suggested that adipocyte play a role in cholesterol efflux activity by expressing the cholesterol transporters ABCA1, and SR-BI. Both adipocyte-transporters were reported to involve in transferring cholesterol from adipocytes to apoAI and mature HDL particles. A study done by Zhang et. al (2010) found that intraperitoneal injection of 3T3-L1 adipocytes into SR-B1 or ABCA1 deficient adipocytes,

* To whom correspondence should be addressed

promotes the cholesterol efflux and lipidation of HDL, thus maintaining the HDL-C level. However, the same author also stresses that inflammation could impair the important functions of adipocytes. These dysfunctional or impaired adipocytes start to secrete high levels of pro-inflammatory cytokines such as TNF-α which disturb its normal regulation in producing adiponectin, an anti-inflammatory adipokine (Aprahamian & Sam, 2011).

A low level of adiponectin is known to be associated with the increment of serum triglycerides, total cholesterol, and low-density lipoproteins levels, as well as thickening of intimal plaque volume (Marso *et al*., 2008; Wang *et al*., 2017). In normal regulation, free cholesterol plays a crucial role in the structural and signaling of mammalian cells as it is one of the components in the cell membranes (Xu *et al*., 2017). However, excess secretion of the free cholesterol could be toxic to the cell as it will be converted into cholesteryl esters and stored in lipid droplets (LDs) (Xu *et al*., 2017). LDs are dynamic cellular organelles that play a role in lipid storage and energy homeostasis (Olzmann & Carvalho, 2019). However, excess accumulation of lipids (triacylglycerides and cholesterol) in the LDs of adipocytes could lead to hypertrophic adipocytes (Verghese *et al*., 2007). When the adipocytes reach their maximum size, the excess lipid, which is commonly cholesterol ester (CE), will be effluxed to high-density lipoproteins (HDL) and transported to the liver (Sun *et al*., 2011; Arvind *et al*., 2019). Excess CE-contained LDs accumulation in macrophages leads to the formation of foam cells, which is one of the factors that promote the progression of atherosclerosis (Xu *et al*., 2018).

HDL is a well-established athero- and cardioprotective agent. Its concentration in plasma is inversely correlated with the risk of developing atherosclerosis and cardiovascular diseases (Saha *et al*., 2012). HDL plays a major role in the removal of excess cholesterol from peripheral tissues to the liver through reverse cholesterol transport (Soumyarani & Jayakumari, 2012). The rate of cellular cholesterol removal by HDL depends on multiple factors, such as cholesterol abundance in the plasma membrane and cholesterol transporters such as ABCA1, ABCG1, and scavenger receptor class B type 1 (SR-B1) receptors. Surprisingly, little is known about the effects of HDL especially its subpopulation on these cholesterol transporters in adipocytes.

The expression of ABCA1, ABCG1, and SR-B1 cholesterol transporters is activated by the transcription factors PPARγ or PPARδ (Oliver *et al*., 2001; Briand *et al*., 2009). The cholesterol pool that is present in adipocytes consists mainly of free cholesterol (unesterified) and its size is dependent on the mass of the adipose tissue and thus is proportional to the cellular TG content (Verghese *et al*., 2007). The cholesterol content in adipose tissue is dependent on the balance between the efflux and influx of cholesterol. Therefore, it is suggested that the rate of efflux to extracellular cholesterol acceptors could affect the cholesterol content of adipose tissue. Maintaining the cholesterol homeostasis of adipose tissue is important for proper metabolic functions (Verghese *et al*., 2007). The output of free cholesterol from adipocytes might affect the homeostasis of cholesterol metabolism (Verghese *et al*., 2007). The dynamic role of adipose tissue in energy balance through its ability to accumulate or release fatty acids and the fact that there is also a correlation between TG and cholesterol content of adipocytes suggest the metabolism of these two lipids could be coupled. Changes in the TG metabolism could affect the transport of cholesterol. Therefore, in this study, it is postulated that incubation of HDL in adipocytes induced by TNF-α could lead to the activation of PPARγ and PPARδ which at the same time increase the expression of cholesterol transporters.

HDL is an immense heterogeneous population of lipoprotein particles. Much has been written on the diversity of HDL populations that differ in size, lipid and protein content, and its effects on the athero- and cardioprotective properties (Gordon *et al*., 2011). HDL particles are classified into two main subpopulations based on their density: HDL2 ($1.063<d<1.125$ g/mL) and HDL3 ($1.125<d<1.21$ g/mL) (Murphy, 2013). HDL2 is a large, light, and lipid-rich type of HDL sub-population (Camont *et al*., 2011). It has been reported to have potent vasodilatory and anti-thrombotic activity (Camont *et al*., 2011). In comparison to HDL2, HDL3 is denser, smaller, and relatively has a cholesterol-poor form. In terms of HDL3 cardioprotective properties, it has been reported that it inhibits LDL oxidation greater than HDL2 (Yoshikawa *et al*., 1997). In addition, HDL3 has better antioxidant properties compared to HDL2 which is possibly due to paraoxonase 1 (PON1) activity (Shuhei *et al*., 2010). Therefore, it is suggested that measurement of HDL heterogeneity and functionality is important rather than looking into the quantity of total HDL (Superko *et al*., 2012).

Therefore, the main question of this study is does the different HDL subpopulations have an impact on reducing the accumulation of lipid droplets and increasing the expression of cholesterol transporters in adipocytes through activation of PPARγ or PPARδ? In addition, is the anti-inflammatory pathway contributing to the above HDLs effects? Therefore, the objectives of this study were to determine the effects of TNF-α on the formation of lipid droplets and the expression of ABCA1, SR-B1, IL-6, NF-κβ (p65), visfatin, adiponectin, PPARδ, and PPARγ and to investigate the effects of total HDL (tHDL) and its subpopulations (HDL2 and HDL3) on these parameters in TNF-α stimulated adipocytes.

MATERIALS AND METHODS

Materials

3T3-L1 preadipocytes were obtained from Zen-Bio Inc (North Carolina, USA). Preadipocyte growth medium-2 (PGMTM-2) BulletKitTM, and AdipoRedTM assay reagent were purchased from Lonza Walkersville Inc (MD, USA). Phosphate-buffered saline (PBS), Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and penicillin/streptomycin antibiotic were purchased from GIBCO (Grand Island, NY). tHDL, HDL2, and HDL3 (1.0 mg) were purchased from Academy Biomedical Inc (Texas, USA). Recombinant Mouse TNF-α was purchased from R&D System Inc. (Minneapolis, USA). (3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide or MTT assay was purchased from Fluka, Germany.

3T3 cell culture and treatment

3T3-L1 preadipocytes were cultured into fully adipocytes according to manufacturer instructions. Briefly, preadipocytes 3T3-L1 were cultured in PGMTM-2 until confluent. After that, cells were induced differentiation by cultured in a preadipocyte growth medium until fully matured. Fully differentiated 3T3-L1 adipocytes (Day 15) were then cultured with a treatment medium containing DMEM, 10% FBS, and 1% penicillin/streptomycin antibiotic in the presence of tHDL, HDL2, or HDL3 with and without TNF-α (10 ng/mL). Cells were maintained at 37°C in a humidified 5% CO_2 incubator.

MTT cell viability assay

Various concentration of tHDL, HDL2 or HDL3 [0.1, 2, 6, 10, 20, 60, and 100 µg/mL (0.1 mg/mL)] were tested for cell viability against 3T3-L1 adipocytes by using MTT assay (Mosmann, 1983). Tests were performed in 96-well plates and incubated for 24 h. Before measuring viability, 20 µL of MTT solution was added to each well and incubated for 4 h at 37 °C. After that, the media was removed and replaced with 100 µL DMSO. The plates were incubated for another 10 to 15 min at room temperature. The absorbance was measured at 540 nm in a plate reader (Tecan Safire2, Austria) to determine the formazan concentration, which is proportional to the number of viable cells.

Quantification of lipid content by AdipoRedTM assay

Lipid content was measured using a commercially available kit (AdipoRed Assay Reagent). Test experiments were done on undifferentiated 3T3-L1 preadipocytes, differentiated 3T3-L1 adipocytes on day 9, and fully mature 3T3-L1 adipocytes on day 15 to see the progress of lipid accumulation in the cells. Before assay, cells were washed with PBS and 100 µL of PBS was added to the wells. Adipored reagent (30 µL) was added to each well. After 10 min, the fluorimeter and fluorescence were measured with an excitation wavelength of 485 nm and emission wavelength of 572 nm.

Protein expression

tHDL, HDL2, and HDL3 at the concentration of 0.1 mg/mL were incubated in TNF-α (10 ng/mL) stimulated adipocytes. After 24 h of incubation, the cell culture medium was collected and stored at -80°C until performing analysis. The protein expression of ABCA1, SRBI, IL-6, NF-κβ (p65), visfatin, and adiponectin were measured by ELISA kit according to the standard protocol provided by the manufacturer.

Gene expression

The gene expression of ABCA1, SR-B1, IL-6, NF-κβ (p65), visfatin, adiponectin, PPARδ, and PPARγ were measured by Quantigene Plex according to the standard protocol provided by the manufacturer. The results were measured using the Bio-Plex system (Bio-Rad Laboratories; Hercules, California). Results were normalized against Hypoxanthine guanine phosphoribosyl transferase-1 (HPRT-1) and Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to obtain the norm ratio.

Statistical analysis

Results are expressed as mean ± SD from 3 independent experiments (n=3). The analysis of variance (ANOVA) with a post-Hoc test was performed. The type of post-Hoc analysis used was Bonferroni. The differences between each HDLs and control were analyzed with Bonferroni post Hoc analysis. All data were analyzed by a statistical package program, SPSS version 20.0. The level of significance was set at p<0.05.

RESULTS

MTT cell viability assay

Incubation of various concentrations of tHDL, HDL2 or HDL3 [0.1, 2, 6, 10, 20, 60, and 100 µg/ml (0.1 mg/mL)] in adipocytes showed no toxicity effects (Data not shown). Therefore 100 µg/ml or 0.1 mg/mL concentration of tHDL, HDL2, or HDL3 was used in this study.

The incubation period of preadipocytes to mature adipocytes

High secretion of lipid droplets is one of the potent characteristics of mature adipocytes. Based on Figure 1, the differentiation of 3T3-L1 from preadipocytes to mature adipocytes took 15 days after growth in the differentiation medium. The accumulation of lipid droplets gradually increased from 30 RFU on the first day of cultured to 777 RFU on day 9. The lipid

accumulation was increased up to 1728 RFU on day 15 as the adipocytes reached their mature state.

Effects of tHDL, HDL2, and HDL3 on ABCA1 and SR-B1 protein and gene expression in inflamed adipocytes

ABCA1 and SR-B1 are ATP binding cassette transporters that play important role in assisting reverse cholesterol transport (RCT). In this study, the ABCA1 protein expression in TNF-α stimulated adipocytes was slightly lower than unstimulated controls but not statistically significant (Figure 2a). Interestingly, the incubation of 0.1 mg/mL tHDL, HDL2, or HDL3 in TNF-α stimulated adipocytes significantly increased the ABCA1 protein expression (p<0.0001) where tHDL showed the highest ABCA1 protein expression when compared to TNF-α alone. This study also found that HDL2 secreted more ABCA1 protein than HDL3, however, there is *no statistically significant difference between them.* This showed that both HDL2 and HDL3 play a similar role in promoting the secreting of ABCA1. At the gene expression levels, no significant changes were observed on the ABCA-1 when the cells were incubated with TNF-α alone and co-incubation with tHDL and its population (HDL2 and HDL3) (Figure 2b). Incubation of tHDL to the inflamed adipocytes significantly increased SR-B1 protein expression (Figure 3a). Interestingly, this study postulated that the positive effects of HDL in promoting the secretion of SR-B1 might be due to HDL3. This is because only HDL3 shows a significant elevation of SR-B1 protein, while HDL2 only shows a slight increment. However, at the gene expression level, tHDL, HDL2, or HDL3 could not increase SR-B1 gene expression in inflamed adipocytes. Only a slight increment of SR-B1 gene expression was observed when the adipocytes were co-incubated with tHDL or HDL3 (Figure 3b).

Effects of tHDL, HDL2, and HDL3 on the secretion of inflammatory markers [IL-6, NF-κβ (p65)] in inflamed adipocyte

Inflammation is one of the factors that impair reverse cholesterol transport. In this study, TNF-α stimulated adipocytes showed higher protein and gene expression of IL-6, p<0.0001 (Figure 4) and NF-κβ (p65), p<0.05 (Figure 5) compared to the unstimulated group. The incubation of tHDL and its subpopulation (HDL2 and HDL3) into the inflamed adipocytes significantly reduced both protein and gene expression of IL-6. In addition, protein secretion of NF-κβ (p65) was also reduced by the presence of tHDL and its subpopulation (HDL2 and HDL3). While at the gene expression level, only a trend of NF-κβ (p65) gene expression reduction was observed when the inflamed adipocytes were incubated with HDLs. It showed that HDL and its subpopulation have protective effects in suppressing the inflammation in the adipocytes induced by TNF-α as shown by the reduction in the protein expression level.

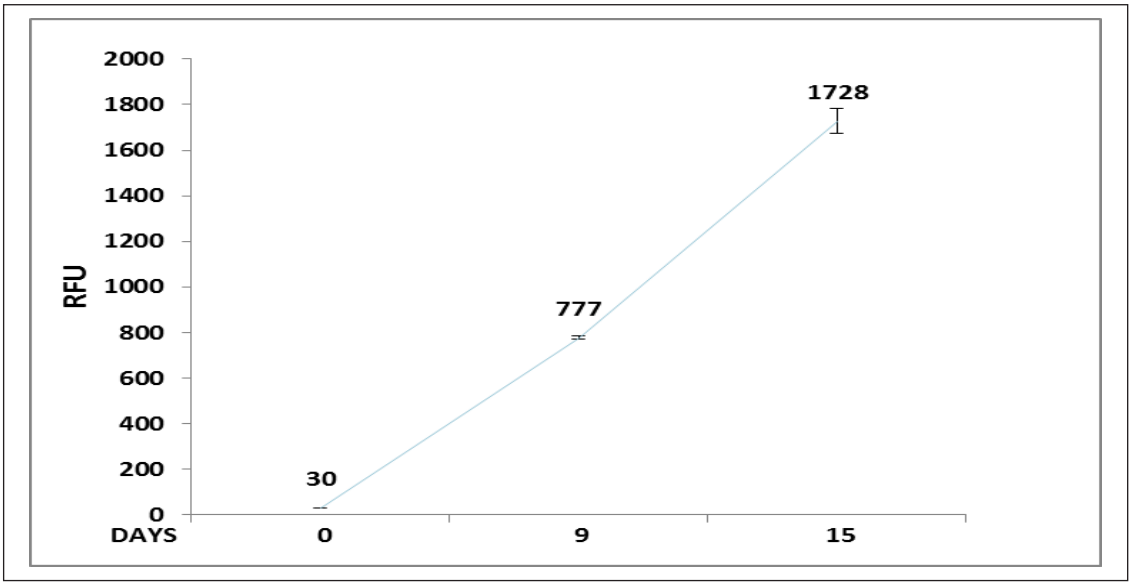

Fig. 1. Lipid droplet accumulation of 3T3-L1 cells indicating the differentiation of preadipocytes into mature adipocytes. Preadipocytes 3T3-L1 cells were cultured in PGMTM-2 until reaching confluency. Then, cells were induced to differentiate by culturing in a preadipocyte growth medium until fully matured. Lipid droplet accumulation on days 0, 9, and 15 was measured by using the AdipoRed Assay Reagent kit.

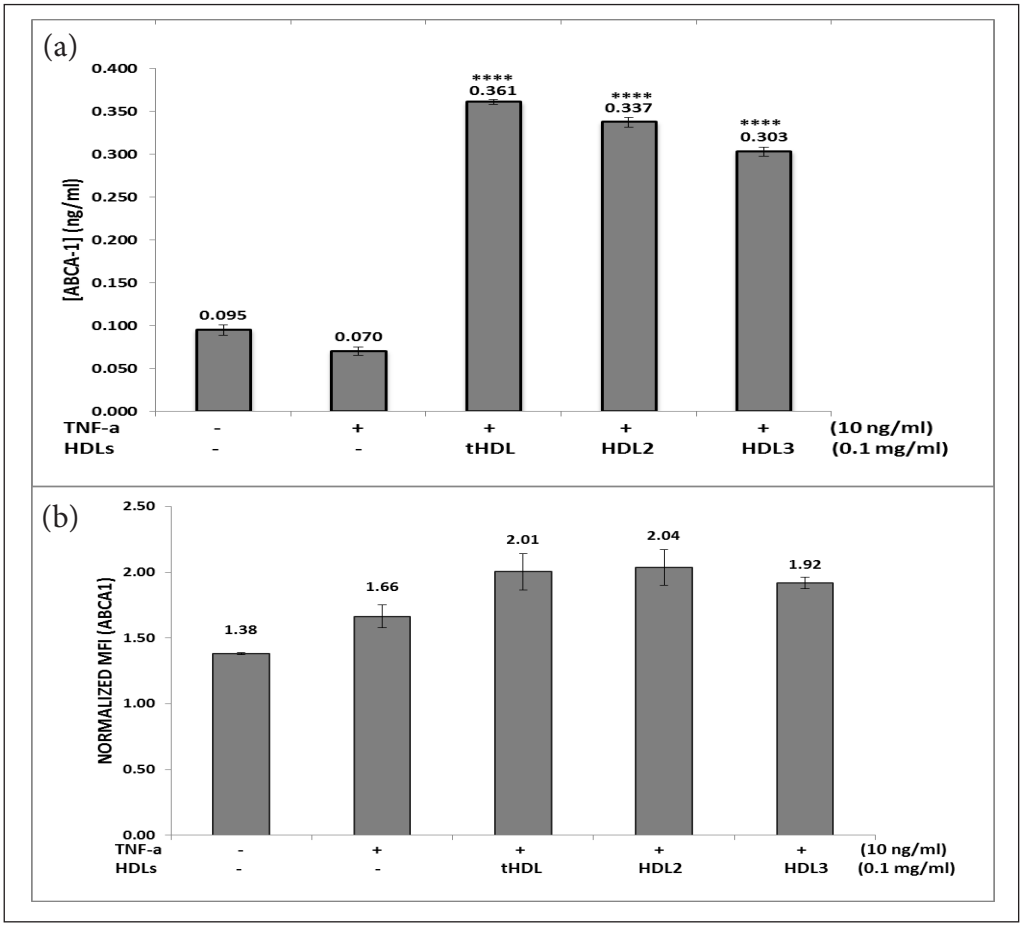

Fig. 2. Effects of tHDL and its HDL2 and HDL3 subpopulations on the (a) protein expression and (b) gene expression of ABCA1. Fully differentiated 3T3-L1 adipocytes (Day 15) were cultured in the treatment medium with 0.1 mg/mL tHDL, HDL2, or HDL3, and 10 ng/mL TNF-α. After 24 h incubation, protein and gene expression of ABCA1 in inflamed adipocytes were measured. GAPDH and HPRT1 were used as the reference genes for the gene expression study. Data are expressed as Mean ± SD from 3 independent experiments (n=3). ****p<0.0001 compared to TNF- α alone.

Effects of tHDL, HDL2, and HDL3 populations on the secretion of adipokines in inflamed adipocytes

Visfatin is an adipokine that is secreted by adipocytes and its high secretion is commonly related to inflammation. In this study, a slight increment of visfatin protein secretion was observed in the inflamed adipocytes compared to adipocytes induced by TNF-α alone (Figure 6). The incubation of tHDL (p<0.01) and its subpopulation (HDL2 & HDL3) significantly reduced protein secretion of visfatin. Interestingly, in between HDL subpopulations, HDL3 (p<0.0001) showed a better effect than HDL2 (p<0.01) in suppressing protein secretion of visfatin.

Adiponectin is another important adipokine secreted by adipocytes. Unlike visfatin, adiponectin plays important role in preventing or suppressing inflammation in adipocytes. In this study, the protein secretion of adiponectin was highly elevated when the inflamed adipocytes were incubated with tHDL, HDL2, and HDL3 (Figure 7). In addition, inflamed adipocytes incubated with both HDL2 (p<0.0001) and HDL3 (p< 0.0001) had higher adiponectin protein expression compared to the tHDL (p<0.01).

Effects of tHDL, HDL2, HDL3 on PPAR-δ and PPAR-δ mRNA expression

PPAR-δ has been postulated to play role in promoting reverse cholesterol transport. In this study, a slight increment of PPAR-δ mRNA expression was observed when the inflamed adipocyte was incubated by tH HDL3 and had a higher increment trend of PPAR-δ mRNA expression compared to HDL2 (Figure 8). There was no increment trend of PPAR-γ exhibited by HDL incubation (Figure 9). However, there was a significant reduction of PPAR-γ showed by HDL2 (p<0.01).

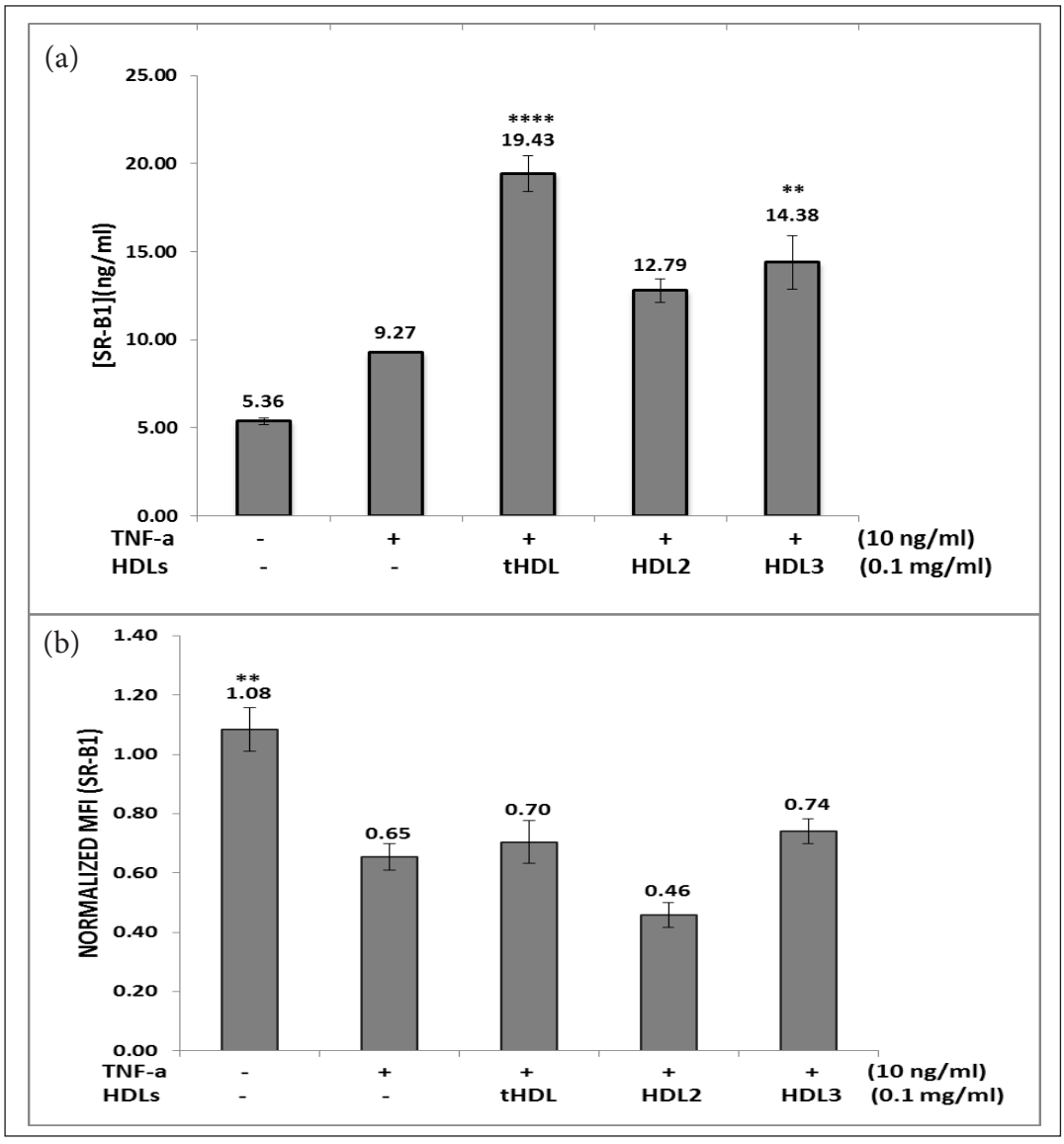

Fig. 3. Effects of tHDL and its HDL2 and HDL3 subpopulations on the (a) protein expression and (b) gene expression of SR-B1. Fully differentiated 3T3-L1 adipocytes (Day 15) were cultured in the treatment medium with 0.1 mg/mL tHDL, HDL2, or HDL3 and 10 ng/mL TNF-α. After 24 h incubation, protein and gene expression of SR-B1 in inflamed adipocytes were measured. GAPDH and HPRT1 were used as the reference genes for the gene expression study. Data are expressed as Mean ± SD from 3 independent experiments (n=3). ****p<0.0001 and **p<0.01 compared to TNF- α alone.

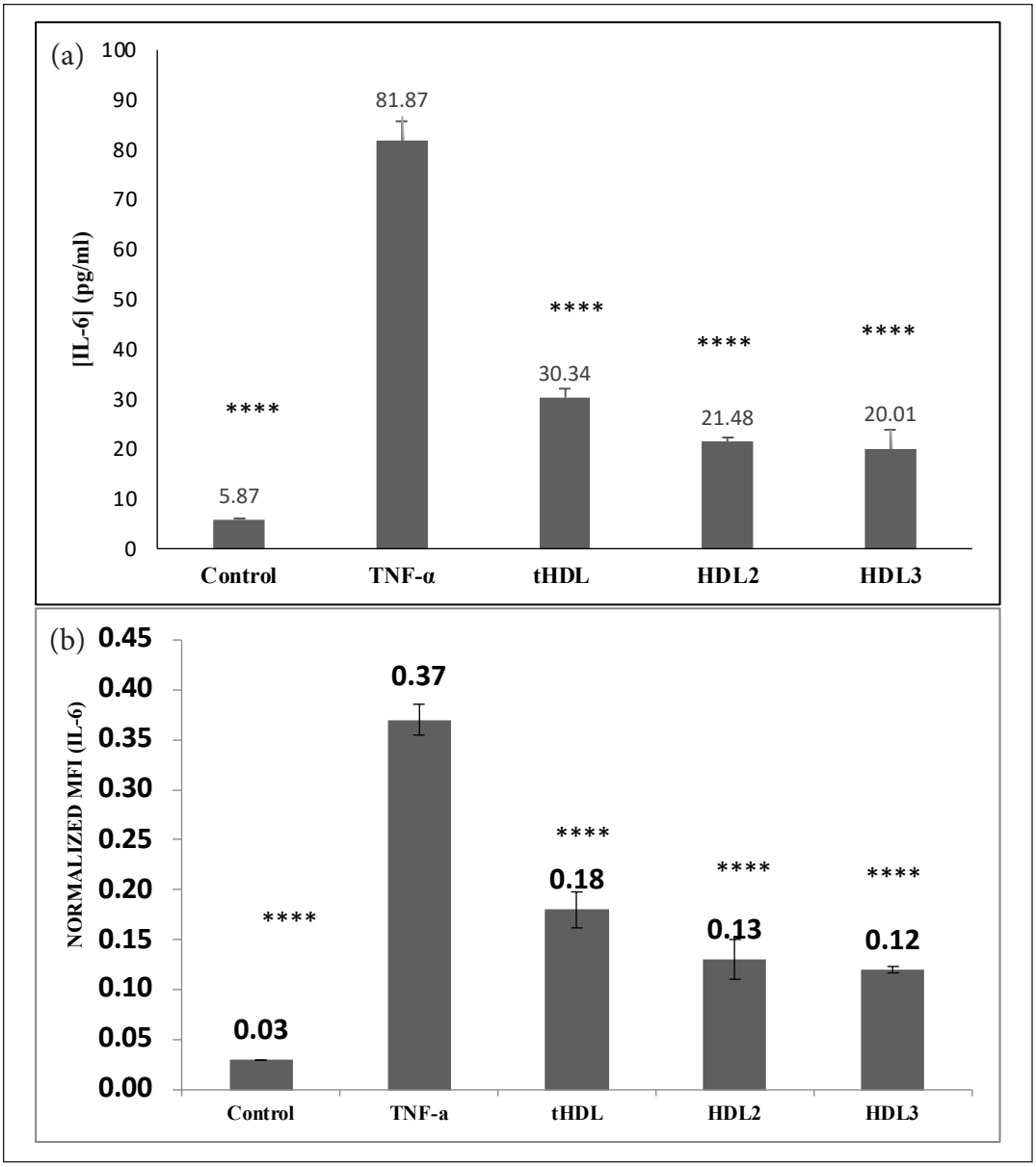

Fig. 4. Effects of tHDL and its HDL2 and HDL3 subpopulations on the (a) protein and (b) gene expression of IL-6. Fully differentiated 3T3-L1 adipocytes (Day 15) were cultured in the treatment medium with 0.1 mg/mL tHDL, HDL2, or HDL3 and 10 ng/mL TNF-α. After 24 h incubation, protein and gene expression of IL-6 in inflamed adipocytes were measured. GAPDH and HPRT1 were used as the reference genes for the gene expression study. Data are expressed as Mean ± SD from 3 independent experiments (n=3). ****p<0.0001 compared to TNF- α alone.

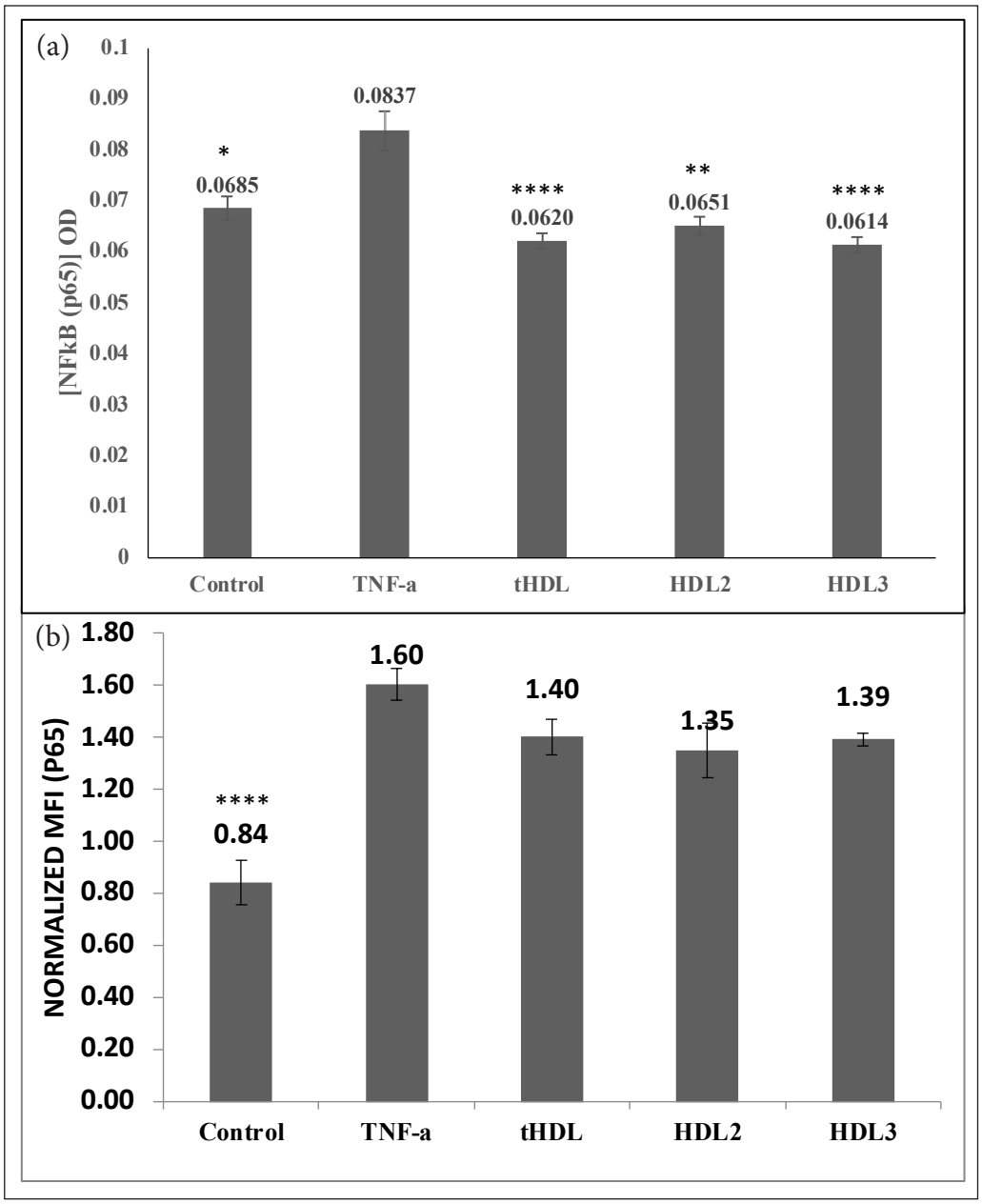

Fig. 5. Effects of tHDL and its HDL2 and HDL3 subpopulations on the (a) protein and (b) gene expression of NF-κβ (p65). Fully differentiated 3T3-L1 adipocytes (Day 15) were cultured in the treatment medium with 0.1 mg/mL tHDL, HDL2, or HDL3 and 10 ng/mL TNF-α. After 24 h incubation, protein and mRNA gene expression of NF-κβ (p65) in inflamed adipocytes were measured. GAPDH and HPRT1 were used as the reference genes for the gene expression study. Data are expressed as Mean ± SD from 3 independent experiments (n=3). ****p<0.0001, **p<0.01, * p<0.05 compared to TNF- α alone.

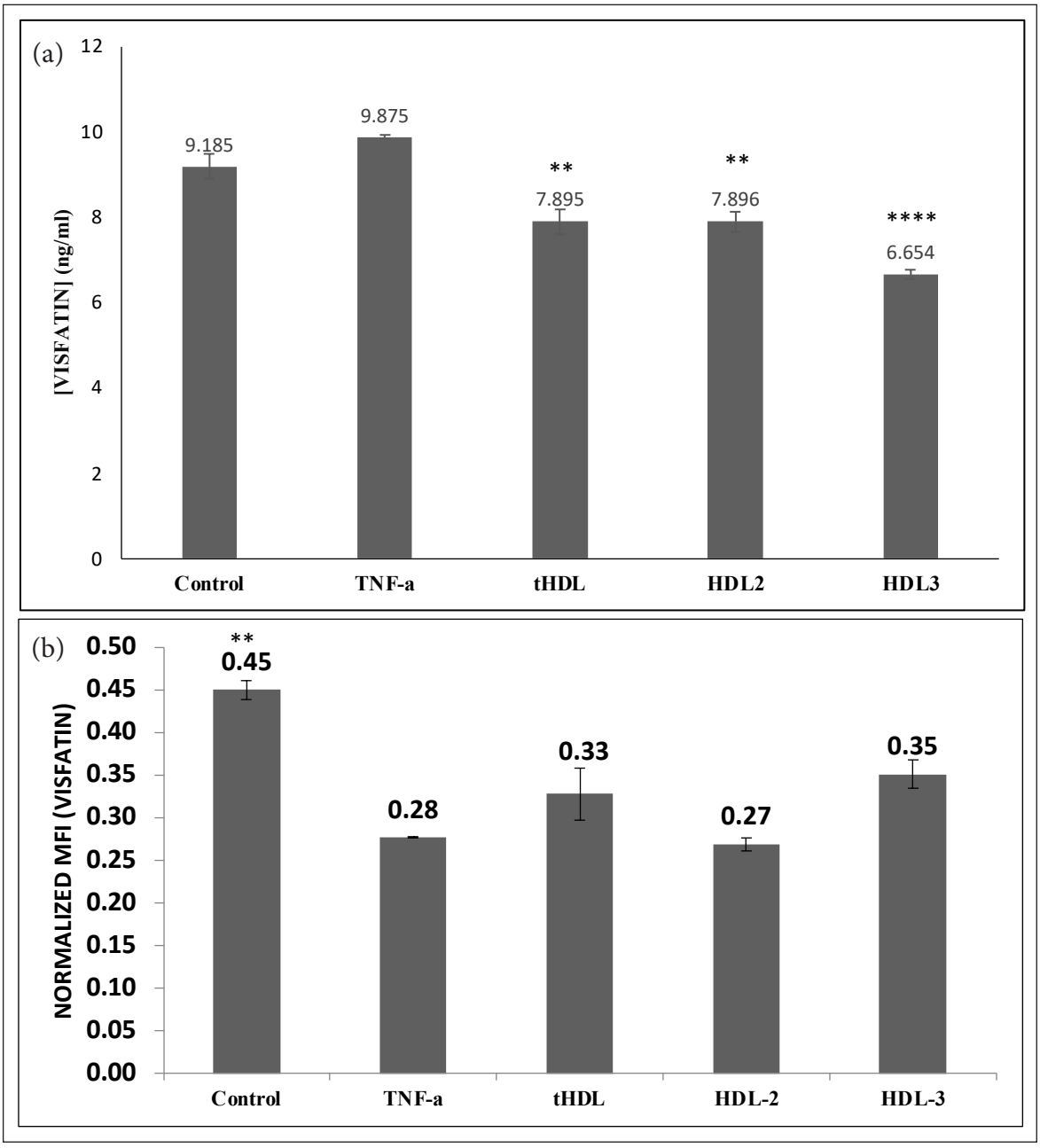

Fig. 6. Effects of tHDL and its HDL2 and HDL3 subpopulations on the (a) protein and (b) gene expression of visfatin. Fully differentiated 3T3-L1 adipocytes (Day 15) were cultured in the treatment medium with 0.1 mg/mL tHDL, HDL2, or HDL3 and 10 ng/mL TNF-α. After 24 h incubation, protein and gene expression of visfatin in inflamed adipocytes were measured. GAPDH and HPRT1 were used as the reference genes for the gene expression study. Data are expressed as Mean ± SD. ****p<0.0001 and **p<0.01compared to TNF- α alone.

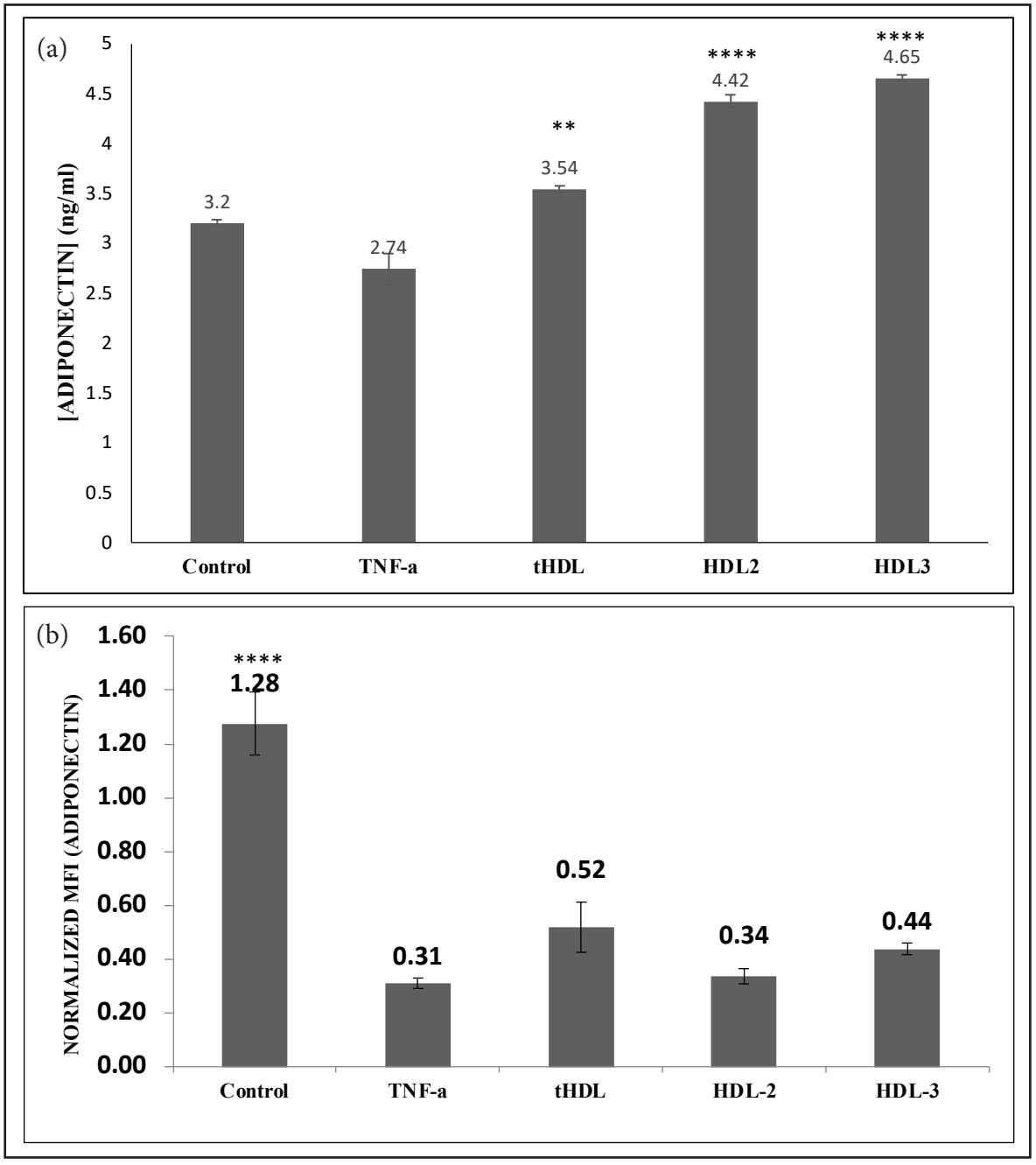

Fig. 7. Effects of tHDL and its HDL2 and HDL3 subpopulations on the (a) protein and (b) gene expression of adiponectin. Fully differentiated 3T3-L1 adipocytes (Day 15) were cultured in the treatment medium containing DMEM, 10% FBS, and 1% penicillin/streptomycin antibiotic in the presence of 0.1 mg/mL tHDL, HDL2, or HDL3 and 10 ng/mL TNF-α. After 24 h incubation, protein and gene expression of adiponectin in inflamed adipocytes was measured. GAPDH and HPRT1 were used as the reference genes for the gene expression study. Data are expressed as Mean ± SD. ****p<0.0001 and **p<0.01compared to TNF- α alone.

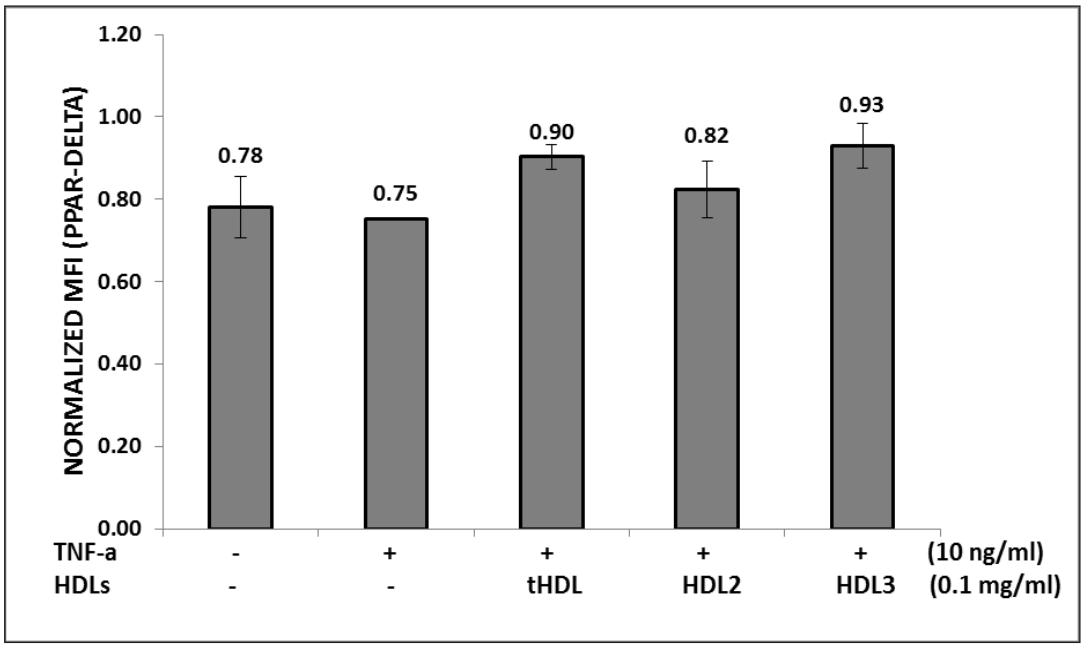

Fig. 8. Effects of tHDL and its HDL2 and HDL3 subpopulations on PPAR-δ gene expression in inflamed adipocytes. Fully differentiated 3T3-L1 adipocytes (Day 15) were cultured in the treatment medium with 0.1 mg/mL tHDL, HDL2, or HDL3 and 10 ng/mL TNF-α. Gene expression of PPAR- δ in inflamed adipocytes was measured by using the Quantigene Plex method after 24 h incubation. Data are expressed as Mean ± SD.

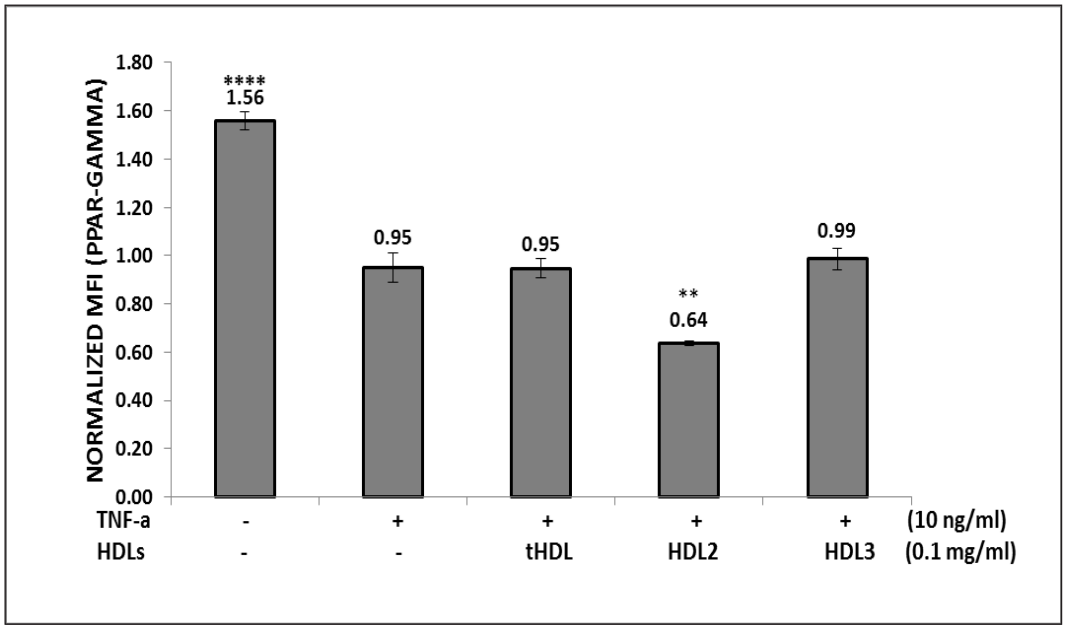

Fig. 9. Effects of tHDL and its HDL2 and HDL3 subpopulations on PPAR-γ gene expression in inflamed adipocytes. Fully differentiated 3T3-L1 adipocytes (Day 15) were cultured in the treatment medium with 0.1 mg/mL tHDL, HDL2, or HDL3 and 10 ng/mL TNF-α. Gene expression of PPAR- γ in inflamed adipocytes was measured by using the Quantigene Plex method after 24 h incubation. Data are expressed as Mean ± SD. ****p<0.0001 compared to TNF- α alone.

DISCUSSION

The 3T3-L1 cell line, a fibroblast-like cell that morphologically resembles a preadipocyte has been used in this study since it could differentiate into an adipocyte-like phenotype when cultured in a suitable media (Rizatti *et al*., 2013). The lipid droplet embedded in the cytoplasm of 3T3-L1 was linearly increased upon the culturing due to the elevation of triglyceride synthesis. In this study, it has been shown that the accumulation of lipid droplets from the preadipocyte state gradually increased on day 9 and subsequently on day 15. At this stage (day 15) the cell undergoes clonal expansion followed by subsequent terminal differentiation. During terminal differentiation, the size of the lipid droplet enlarges and coalesces to form one large lipid vacuole that indicated the mature state of the adipocyte (Niemela *et al*., 2008). In adipogenesis, a lot of transcription factors are subsequently activated one of them is peroxisome proliferator-activated receptor-γ (PPAR-γ). Activation of PPAR- γ leads to the activation of adipocyte-specific genes and trigger the growth arrest of adipocyte which are crucial for adipocyte differentiation (Niemela *et al*., 2008). The significant reduction of PPAR- γ by HDL2 in the inflamed adipocyte in this study might be because the HDL2 suppressed the adipocyte differentiation induced by the TNF-α.

In a previous study, a reduction of cholesterol transporter such as ABCA1 and SR-B1 in inflamed adipocytes commonly led to a reduction of cholesterol efflux activity by the adipocytes (Zhang *et al*., 2010). In a similar study, it is reported that cholesterol efflux activity through ABCA1 and SR-B1 receptors was reduced with total HDL (tHDL) incubation., In this study, we investigated the protein and gene expression of these cholesterol transporters in TNF-α stimulated adipocytes incubated with tHDL and its subpopulations (HDL 2 & HDL3).

Adipocytes support the transfer of cholesterol to HDL *in vivo* as well as *in vitro* by the action of ABCA1 and SR-BI (McGillicuddy *et al*., 2011). It was reported that the expression of cholesterol transporter (ABCA1 & SR-B1) is reduced in partially differentiated inflamed adipocytes (Zhang *et al*., 2010). However, in this study, only ABCA1 finding is conquered to that Zhang *et al*., but not SR-B1 protein expression. In this study, the SR-B1 expression in TNF-α incubated adipocytes have a higher trend of expression, but it is not significant. The difference in the result may be due to adipocytes used in this study being fully differentiated not partially differentiated has been used by Zhang *et al*. (2010).

In this study, incubation of 0.1 mg/mL tHDL, HDL2, and HDL3 in inflamed adipocytes significantly increase the ABCA1 protein and gene expression, where tHDL showed the highest ABCA1 expression. While in between subpopulation, HDL2 exhibit higher ABCA1 protein expression compared to HDL3. It has been reported that lipid-rich HDL2 has a higher amount of Apo-AI compared to HDL3. Apo-AI promotes cholesterol efflux by binding to the cellular cholesterol efflux pumps, ABCA1 (Eren *et al*., 2012). ABCA1 mediates lipidation of lipid-poor apolipoprotein (Apo-AI) with phospholipids and free cholesterol to produce nascent HDL which is a crucial step for the formation of mature HDL (Yin *et al*., 2010). Numerous studies show that the antiatherogenic and antioxidant properties of HDL are mainly due to the presence of Apo-AI and HDL2 exerts the most protective role against CAD compared to another HDL subpopulation (Eren *et al*., 2012). Lesser Apo-AI content in HDL3 may contribute to the lesser ABCA1 expression compared to HDL2. Interestingly, HDL3 is also causing a significant increment of ABCA1 expression in this study even though not as high as HDL2. Elevation of ABCA1 expression by HDL may then promote reverse cholesterol transport in adipocytes as shown in this study.

However, in this study, HDL2 does not promote the protein secretion of SR-B1, another important cholesterol transporter in reverse cholesterol transport. Interestingly, HDL3 shows prominent activity in promoting the protein secretion of SR-B1. There are conflicting facts on which one of the HDLs subpopulations has prominent activity in promoting reverse cholesterol transport. Even though a lot of publications emphasize that HDL2 plays a more prominent role in reverse cholesterol transport, however, a study done by Martin *et al*. (2015) found that HDL3 plays a more crucial role in reverse cholesterol transport and contains more ApoA1 than HDL2. The same article also states that 75% of HDL cholesterol in circulation exists in the form of the HDL3 subpopulation (Martin *et al*., 2015). Interestingly, this study supports the finding obtained by Martin *et al*. (2015), in which HDL3 can promote the secretion of both ABCA1 and SR-B1 in the inflamed adipocytes compared to HDL2 that only promote ABCA1. In addition, the increment of the SR-B1 secretion by the HDL3 was responsible for suppressing the secretion of inflammatory markers [IL-6 and NF-kB (p65)] and visfatin and increasing adiponectin secretion in the inflamed adipocyte which was better than HDL2.

SR-B1 has been known to have anti-inflammatory properties especially in suppressing or reducing the inflammation promoted by the LPS. *In vivo* study conducted by Cai *et al*. (2012) showed that SR-BI-null mice have a higher pro-inflammatory response to LPS compared to wild-type (WT) mice. A similar investigator also proved that SR-BInull primary macrophages exhibited higher inflammatory cytokine response compared to the control macrophages. Overexpression of SR-BI in the

macrophages attenuated the inflammatory response induced by LPS. This previous study supported our postulation in which significant increments of SR-B1 expressed by HDL3 play a crucial role in suppressing inflammation. Interestingly, a high elevation of pro-inflammatory cytokines (IL-6 and TNF-α) was observed in the mice that received SR-BI-null cells compared to the mice that received WT cells (Cai *et al*., 2012). This might explain why the inflamed adipocytes have lower secretion of IL-6 when induced by HDL3, as HDL3 promoted the secretion of SR-B1. In addition, disruption of SR-B1 expression also led to the activation of the NF-kB signaling pathway. Macrophages isolated from SR-BI-null mice showed higher activation of NF-kB compared to the macrophages isolated from wild-type mice (Guo *et al*., 2009). Besides, according to Guo *et al*. (2009), SR-BI could suppress TLR4-mediated NF-κB activation.

Increase secretion of visfatin and decrease secretion of adiponectin are some of the characteristics of inflamed adipocytes. In this study, the presence of HDLs in the inflamed adipocyte significantly reduced the secretion of visfatin and significantly increased the secretion of adiponectin. Result obtained in this present study is concurrent with the study conducted by Song *et al*. (2016), in which HDL reduced the secretion of visfatin induced by oxLDL in 3T3-L1 differentiated adipocytes, by upregulating the SR-BI expression (Song *et al*., 2016).

In this study, we also found that the incubation of 100 µg/mL HDL especially HDL3 in inflamed adipocytes increased the expression of PPAR-δ. According to Vrins *et al*. (2009), the activation of PPAR-δ increases the level of HDL and enhanced the cholesterol efflux of THP1 human monocytes to apolipoprotein (Apo) A-I (Vrins *et al*., 2009). Besides, PPAR-δ is also involved in stimulating the proliferation of preadipocytes by inducing the activity of PPAR-α which significantly promotes adipogenesis (Luquet *et al*., 2005). The expression of PPAR-δ was also higher in inflamed adipocytes without HDL treatment. The inflammation induced by TNF-α in the adipocyte enhances the expression of PPAR-δ through the stress kinase signaling pathway. While the expression of PPAR-α was inhibited by the presence of TNF-α and the incubation of HDL in the inflamed adipocytes did not increase the expression of PPAR-α. Therefore, it can be concluded that HDL promotes cholesterol efflux in inflamed adipocytes by enhancing the expression of ABCA1 through the PPAR-δ pathway.

CONCLUSIONS

This study exhibits an increase in cholesterol transporters expression by HDL and its subpopulation (HDL 2 and HDL3) which subsequently leads to reduced secretion of inflammatory markers (IL-6, NF-kB (p65), and visfatin). It is also responsible for the increase in adiponectin secretion in inflamed mature adipocytes. Findings from this study also suggested that the beneficial effects of HDL3 as an anti-inflammatory are superior to HDL2. HDL especially HDL3 exhibits beyond its reverse cholesterol transporter property by exhibiting anti-inflammatory effects thru deactivation of NF-κβ (p65). This may contribute to reducing the progression of obesity-related complications.

ACKNOWLEDGEMENTS

This research was funded by the Fundamental Research Grant Scheme, Ministry of Higher Education Malaysia [Grant code: 600-RMI/FRGS 5/3 (35/2013)] and Long Research Grant Scheme, LRGS P3, Ministry of Higher Education Malaysia [Grant code: RMI/ST/LRGS5/3(2/2011)-2]. The study was carried out at the Institute of Pathology, Medical & Forensic Laboratory, and Institute of Medical Molecular Biotechnology, Universiti Teknologi MARA (UiTM), Malaysia.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

REFERENCES

Aprahamian, T.R. & Sam, F. 2011. Adiponectin in cardiovascular inflammation and obesity. *International Journal of Inflammation*, **2011**: 376909. https://doi.org/10.4061/2011/376909

Arvind, A., Osganian, S.A., Cohen, D.E. & Corey, K.E. 2019. Lipid and lipoprotein metabolism in liver disease [WWW Document]. Endotext. URL https://www.endotext.org/MDText (accessed 15.10.22).

Briand, F., Naik, S.U., Fuki, I., Millar, J.S., Macphee, C., Walker, M., Billheimer, J., Rothblat, G. & Rader, D.J. 2009. Both the peroxisome proliferator-activated receptor (PPAR) delta agonist, GW0742, and ezetimibe promote reverse cholesterol transport in mice by reducing intestinal re-absorption of HDL-derived cholesterol. *Clinical and Translational Science*, **2(2)**: 127–133. https://doi.org/10.1111/j.1752-8062.2009.00098.x

Cai, L., Wang, Z., Meyer, J.M., Ji, A. & Van Der Westhuyzen, D.R. 2012. Macrophage SR-BI regulates LPS-induced pro-inflammatory signaling in mice and isolated macrophages. *Journal of Lipid Research*, **53(8)**: 1472-1481. https://doi.org/10.1194/jlr.M023234

Camont, L., Chapman, M.J. & Kontush, A. 2011. Biological activities of HDL subpopulations and

their relevance to cardiovascular disease. *Trends in Molecular Medicine*, **17(10)**: 594-603. https://doi.org/10.1016/j.molmed.2011.05.013

Das, U.N. 2001. Is obesity an inflammatory condition? *Nutrition*, **17(11–12)**: 953–966. https://doi.org/10.1016/s0899-9007(01)00672-4

Eren, E., Yilmaz, N. & Aydin, O. 2012. High density lipoprotein and it's dysfunction. *The Open Biochemistry Journal,* **6**: **78-93**. https://doi.org/10.2174/1874091X01206010078

Gordon, S.M., Hofmann, S., Askew, D.S. & Davidson, W.S. 2011. High density lipoprotein: it's not just about lipid transport anymore. *Trends in Endocrinology & Metabolism,* **22(1)**: 9-15. https://doi.org/10.1016/j.tem.2010.10.001

Greenberg, A.S., Coleman, R.A., Kraemer, F.B., McManaman, J.L., Obin, M.S., Puri, V., Yan, Q.W., Miyoshi, H. & Mashek, D.G. 2011. The role of lipid droplets in metabolic disease in rodents and humans. *The Journal of Clinical Investigation*, **121(6)**: 2102-2110.

Guilherme, A., Virbasius, J.V., Puri, V. & Czech, M.P. 2008. Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. *Nature Reviews Molecular Cell Biology*, **9(5)**: 367-377. https://doi.org/10.1038/nrm2391

Guo, L., Song, Z., Li, M., Wu, Q., Wang, D., Feng, H., Bernard, P., Daugherty, A., Huang, B. & Li, X. A. 2009. Scavenger receptor BI protects against septic death through its role in modulating inflammatory response. *Journal of Biological Chemistry*, **284(30)**: 19826-19834. https://doi.org/10.1074/jbc.M109.020933

Gutierrez, D.A., Puglisi, M.J. & Hasty, A.H. 2009. Impact of increased adipose tissue mass on inflammation, insulin resistance, and dyslipidemia. *Current Diabetes Reports*, **9(1)**: 26–32.

Huang, J.P., Hsu, S.C., Meir, Y.J.J. Hsieh, P.S., Chang, C.C., Chen, K.H., Chen, J.K. & Hung, L.M. 2018. Role of dysfunctional adipocytes in cholesterol-induced nonobese metabolic syndrome. *Journal of Molecular Endocrinology*, **60(4)**: 309-323. https://doi.org/10.1530/JME-17-0194

Luquet, S., Gaudel, C., Holst, D., Lopez-Soriano, J., Jehl-Pietri, C., Fredenrich, A. & Grimaldi, P.A. 2005. Roles of PPAR delta in lipid absorption and metabolism: a new target for the treatment of type 2 diabetes. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, **1740(2)**: 313-317. https://doi.org/10.1016/j.bbadis.2004.11.011

Marso, S.P., Mehta, S.K., Frutkin, A., House, J.A., McCrary, J.R. & Kulkarni, K.R. 2008. Low adiponectin levels are associated with atherogenic dyslipidemia and lipid-rich plaque in nondiabetic coronary arteries. *Diabetes Care,* **31(5)**: 989-994. https://doi.org/10.2337/dc07-2024

Martin, S.S., Khokhar, A.A., May, H.T., Kulkarni, K.R., Blaha, M.J., Joshi, P.H., Toth, P.P., Muhlestein, J.B., Anderson, J.L., Knight, S. & Li, Y. 2015. HDL cholesterol subclasses, myocardial infarction, and mortality in secondary prevention: the lipoprotein investigators collaborative. *European Heart Journal*, **36(1)**: 22-30. https://doi.org/10.1093/eurheartj/ehu264

McGillicuddy, F.C., Reilly, M.P. & Rader, D.J. 2011. Adipose modulation of high-density lipoprotein cholesterol implications for obesity, high-density lipoprotein metabolism, and cardiovascular disease. *Circulation*, **124(15)**: 1602-1605. https://doi.org/10.1161/CIRCULATIONAHA.111.058453

Mosmann, T. 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, **65(1-2)**: 55-63. https://doi.org/10.1016/0022-1759(83)90303-4

Murphy, A.J. 2013. High density lipoprotein: assembly, structure, cargo, and functions. *ISRN Physiology*.

Niemelä, S., Miettinen, S., Sarkanen, J.R., & Ashammakhi, N. 2008. Adipose tissue and adipocyte differentiation: molecular and cellular aspects and tissue engineering applications. *Topics in Tissue Engineering*, **4(1)**: 26.

Oliver, W.R., Shenk, J.L., Snaith, M.R., Russell, C.S., Plunket, K.D., Bodkin, N.L. & Xu, H.E. 2001. A selective peroxisome proliferator-activated receptor δ agonist promotes reverse cholesterol transport. *Proceedings of the National Academy of Sciences,* **98(9)**: 5306-5311. https://doi.org/10.1073/pnas.091021198

Olzmann, J.A. & Carvalho, P. 2019. Dynamics and functions of lipid droplets. *Nature Reviews Molecular Cell Biology*, **20(3):** 137-155. https://doi.org/10.1038/s41580-018-0085-z

Rizzatti, V., Boschi, F., Pedrotti, M., Zoico, E., Sbarbati, A. & Zamboni, M. 2013. Lipid droplets characterization in adipocyte differentiated 3T3-L1 cells: size and optical density distribution. *European Journal of Histochemistry*, **57(3)**: 24. https://doi.org/10.4081/ejh.2013.e24

Ruiz Estrada, M.A., Swee Kheng, K. & Ating, R. 2019. The Evaluation of Obesity in Malaysia. SSRN Electron. https://doi.org/10.1007/s11892-009-0006-9

Saha, S., Graessler, J., Schwarz, P.E., Goettsch, C., Bornstein, S.R. & Kopprasch, S. 2012. Modified high-density lipoprotein modulates aldosterone release through scavenger receptors via extracellular signal-regulated kinase and janus kinase-dependent pathways. *Molecular and Cellular Biochemistry*, **366(1-2)**: 1-10. https://doi.org/10.07/s11010-012-1274-2

Shuhei, N., Söderlund, S., Jauhiainen, M.& Taskinen, M.R. 2010. Effect of HDL composition and

particle size on the resistance of HDL to the oxidation. *Lipids in Health and Disease*, **9(1)**: 104. https://doi.org/10.1186/1476-511X-9-104

Song, G., Wu, X., Zhang, P., Yu, Y., Yang, M., Jiao, P., Wang, N., Song, H., Wu, Y., Zhang, X. & Liu, H. 2016. High-density lipoprotein inhibits ox-LDL-induced adipokine secretion by upregulating SR-BI expression and suppressing ER Stress pathway. *Scientific Reports*, **6**:30889. https://doi.org/10.1038/srep30889

Soumyarani, V.S. & Jayakumari, N. 2012. Oxidatively modified high density lipoprotein promotes inflammatory response in human monocytes–macrophages by enhanced production of ROS, TNF-α, MMP-9, and MMP-2. *Molecular and Cellular Biochemistry*, **366 (1-2)**: 277-285. https://doi.org/10.1007/s11010-012-1306-y

Sun, K., Kusminski, C.M. & Scherer, P.E. 2011. Adipose tissue remodeling and obesity. *The Journal of Clinical Investigation*, **121(6)**: 2094-2101. https://doi.org/10.1172/JCI45887

Superko, H.R., Pendyala, L., Williams, P.T., Momary, K.M., King III, S.B. & Garrett, B.C 2012. High-density lipoprotein subclasses and their relationship to cardiovascular disease. *Journal of Clinical Lipid*, **6(6)**: 496-523. https://doi.org/10.1016/j.jacl.2012.03.001

Verghese, P.B., Arrese, E.L., & Soulages, J.L. 2007. Stimulation of lipolysis enhances the rate of cholesterol efflux to HDL in adipocytes. *Molecular and Cellular Biochemistry*, **302(1-2)**: 241-248. https://doi.org/10.1007/s11010-007-9447-0

Vrins, C.L., van der Velde, A.E., van den Oever, K., Levels, J.H.M., Huet, S., Oude Elferink, R.P.O., Kuipers, F. & Groen, A.K. 2009. Peroxisome proliferator-activated receptor delta activation leads to increased transintestinal cholesterol efflux. *Journal of Lipid Research*, **50(10)**: 2046–2054. https://doi.org/10.1194/jlr.M800579-JLR200

Wang, Y., Wang, X., Guo, Y., Bian, Y., Bai, R. Liang, B. & Xiao, C. 2017. Effect of adiponectin on macrophage reverse cholesterol transport in adiponectin-/-mice and its mechanism. *Experimental and Therapeutic Medicine,* **13(6)**: 2757-2762. https://doi.org/10.3892/etm.2017.4321

Wellen, K.E. & Hotamisligil, G.S. 2003. Obesity-induced inflammatory changes in adipose tissue. *The Journal of Clinical Investigation*, **112(12)**: 1785-1788. https://doi.org/10.1172/JCI20514

Xu, S., Zhang, X. & Liu, P. 2018. Lipid droplet proteins and metabolic diseases. *Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease*, **1864(5)**: 1968-1983. https://doi.org/10.1016/j.bbadis.2017.07.019

Xu, Y., Du, X., Turner, N., Brown, A.J. & Yang, H. 2019. Enhanced acyl-CoA: cholesterol acyltransferase activity increases cholesterol levels on the lipid droplet surface and impairs adipocyte function. *Journal of Biological Chemistry*, **294(50)**: 19306-19321. https://doi.org/10.1074/jbc.RA119.011160

Yin, K., Liao, D.F., & Tang, C.K. 2010. ATP-binding membrane cassette transporter A1 (ABCA1): a possible link between inflammation and reverse cholesterol transport. *Molecular Medicine*, **16(9)**: 438. https://doi.org/10.2119/molmed.2010.00004

Yoshikawa, M., Sakuma, N., Hibino, T., Sato, T., & Fujinami, T. 1997. HDL3 exerts more powerful anti-oxidative, protective effects against copper-catalyzed LDL oxidation than HDL2. *Clinical Biochemistry*, **30(3)**: 221-225. https://doi.org/10.1016/s0009-9120(97)00031-3

Zhang, Y., McGillicuddy, F.C., Hinkle, C.C., O'Neill, S., Glick, J.M., Rothblat, G. H. & Reilly, M.P. 2010. Adipocyte modulation of high-density lipoprotein cholesterol. *Circulation*, **121(11)**: 1347. https://doi.org/10.1161/CIRCULATIONAHA.109.897330