Dietary Curcumin Intervention Targets Both White Adipose Tissue Inflammation and Brown Adipose Tissue Thermogenesis

by

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A thesis submitted in conformity with the requirements
for the degree of Master of Science

Department of Physiology
University of Toronto

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2017

Abstract

Increased adipose tissue inflammation and reduced energy expenditure are among the key causative factors of obesity and its related metabolic disorders. Therapeutic tools targeting these two pathological events are desired. Previous studies have mainly attributed the body-weight lowering effect of curcumin and other dietary polyphenols to their anti-inflammatory properties in adipose tissues and elsewhere. We found that in high fat diet (HFD)-fed mice, dietary curcumin intervention reduced not only epididymal adipose tissue macrophage infiltration but also the ratio of M1-like versus M2-like macrophages. *In vitro* curcumin treatment also reduced pro-inflammatory cytokine expression in macrophages and adipocytes. Furthermore, curcumin intervention in HFD-fed mice increased energy expenditure and body temperature in response to cold challenge, associated with increased brown adipocyte uncoupling protein 1 expression. We conclude that curcumin-intervention plays a dual modulatory role in preventing metabolic disorders by attenuating white adipose tissue inflammation and increasing brown adipocyte activity.

Acknowledgments

My foremost sincere gratitude and thanks to my supervisor, Dr. Tianru Jin, for accepting my application of undergraduate internship and taking me as his graduate student, providing me chances and opportunities to learn and study in his lab. With his help, the great capacity of working and thinking independently and academically was developed. Also, Thanks for the financial support from him, covering most of the expenses during my graduate study and ADA conference attending. For these and many other reasons, I would to present my most gracious appreciation to Dr. Jin.

Secondly, I would like to thank Dr. Weijuan Shao for her valuable advice and the most precious encouragements. Dr. Shao helped me contact with Dr. Jin so that I could have the opportunity to start my research life in the University of Toronto when I was still an undergraduate student in Peking University in China. Many thanks to her for equipping me with essential laboratory techniques. Her care about my life in Toronto is also appreciated. Thank you!

There are many thanks to Dr. George Fantus and Dr. Daniel Winer not only for being my supervisory committee but also for giving my valuable advices. My research had several hard times and it was tough to push forward. Thanks for the help from you to broaden my perspective on the research, leading to a new angle.

I would like to thank Dr. Patricia Brubaker, Dr. Denise Belsham and Dr. Heyu Ni for being my thesis defense examiner. Their support and suggestions that were extremely helpful and added valuable improvements to my thesis.

I would like to thank Dr. Zongping Feng and Dr. Hongshuo Sun for the excellent teaching and course guidance. I learnt to read articles and think in a more scientific and critical way on the courses.

I would like to thank Dr. Wilfred Ip, Dr. Alex Chiang and Dr. Joanna Xiong in the lab helped me a lot to get familiar with the study and life in Canada. Thanks for teaching me mice pancreatic perfusion, islet isolation, hepatic perfusion and other laboratory techniques.

Thanks for the guidance from Dr. Dehua Cui, Dr. Nanping Wang, Dr. Chunyan Zhou, Dr. Yuedan Wang, Dr. Weiping Wang, Dr. Zhuqing Jia during my undergraduate study. Great

laboratory techniques and research academic experience were obtained early in my university life.

I wish to thank all the volunteers, students and post-docs I have met and worked with during my study. Thanks for giving me so much help around the lab.

Very special thanks to my dearest friends. Many thanks to Jingqiong Wang for all the understanding, support and encouragement she has offered. Also, I would like to thank Sijie Wang for chemical synthesizing and purifying curcumin in his lab for me. Hopefully we can meet in Japan in September 2017. Many special thanks to Dr. Shuai Cao, Dr. Peng Wang, Dr. Nan Wang, Congcong Zhu, Siyuan Guo, Zimeng Zhang, Liuqingqing Yang, Xiaoshen Ma and all other friends of mine.

Finally, I would like to thank my parents Jincai Song and Li Sun. They are not in my study field and may not able to help me with my academic achievements, however, their grace of parenting me for over twenty years is appreciated. In Chinese traditions, children usually do not travel far from their parents. I realized that it has been three years since the last time I stay with my father. Thanks for the understanding and supporting me to study abroad. I cannot possibly express the deep gratitude with any more words. I will be back to China to see my parents and other family members, sisters and brothers after graduation.

This work was supported by an operating grant by the Canadian Institutes of Health Research (MOP 97790) to Tianru Jin. I am supported by a Banting and Best Diabetes Centre (BBDC) graduate studentship. Lili Tian is supported by a BBDC post-doctoral fellowship. Kejing Zeng is the recipient of the Scholarship for International Program for Ph.D. candidates, Sun Yat-Sen University.

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List of Abbreviations

AGIs	Alpha-glucosidase inhibitors
ARG1	Arginase 1
ATP	Adenosine triphosphate
AUC	Area under the curve
BAT	Brown adipose tissue
BMI	Body mass index
BSA	Bovine serum albumin
CCL	Chemokine (CC motif) ligands
COX	Cyclooxygenase
CRP	C-reactive protein
CtBP1/2	C-terminal-binding protein 1/2
Cur	1% Curcumin mixed high fat diet / Curcumin treatment
CXCL	Chemokine (C-X-C motif) ligand
DM	Diabetes mellitus
DMEM	Dulbecco's modified Eagle's medium
ELISA	Enzyme-linked immunosorbent assay
FFA	Free fatty acid
FGF21	Fibroblast growth factor 21
FIZZ1	(cysteine-rich secreted protein) Found in inflammatory zone 1
FoxC2	Forkhead box C2
GDM	Gestational diabetes mellitus
GLP-1	Glucagon-like peptide-1
hERG	Human ether-a-go-go-related gene
HFD	High fat diet
i.p.GTT	Intraperitoneal glucose tolerance test
i.p.ITT	Intraperitoneal insulin tolerance test
i.p.PTT	Intraperitoneal pyruvate tolerance test
ICAM-1	Intercellular Adhesion Molecule 1
IDDM	Insulin-dependent diabetes mellitus
IFNγ	Interferon γ
IKKβ	Inhibitor of nuclear factor kappa-B kinase subunit beta
IL	Interleukin
iNOS	Cytokine-inducible nitric oxide synthases
IRS-1	Insulin receptor substrate-1
JNK	c-Jun N-terminal kinases
KLF4	Kruppel-like factor 4
LFD	Low fat diet
LPS	Lipopolysaccharide
LUC	Luciferease
mBAT	Mouse brown adipocyte

MCP-1/CCL2	Monocyte chemoattractant protein 1
MTT	Thiazolyl blue tetrazolium bromide
MYF5	Myogenic factor 5
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NIDDM	Non-insulin-dependent diabetes mellitus
NMR	Nuclear magnetic resonance
NSAIDs	Non-steroidal anti-inflammatory drugs
PAI-1	Plasminogen activator inhibitor-1
PCR	Polymerase chain reaction
PEI	Polyethyleneimine
PGC-1α	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PPARα/γ	Peroxisome proliferator-activated receptor α/γ
PRDM16	PR domain containing 16
Qcunp	Quercetin-decorated liposomes of curcumin
RER	Respiratory exchange ratio
STAT1	Signal transducer and activator of transcription 1
T1D	Type 1 diabetes
T2D	Type 2 diabetes
TBX1	T-box protein 1
TLR4	Toll-like receptor 4
TMEM26	Transmembrane protein 26
TNFα	Tumor necrosis factor alpha
UCP1	Uncoupling protein 1
VCAM-1	Vascular cell adhesion protein 1
WAT	White adipose tissue
WHO	World Health Organization

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General Introduction

1 Diabetes, its prevention, and treatment

1.1 Historical recognition of diabetes as a metabolic disease

It has been 3000 years since the first time that diabetes was described by ancient physicians[1]. Ancient Indian physicians observed that the urine from diabetic people attracted ants. They called this urine as "honey urine"[1]. In ancient China, the diabetes condition was described as "Xiao Ke" or "Emaciation with extreme thirsty symptom" in ***Huangdi Neijing***, a Chinese traditional medical text written in 111AD.

In around 230 BC, the term "Diabetes" was developed by Apollonius of Memphis. The Greek meaning of "Diabetes" is "to pass through"[1] since they believed that the "honey urine" from diabetic patients resulted from a disease of the kidney.

Among all symptoms of diabetes recorded by various physicians throughout the history, "thirsty" and "weight loss" were the most mentioned. Aretaeus of Cappadocia, a Greek physician, described diabetes mellitus in his work ***On the Causes and Indications of Acute and Chronic Diseases***:

> *Diabetes is a dreadful affliction, not very frequent among men, being a melting down of the flesh and limbs into urine. The patients never stop making water and the flow is incessant, like the opening of the aqueducts.*

Even it has been hundreds of years since diabetes was recognized and various therapies have been developed during the history, mechanisms underlying the disease remained unclear until around 1900s. In 1889, the Polish-German physician Oskar Minkowski and his colleague Joseph von Mering removed pancreas from a healthy dog. The dog died shortly after the surgery. Interestingly, sugar was found in the dog's urine, suggesting the relationship between the function of pancreas and diabetes[2]. Before conducting this experiment, Paul Langerhans identified island-like tissue clumps in the pancreas under the microscope in German. Later, Edouard Laguesse, a French pathologist and histologist, suggested that these clumps may produce some vital secretions that regulate food digestion. Diabetes was attributed to the lack of

these secretions. The name “insulin” is from Latin *insula* (meaning “island”) as they were thought to be produced by the “tissue islets”.

In the year of 1921, Frederick Banting and Charles Best repeated the above experiment on dogs with the help of John Macleod at the University of Toronto. Furthermore, they tried to cure the dog with the extraction of healthy dog pancreas. Marjorie, which was the dog that received the extraction injection, could remain alive. The next year, Leonard Thompson, the first person who received canine insulin injection[3], was saved from diabetes mellitus.

The Nobel Prize in Physiology or Medicine was awarded to Frederick Banting and John Macleod in 1923, only two years after the above discovery. The patent for insulin was sold to the University of Toronto for $1.5 which made it possible that the production and utilization of insulin spread all over the world fast.

1.2 General Information on diabetes and its classification

Diabetes mellitus (DM), referred as diabetes hereafter, is one of the most common chronic diseases in the world. The number of people living with diabetes has increased from 108 million in the year of 1980 to 422 million in 2014[4]. It will be the seventh leading cause of death in 2030 all around the world, predicted by the World Health Organization (WHO). Diabetes is a metabolic disorder characterized by the long-term high plasma glucose level (2-hour after diet blood glucose level is higher than 11.1 mmol/L or fasting glucose is higher than 7.0 mmol/L)[5]. The classical symptoms of diabetes are polyphagia, polydipsia, polyuria and unexplained loss-of-weight[6]. Lack of in-time treatment will cause numerous complications such as a headache, blurred vision and slow-healing wounds and some other acute-complications, resulted from microcirculatory disturbance. Long-term hyperglycemia may lead to cardiovascular diseases, kidney diseases, retinopathy and diabetic feet. The primary causes of blindness, kidney failure, heart attacks, stroke and lower limb amputation are also diabetes mellitus[6].

There are three major types of diabetes, classified based on the pathogenesis: type 1 diabetes (T1D), type 2 diabetes (T2D) and gestational diabetes.

Type 1 diabetes, also known as insulin-dependent diabetes mellitus (IDDM), is characterized by the lack of insulin produced by islet β-cells. Most IDDM subjects are children or juvenile so traditionally it was named as “Juvenile diabetes”. T1D is caused by β-cell deficiency due to

genomic mutations or immune-mediated autoimmune diseases. It could be an inherited disease with multiple gene mutations[7]. Although the pathogenic mechanism is not fully understood, most T1D patients can live healthy with insulin administration.

Type 2 diabetes, also known as non-insulin-dependent diabetes mellitus (NIDDM), is considered as an acquired disease. T2D can be described as a comparative absence of insulin. It is a metabolic disorder with decreased insulin sensitivity and increased blood glucose without pathological changes in the pancreas at its onset stage. Insulin secretion becomes insufficient with the progression of the disease. Epidemiology studies indicated that obese individuals have a higher risk of diabetes development. Tissue inflammation is another causative factor of T2D independently from obesity. Recent studies have shown that T2D is highly associated with adipose tissue inflammatory response and its crosstalk with the liver tissue[8].

Gestational diabetes mellitus (GDM) is the third common type of diabetes. The inadequate insulin secretion and decreased insulin sensitivity can also be observed in GDM individuals during pregnancy. Though most of the GDM are transient, 5-10% of GDM patients will eventually develop T2D. The risk of macrosomia, congenital heart, intrauterine fetal distress of babies is elevated without careful medical supervision and treatment throughout the pregnancy.

1.3 Diabetes prevention and treatment

Diabetes mellitus is a chronic metabolic disease. To maintain euglycemic and to prevent obesity are vital means in the prevention of diabetes. Although there is no known established prevention method to T1D[7], however, T2D could be prevented or treated with diet control, exercise and normal-weight maintenance. Those approaches are collectively known as “Lifestyle modifications”[9].

The proper utilization of anti-diabetic agents is dependent on disease type, age, gender and some other criteria, aiming at maintaining euglycemia. The currently approved anti-diabetic drugs are summarized in Table 1. Insulin injection is a known effective measure to reduce hyperglycemia in all diabase types, especially in T1D. The treatments of T2D include methods to increase insulin production and secretion, increase glucose transportation and uptake, to increase insulin sensitivity or to reduce glucose uptake in gut and glucose resorption by the kidney. Biguanide reduces hepatic glucose neogenesis and increases glucose uptake[10]. Thiazolidinedione sensitizes

Table 1. List of currently approved anti-diabetic drugs

Category	Name	Mechanism
Insulin	Animal Insulin Semisynthetic Human Insulin Recombinant Human Insulin	Promotes glucose disposal
Biguanide	Metformin	Reduces hepatic glucose neogenesis and increase glucose uptake
DPP4 inhibitor	Sitagliptin Retagliptin Vildagliptin Saxagliptin Algogliptin Ligelieting Teneligliptin Gemigliptin Anagliptin Omarigliptin Trelagliptin	Increases incretin levels and reduces glucagon release
SGLT2 inhibitor	Dapagliflozin Canagliflozin Empagliflozin Luseogliflozin Tofogliflozin Ipragliflozin Hengliflozin	Prevents glucose reabsorption from the glomerular filtrate in the kidneys
GLP-1 receptor agonists	Liraglutide Dulaglutide Albiglutide Insulin Glargine Insulin Degludec	Promote endogenous insulin secretion
Thiazolidine dione	Rosiglitazone Pioglitazone	Sensitizies insulin signal through PPAR dependent signaling pathways
Sulfonylurea	Acetohexamide, Glibenclamide Glimepiride	Promote endogenous insulin secretion
AGIs	Acarbose Miglitol Voglibose	Inhibits glucose digestion and absorption

insulin signal through the peroxisome proliferator-activated receptor (PPAR) dependent signaling pathways[11]. Sulfonylurea and recently developed Glucagon-like peptide-1 (GLP-1) agonists[12] promote endogenous insulin secretion by β-cells. Alpha-glucosidase inhibitors (AGIs) inhibits glucose digestion and absorption in gut[10].

2 Obesity and Inflammation

There is a dramatic increase in the incidence of obesity during the past two decades. Importantly, the increase applies to both adults and children. Obesity-related complications such as T2D, non-alcoholic fatty liver disease (NAFLD) and atherosclerosis are threatening our lives and bringing enormous economic burdens globally. Epidemiological and laboratory studies have revealed that obesity is related to be development of insulin resistance and T2D. Studies have also shown that metabolic and immune systems were closely related due to the demand of energy storage and host defense[13]. Evidently, obese individuals show elevated inflammatory cytokine levels in their plasma[14], such as C-reactive protein (CRP), interleukin-6, and plasminogen activator inhibitor-1 (PAI-1)[15]. These cytokines are also related to the development of insulin resistance and T2D[15,16].

Liver and adipose tissues are tightly involved in both metabolic and immune responses. Chronic metabolic disorders, including the lack of or the excessive of nutrients, lead to the development of disorders in the immune system[17]. Recent studies have also revealed that high fat diet (HFD) feeding alters expression of diet related neuropeptides including neuropeptide Y in hypothalamus. It is believed that hypothalamus plays a vital role in regulating inflammation in HFD-fed mice[18]. Saturated fatty acids induce neuroinflammation in the arcuate nucleus of the hypothalamus, which consequently affects energy homeostasis[19]. During the progression of metabolic diseases, the cross talk between these tissues plays important roles[20].

Other than the energy storage site, the adipose tissue is also an endocrine organ that produces peptides as well as adipo-cytokines in regulating inflammatory responses and other physiological processes. A well-known inflammatory reaction in the white adipose tissue is macrophage infiltration[21]. The over-expanded adipocytes recruit macrophage which expresses pro-inflammatory cytokines such as IL-1β, IL-6, TNF-α, and MCP-1[14,22]. MCP-1 receptor knock-out mice showed less macrophage infiltration and pro-inflammatory cytokines production[23]. Moreover, the massive capillary network in the adipose tissue promotes tissue inflammation. Studies have demonstrated that the vascular endothelial cell from mice receiving HFD produced

intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion protein 1 (VCAM-1), E-selectin, P-selectin that promoting inflammatory cells infiltration[24].

In the adipose tissue, studies have demonstrated that certain immune cytokines can activate c-Jun N-terminal kinases (JNK) and IKKβ/NF-κB signaling pathways, which in turn enhance tissue inflammatory responses[25]. The activated JNK phosphorylates c-Jun, consequently, triggers insulin resistance through insulin receptor substrate-1 (IRS-1) phosphorylation (Ser 307 in rat IRS-1 or Ser 312 in human IRS-1)[26,27]. Thus, anti-inflammatory agents are considered as potentially effective methods to improve insulin sensitivity[28].

3 Macrophages, their subtypes, and functions

Macrophages play crucial roles in both innate and adaptive immune responses. Macrophage infiltration activates inflammatory reaction in the adipose tissue. It has been demonstrated that high-calorie intake can induce macrophage infiltration[29]. Macrophages that are recruited to inflammatory sites in the response to inflammatory cytokines (such as MCP-1) play an important role during the progression of adipose tissue inflammation[30]. The inflammatory reaction, on the one hand, is essential for pathogen clearance, and will cause tissue damage. Thus at different stages of diseases, different macrophages are induced to either promote or inhibit inflammatory response[31]. Various stimuli drive macrophages to differentiate into a spectrum of subtypes, M1-like and M2-like are the major types of macrophages in adipose tissues. The differentiation of M1-like macrophages, or classically activated macrophages, is induced by pro-inflammatory factors, such as lipopolysaccharide (LPS) and interferon γ (IFNγ). The M1-like macrophage expresses pro-inflammatory cytokines such as IL-6, IL-12, MCP-1, TNFα and iNOS to adequately clear pathogens. M1-like macrophages take the vital role at the early stage of inflammatory responses. M2-like macrophages or alternatively-activated macrophages, however, are induced by Th-2 cytokines, such as IL-4 and IL-13. The activated M2-like macrophages do not express pro-inflammatory cytokines like TNFα, IL-6, IL-12 and IL-23, instead, they produce IL-10[32]. Studies have shown that M2-like macrophages express anti-inflammatory proteins, playing a role on inflammation suppression and tissue repairing[32].

The chemokine expression is different in the two types of macrophages as well. LPS activated M1-like macrophage showed increased expression of NF-κB-dependent CXCL1, 2, 3, 5, 8, 9, 10 and CCL2, 3, 4, 5, 11, 17[33]. The production of CXCL9, 10 and CCL5 activates signal transducer

and activator of transcription 1 (STAT1)[34]. The M2-like macrophage differentiation inhibits chemokine expression by M1-like cells. The IL-4 and IL-10 produced by M2-like macrophages inhibit CXCL9, 10 and CCL5 expression[35] via STAT3 activation[36] and NF-κB pathway inhibition[37]. Moreover, M2-like macrophages not only inhibit pro-inflammatory cytokines, IL-4 and IL-10 but also induce the expression of CCL2 (aka. MCP1)[38], which is a Th2 related chemokine.

In obese individuals, increased free fatty acid (FFA) activates Toll-like receptor 4 (TLR4)[39], leading to M1-like macrophage recruitment. Pro-inflammatory cytokines and chemokines produced by these M1 cells reduce insulin sensitivity and recruit more M1-like cells. Under the condition of insulin resistance, M2-like macrophage also plays a role against M1-like macrophages. IL-10 produced by M2-like cells inhibits inflammation and enhances insulin sensitivity. Furthermore, in the 3T3-L1 cell model, TNFα was shown to induce insulin resistance while IL-10 treatment was shown to reverse these process[40,41].

4 Energy balance, brown adipose tissue and uncoupling protein 1 (UCP1)

There are two main kinds of adipose tissue: brown adipose tissue (BAT) and white adipose tissue (WAT) in mammals. White adipose tissues are located subcutaneously and around organs. A white adipocyte contains one large lipid droplet with a few mitochondria. It stores energy in the form of triglyceride. White adipocytes produce adipokines such as adiponectin, leptin and resistin[42]. The expansion and proliferation of white adipocytes are highly related with obesity, inflammation and metabolic disorders.

Brown adipose tissues are located at supraclavicular, paravertebral and periadrenal regions in humans. In rodents, BAT can be found in the interscapular region. BAT plays the crucial role in body temperature maintenance and thermogenesis, and its function can be controlled by the sympathetic nervous system[43]. In brown adipocytes, there are multiple small lipid droplets with large amounts of mitochondria. Moreover, the uncoupling protein 1 (UCP1) is highly expressed in the inner membrane of the mitochondria, specifically in brown adipocytes[44]. It uncouples electron transport chain from oxidative phosphorylation and produces heat instead of synthesizing ATP to increase energy expenditure[45].

In addition to the brown adipose tissue and the white adipose tissue, a new type of adipocytes called “beige adipocyte” was discovered recently[46]. Beige adipocytes are derived from the white adipose tissue as an energy storage site. Besides, it can also produce heat by consuming more energy. The beige adipocyte in inguinal subcutaneous adipose tissue is activated via long-term cold stimulation, β-adrenergic receptor activation or PPARγ activation. This process is usually called “beiging” or “browning”[46,47].

Induced mouse beige adipocytes produce UCP1 similar to brown adipocytes. Moreover, beige adipocytes express CD137, TMEM26 and TBX1, which cannot be found in brown adipocytes. CD137 was considered as a specific marker for beige cell[48]. By analyzing adult human brown adipocytes, Wu *et al.* demonstrated that they not only highly express UCP1, but also beige cell specific markers (CD137, TMEM26, TBX1), indicating that most of the adult human brown adipocyte might be beige cells instead of brown adipocytes[46]. Studies on beige adipocytes indicated that impaired beige cell function resulted in mice metabolic disorders and adipose accumulation[49].

Brown adipocytes are differentiated from $MYF5^+$ cells that originated from muscle cells in embryos[50]. Brown adipocyte differentiation involves multiple transcription factors including PRD1-BF-1-RIZ1 homologous domain-containing protein-16 (PRDM16), PPARγ, PPARγ coactivator-1α (PGC-1α), C-terminal-binding protein 1/2 (CtBP1/2) and forkhead box C2 (FoxC2)[51]. The transcription factor PRDM16 is the key factor for brown adipocyte differentiation. It promotes thermogenic gene expression and inhibits white adipocyte-related gene expression[52]. Moreover, in $MYF5^+$ cells, the expression of PRDM16 inhibits myocyte related gene expression and muscle cell differentiation. The overexpression of PRDM16 in white adipocyte promotes $MYF5^-$ cells to be differentiated into beige adipocytes[53].

Puigserver, P. and Spiegelman, B M showed PPARγ increases thermogenic gene expression (UCP1, PGC-1α) via elevating PRDM16 expression to induce cell beiging[54]. FoxC2 activates β-adrenergic receptors and cAMP-PKA signaling pathway to promote the cell beiging process[55]. PGC-1α plays an important role in regulating mitochondrial function. The inhibition of PCG-1α during cell differentiation does not affect brown adipocyte differentiation but reduces thermogenic gene expression[56].

Following the recognition of various transcription factors that are involved in brown adipocyte differentiation and function, scientists may eventually be able to promote brown adipocyte differentiation and to enhance brown adipocyte activity for the prevention and treatment of obesity and its related metabolic disorders.

5 Dietary polyphenols and the curry compound curcumin

5.1 Three most studied dietary polyphenols

Enormous efforts have been made to search for agents or methods from various aspects to reduce obesity. Natural products have drawn considerable attention for their attenuating effect on inflammation and insulin resistance[57-60]. Anthocyanin, resveratrol and curcumin are the three most studied dietary polyphenols for the past two decades[61] (Figure 1).

Anthocyanin is a water-soluble plant pigment stored in the vacuole of plant cells (Figure 1A). Studies have shown that anthocyanin exerts many beneficial effects, including DNA damage protection[62], anti-oxidation[63] and anti-inflammation[64]. It was also demonstrated that dietary anthocyanin intervention could inhibit body weight gain, platelet hyperactivity, and hypertriglyceridemia[65]. HFD induced hyperglycemia, hyperinsulinemia and hyperleptinemia were also shown to be prevented by anthocyanin intervention in mice[66]. Platelets are involved in immune responses, atherosclerosis, angiogenesis, lymphatic vessel development, and liver regeneration[67]. The optimal platelet function can also be maintained by anthocyanin intervention in HFD rats[65]. The platelet granule promoted platelet adhesion and cardiovascular thrombi formation. It was also shown that the activation of PI3K-Akt signaling pathway in platelets could be inhibited by anthocyanin the platelet granule secretion was attenuated consequently[68]. Thus, the anthocyanin possesses cardiovascular protective effect.

Resveratrol is a non-flavonoid phenol which is primarily found in the skin of fruits such as grapes, raspberries and blueberries (Figure 1B)[69]. In 1999, It was reported that resveratrol dietary intervention up-regulated plasma adenosine and exhibited myocardio-protective effect[70]. Resveratrol was also shown to possess therapeutic and prevention potential for cardiovascular diseases[71] and joint disorders[72]. As a cyclooxygenase (COX) inhibitor, the anti-inflammatory function of resveratrol was also tested by many research groups[73,74].

Lipophilic polyphenol curcumin is one of the dietary hopanoids (Figure 1C). It is the principal curcuminoid of turmeric, a member of the ginger family[75]. Experimental animal studies have shown the capability of curcumin to mitigate inflammatory diseases, cancer, neurodegenerative diseases, diabetes, obesity, and atherosclerosis[57]. It has been reported that dietary curcumin reduces macrophage infiltration in adipose tissue (adipose tissue infiltrated macrophage, ATM) in mouse models[76]. Furthermore, extensive experimental animal studies, including a few from our group[58,60,76-79], and a handful of clinical studies have shown a plasma glucose and body weight lowering effect and insulin-sensitizing effect of turmeric or curcumin. Importantly, a recent small-scale clinical trial indicated that a 9-month dietary curcumin intervention in pre-diabetic subjects significantly lowered the number of individuals who eventually developed T2D[80]. Thus, the nutraceutical curcumin possesses the great potential not only in the treatment but also the prevention of diabetes development. During the past two decades, scientists have mainly attributed the metabolic beneficial effects of curcumin (and many other dietary polyphenols) to its anti-inflammatory and anti-oxidative properties.

5.2 Chemical properties of curcumin

Curcumin is extracted from the turmeric. It hasthree forms: curcumin, dimethoxy curcumin and bisdemethoxy curcumin[57] (Figure 1C). Most commercially available “curcumin” that has been utilized in various studies is the mixture of the three forms[81]. Although there are studies investigating the application on diabetes and its associated disorders with different curcuminoids[57], the difficulties of identifying the function of a particular structure cannot be ignored.

Studies with nuclear magnetic resonance (NMR) spectroscopy have indicated that curcumin contains a β-diketone moiety that induces keto-enol tautomers in solution, and the enol tautomer is more likely to appear at pH 3-9[82]. The enol tautomer results in a hydrogen-bonded structure and the calculated log P for this tautomer is between 2.3 and 3.2[83]. The low water solubility has created the difficulty, to date, in developing curcumin as an ideal pharmaceutic agent.

In addition, curcumin emits fluorescence in certain conditions. During our study, the auto-fluorescence was observed in living cells only 10 minutes after it was added to the culture medium. The fluorescence disappears after 10 hours, prompting us to wonder whether curcumin

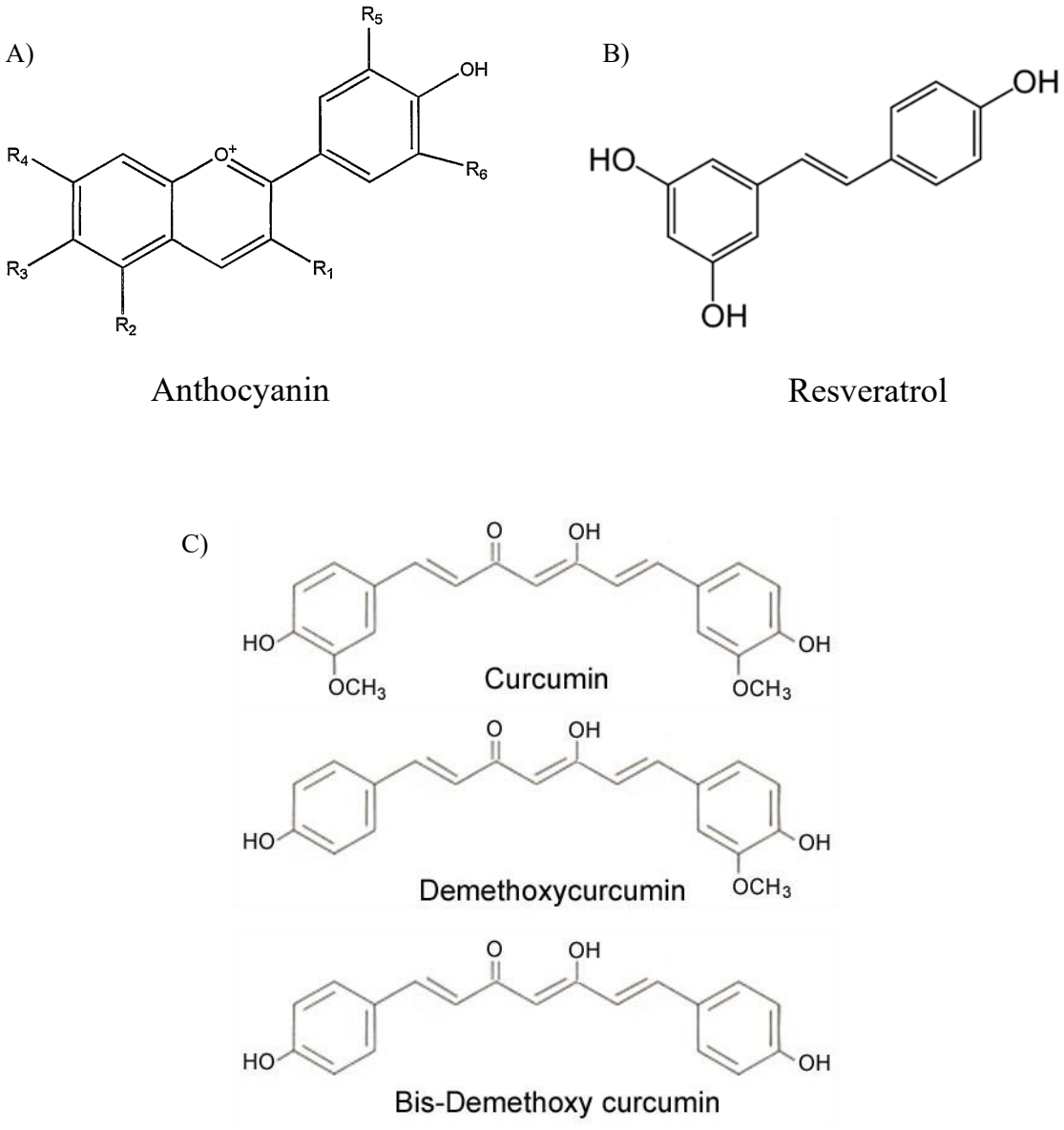

Figure 1. The chemical structures of the three most studied dietary polyphenols. (A) Anthocyanin. (B) Resveratrol. (C) Curcumin in three forms.

interferes with the lipid layers of the cell membrane. Due to the keto-enol tautomerism, the absorption maximum in organic solvents is ~408-430 nm while the emission peak is 460-560 nm. Thus, curcumin exhibits photophysical and photochemical properties with low yield and short halflife[84]. The potential fluorescence emitted by curcumin could interfere with the readouts of some assays that must be taken into consideration.

Another study suggested that the chemical stability of curcumin in aqueous buffers is not as long as an ideal drug[85]. The half-life of curcumin in aqueous buffer at neutral pH 7.5 and room temperature is approximately 20 minutes[86]. Curcumin is mainly degraded by oxidation[86] which contributes to its anti-oxidation property. However, studies have also shown that this instability can be improved with lipid or nanoparticle capsules[87].

5.3 Pharmacokinetics properties of curcumin

Although curcumin has been developed as a drug, somehow, bioavailability and pharmacokinetic properties of curcumin have been under intensive investigations[88]. A study on rats measuring plasma curcumin using validated liquid chromatography technique concluded that the oral bioavailability in rats is about 1%[89]. Another curcumin dose-tolerance study suggested that in human, curcumin only appears in serum with an oral dose up to 12g/day and the bioavailability from the gut limits the absorption of oral dosing of curcumin[90]. Methods to improve the absorption is being studied by many research groups and substantial progress have been achieved[91,92].

The distribution of curcumin through the body primarily affects pharmacological effects. It has been reported that curcumin is distributed throughout the whole body among different tissues[83,93]. These findings supported the concept that curcumin has multiple targets. However, whether curcumin molecules are degraded or modified upon they reach their targets and what are the bioactive compounds remains to be further investigated.

5.4 Potential toxicological effects

Although curcumin is a relatively safe compound in food, the potential toxic side effect of curcumin cannot be fully ignored. Studies have shown that curcumin inhibits human ether-a-go-go-related gene (hERG) potassium channels in whole-cell patch-clamp experiments in HEK293 cells[94]. This inhibition is considered as a potential cardiac toxic property of a drug. Besides, since

curcumin behaves as a metal chelation, iron deficiency could be induced by intensive curcumin administration with poor iron diet[95].

5.5 Cell membrane inference effects

Other studies showed that due to the keto-enol tautomerism, the absorption maximum in organic solvents is ~408-430 nm while the emission peak is 460-560 nm. Thus, curcumin exhibits photophysical and photochemical properties with low yield and short lifetime[96]. The depth of curcumin binding in the membrane bilayer structure was identified by fluorescence anisotropy measurements[97], using brominated derivatives of carboxylic acids. The acid acts as fluorescence quenchers that can be detected only at the specific depth of the bilayer as designed. The study implied that curcumin is localized deep in the hydrophobic acyl chain of the cell bilayer phospholipid membrane[98].

There are several studies on the influence of certain anti-inflammatory drugs on cell membrane fluidity[99,100] since the cellular function could be affected by the membrane fluidity[101]. It was suggested that the decreased membrane fluidity leads to the modification of membrane proteins *in vivo*, presumably because of the changes in the dynamic and orientation of membrane proteins floating within the lipid bilayer[102-104]. The modification of membrane physical characteristics, such as the fluidity and permeability would influence membrane enzyme and membrane-bond receptor activities, ion channels and consequently cell homeostasis and biological function[105,106].

The therapeutic effect of non-steroidal anti-inflammatory drugs (NSAIDs) was partly attributed to its modification on membrane phospholipid bilayer physical characteristics[101,104] Studies have shown that NSAIDs increased membrane fluidity in a dose-dependent manner[101]. In addition, NSAIDs increases the intracellular Ca^{2+} level by permeabilizing the calcium-loaded liposomes on gastric mucosal cell membrane[107]. Thus, it was proposed that the membrane fluidity affection of NSAIDs may play a major role in its therapeutic effects[101].

Curcumin, as described above, may interact with the in the lipophilic phase of the cell membrane, affecting cell fluidity as well[108]. Since curcumin has lipophilic properties, curcumin molecules may be exclusively distributed in cell membranous structures including the plasma membrane, endoplasmic reticulum and nuclear envelope[109]. However, the entry of cell nuclei was not observed[109]. Curcumin was localized in the hydrophobic hydrocarbon region of the

membrane bilayers[98] and the hydrocarbon region was considered to be significant in determining protein-lipid interactions and providing hydrophobic matrix area for certain proteins[110]. Similar to NSAIDs, curcumin increases cell membrane fluidity in a dose-dependent manner as well[108]. In addition, the membrane surface was also expanded by 1.8% while a non-bilayer structure was postulated as a mechanism of the appearance of the unbalanced cell membrane[108]. The non-bilayer structure requires lower energy for lipid transition allowing molecules to energy the cell more easily and faster through the cell membrane. In addition, curcumin increases the permeability of the mitochondrial membrane, decreases mitochondrial membrane potential and interference with the energy coupling system[108].

5.6 Curcumin clinical trials

Even curcumin was shown to be effective against various diseases in rodent models, clinical trials we not always succeed. The study focusing on the bioactivity of curcumin revealed that its absorption from the gut was limited. Curcumin is detectable in plasma only when subjects received a single dose of 10 or 12g[90]. The systemic exposing of curcumin is required for therapeutic utilities and methods to increase the absorption of orally dosed curcumin are under the development[111,112]. Scientists are also developing new delivery methods that enhance the curcumin bioactivity *in vivo*. A self-microemulsifying system[91] as well as a tri-component hybridization system[92] was proved to be effective in enhancing oral absorption of curcumin.

Two clinical studies also provided evidence that dietary curcumin can bring the beneficial effect in AD subjects[113,114]. However, another clinical trial performed with AD patients showed limited improvement after a 24-week of curcumin administration (2g/day or 4g/day)[115].

A clinical study on pancreatic cancer showed that despite the low absorption, and oral dose of curcumin induced marked tumor regression and one patient showed significant increase of serum levels of several cytokines (IL-6, IL-8, and IL-10)[116].

A 9-month clinical trial demonstrated that oral curcumin intervention improved β-cell function in prediabetic subjects along with improved insulin sensitivity. Moreover, curcumin intervention significantly prevented T2D development. 16.4% of subjects in the placebo-treated group have developed T2D while 0% from the curcumin intervention group became diabetic[80].

5.7 Curcumin carrier study

The clinical use of curcumin is limited due to the low water solubility and bioavailability. The investigation of carriers that help to enhance the curcumin absorption is highlighted. The methods that have been developed to date are summarized in below:

Curcumin nanoparticles are solid colloid particles with diameter ranged from 10-1000nm. The nanoparticle has high bioavailability and water solubility which is helpful with hydrophobic reagents' delivery[117]. A study showed that the bioavailability of curcumin entrapped in nanoparticles was significantly increased[117].

The solid dispersion of curcumin improved the curcumin function. In curcumin solid dispersion, curcumin is gathered in high polymer carriers in crystallized or single-molecular form. After orally administrating the modified curcumin in rats, the plasma concentration was 86-fold of increased than curcumin powder[118].

There are curcumin inclusion complexes in which curcumin molecule is entirely or partially packed within cyclodextrin. Sun, Y proved that the complexation of Cur and hydroxypropyl-beta-cyclodextrin (HP-beta-CD) presented higher solubility and curcumin stability[119].

Curcumin liposomes are lipid bilayer miniature globular carriers with curcumin packed inside. It helps curcumin to distribute into water and extends the effective time. Significant efficacy improvement was observed both *in vitro* and *in vivo* with Quercetin-decorated liposomes of curcumin (QCunp)[120].

Curcumin self-microemulsion delivery system has been developed in which curcumin molecules presented as isotropic mixtures of oils, surfactants, and co-solvents/co-surfactants, emulsifying with gentle agitation. Rabbits received oral administration of self-microemulsifying formulation containing curcumin; the plasma concentration is 10-fold higher than the rabbits received powder curcumin[91].

5.8 Dietary polyphenol intervention and lifestyle modification

Lifestyle modifications including dietary-intervention are alternative and promising tools for the treatment and prevention of obesity and its related metabolic disorders[121]. Dietary polyphenols including resveratrol, anthocyanin and curcumin have been found to improve insulin signaling,

reduce body weight gain, and prevent T2D development in animal models or pre-diabetic human subjects[61,80,122]. Previous investigations have attributed these effects of dietary polyphenols mainly to their anti-inflammation properties, secondary to their body weight lowering effect. Our laboratory has demonstrated recently that curcumin can improve insulin signaling independent of its anti-inflammation property[59] and this multiple target polyphenol can stimulate the production and function of the hepatic hormone fibroblast growth factor 21 (FGF21)[60].

Hypothesis and research aims

1 Hypothesis

The metabolic beneficial effect of dietary intervention with natural polyphenols has been drawing more and more attention of biomedical researchers. We have learned for a long time that dietary polyphenol intervention reduces body weight gain in response to HFD consumption and/or improves insulin sensitivity in animal models, or in human subjects[61,76,123]. However, our knowledge on the mechanistic insights of those polyphenols is limited. Despite enormous effectors with the generation of nearly 10 thousand publications, scientists have mainly attributed the beneficial effects, including the metabolic beneficial effects of plant polyphenols to their anti-inflammation and anti-oxidative effects.

However, a few recent studies conducted in the Jin's lab raised the question that at least for some polyphenols, such as curcumin, the body weight lowering effect as well as insulin signaling improvement effect could be both dependent and independent of the anti-inflammation[59,60]

In 2015, Tian and colleagues in our laboratory tested the hypothesis whether one of the dietary polyphenols, curcumin can exert the insulin signaling improvement effect, independently of its anti-inflammation effect[59]. In this study, they generated insulin resistance in C57BL/6 mice with dexamethasone injection consecutively for five days, in the absence and presence of curcumin gavage. Dexamethasone caused insulin resistance without affecting body weight and inflammation status, while concomitant curcumin intervention attenuated insulin resistance induced by dexamethasone injection, demonstrated by conducting intraperitoneal insulin tolerance test (i.p.ITT) as well as liver tissue PKB and GSK3 activation in response to insulin injection[59]. This is likely to be the first experimental evidence that curcumin can improve insulin signaling independently of its anti-inflammatory and body weight lowering effect.

In conducting the above investigation, Tian and colleagues also observed that short-term curcumin intervention increased the expression of fibroblast growth factor 21 (Fgf21) in the liver, as well as plasma Fgf21 levels[59]. Fgf21 is an endocrine hormone that improves metabolic profile of obese animals. Zeng and colleagues in our laboratory then conducted both *in vitro* and *in vivo* investigations, showing that *in vitro* curcumin treatment stimulated Fgf21 mRNA and protein expression in mouse and human hepatocytes, and the stimulation was also observed in

mouse liver with 4-day curcumin gavage. More importantly, increased hepatic Fgf21 expression was found in obese mice, either induced by HFD consumption or due to the genetic defect (i.e. ob/ob mice)[124]. Zeng and colleagues found that long-term (12 weeks) dietary curcumin intervention attenuated HFD induced Fgf21 elevation and improved Fgf21 resistance[60]. These two recent studies above in our laboratory added to our knowledge on the metabolic beneficial effect of the curry compound curcumin[59,60].

My study focuses on the adipose tissue, aiming to further expand our mechanistic understanding of the metabolic beneficial effect of dietary curcumin intervention. On one hand, we recognize the importance of the anti-inflammatory effect of curcumin in white adipose tissue. On the other hand, as curcumin and other dietary polyphenols are known to target multiple tissues/organs/cell lineages, I asked whether curcumin could exert its effect on BAT function.

My general hypothesis for this study is that curcumin intervention targets both WAT inflammation and BAT function. Furthermore, I hypothesize that the attenuation effect of curcumin intervention on WAT inflammation is achieved by affecting macrophage polarity and the regulation of anti-inflammatory and pro-inflammatory cytokines by both macrophages and adipocytes. I propose to conduct the following aims to test this hypothesis.

2 Research Aims

2.1 Aim 1 Whether curcumin intervention attenuates adipose tissue inflammation via regulating macrophage polarity and pro/anti-inflammation cytokine expression

Adipose tissue inflammation is a complex process, involving local pro-inflammatory cytokine expression and inflammatory immune cells recruitment[15]. The first aim of this study was to determine how curcumin reduces inflammation in white adipose tissue.

Our project started with the identification of epididymal adipose tissue infiltrated immune cell type and numbers in mice received long-term HFD consumption and curcumin intervention. The immune cell infiltration and the ratio of pro-inflammatory cells to immune regulatory cells (anti-inflammatory cells) were analyzed. Also, the expression and function of pro-inflammatory cytokines from both adipose tissue and infiltrated immune cells were assessed.

With our macrophage cell model Raw264.7, we could investigate the direct effect of curcumin on macrophage differentiation (M1-like versus M2-like) *in vitro*. With these findings, we tested our first hypothesis that curcumin attenuates adipose tissue inflammation by targeting both macrophage and white adipocytes.

2.2 Aim 2 Whether curcumin intervention increases energy expenditure

As attenuated body weight gain can also be attributed to the increase of body energy expenditure, the adipose tissue thermogenesis is considered as one of the key factors in regulating body energy balance[125,126]. I, therefore, proposed that curcumin directly stimulates BAT UCP1 expression. In our study, I conducted metabolic cage analyses on oxygen consumption, carbon-dioxide production, respiration exchange rate, energy expenditure and physical activity in mice under HFD feeding, with and without curcumin intervention.

I have also conducted a separated animal study in which mice were fed with LFD, HFD or HFD with curcumin. The mice were exposed to 4℃ for 48 hours. The mouse rectal temperature was recorded referred as the mouse core temperature[127]. The function of curcumin on regulating body temperature was tested.

Hepatic Fgf21 was shown to be stimulated by curcumin treatment *in vitro* and *in vivo* with curcumin intervention. Fgf21 is also expressed in adipocytes. Recent studies suggested that Fgf21 facilitated white adipocyte beiging[128] and brought metabolic beneficial effects to the experimental animals[129,130]. In conducting the study on the 2nd aim, I have also assessed the effect of curcumin on adipose tissue or mature adipocyte Fgf21 expression and whether Fgf21 treatment affects Raw264.7 cell differentiation.

3 Significance of the study

To reduce adipose tissue inflammation and to increase energy expenditure are desired to attenuate obesity and its related metabolic disorders. Based on our knowledge, pharmacological agents for targeting both events are currently unavailable. Knowledge obtained from conducting this study may lead to the development of novel treatment for obesity and related disorders. Furthermore, the study will deepen our general knowledge on the beneficial effect of dietary polyphenols and broaden our view on lifestyle modification, its implication in metabolic disease treatment and prevention.

Materials and Methods

1 Reagents

Curcumin was purchased from Sigma Aldrich (St. Louis, MO) for the *in vitro* cell culture experiments or from Organika Health Products (Richmond, B.C, Canada, a 95% standardized curcumin extract) for the *in vivo* dietary intervention in mice. For the *in vivo* study, 10g curcumin was mixed with 1 kg HFD as we documented previously. Free glycerol reagent (Sigma F6428) and Glycerol Standards (Sigma G7793) were purchased from Sigma-Aldrich. *E. coli* lipopolysaccharide (LPS) was purchased from Sigma-Aldrich (L2880). Murine IFNγ (AF-315-05) was purchased from Peprotech. Human recombinant FGF21 was from Novoprotein Scientific Inc. (Summit, NJ). The PPARα antagonist GW6471 (G5045) and the agonist Wy14643 (C7081) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The PPARγ antagonist GW 9662 (M6191) and the agonist Rosiglitazone (R2408) were purchased from Sigma-Aldrich (DTT-RO ROCHE) and Triton X-100 (X100) were purchased from Sigma-Aldrich. Luciferin

2 Animal work

Male C57BL/6J mice purchased from Charles River Laboratories (St. Laurent, QC, Canada) were housed under the conditions of constant temperature (22°C, unless otherwise specified) and a 12h light/dark shift with free access to food and water. The animal experiments and protocols were approved by the University Health Network Animal Care Committee and performed in accordance with the guidelines of the Canadian Council of Animal Care.

2.1 Long-term curcumin-fed mice

Forty-Five C57/BL6 mice obtained from Jackson laboratory were separated randomly into three groups, feeding the different type of food: chow diet group (LFD), high-fat diet group (HFD) and 1% curcumin high-fat diet group (Cur). The age and body weight of mice were matched. The high-fat diet is 60% fat calories from Bio-Serv (Product# F3282). The body weight and amount of food intake were recorded weekly. Intraperitoneal glucose tolerance test (i.p. GTT), intraperitoneal insulin tolerance test (i.p.ITT) and intraperitoneal pyruvate tolerance test (i.p.PTT) were performed after 10 weeks of special food feeding. Mice were sacrificed at the 18th week and tissues were collected.

2.2 Metabolic tolerance tests

Mice fasted overnight for glucose tolerance tests, or fasted for 6 h for insulin or pyruvate tolerance tests. Following the fasting, glucose (2 g/kg), insulin (0.65 U/kg) or pyruvate (2 g/kg) was i.p. injected. Blood samples collected from tail vein were used for glucose measurements.

2.3 Insulin and FGF21 ELISA

Plasma insulin and FGF21 was measured using ELISA kits followed by the instructions. The insulin was measured using Ultra sensitive mouse insulin immunoassay kit (Toronto BioScience, Cat# 32380). The Fgf21 Immunoassay Kit (catalog number 32180) provided by Antibody and Immunoassay Services (The University of Hong Kong).

2.4 Metabolic cage analyses

HFD or Cur mice were housed individually in metabolic cages. Sufficient food and water were provided. After 24 hours, data were collected and analyzed using a lab animal monitoring system (Columbus Instruments).

2.5 Short-term curcumin-fed mice, cold exposure and body temperature recording

Twenty-Four C57/BL6 mice obtained from Jackson laboratory were separated randomly into three groups, feeding different types of food: chow diet group (LFD), high-fat diet group (HFD) and 1% curcumin high-fat diet group (Cur). The age and body weight of mice were matched. The cold challenge was performed after 2, 4 and 6 weeks of feeding. Mice were put at 4°C environmental chambers, and rectal temperature was recorded using an electronic thermometer equipped with a rectal probe (HH63K Stick Type Temperature Transducer and Thermometer, Omega, Quebec, Canada). The rectal temperatures were taken every 4 hours during the cold challenge period. The probe was lubricated with 50% glycerol before use to reduce possible damage and mice stress.

3 Measurement of liver triglyceride content

Weight ~100 mg of liver into 1.5 mL tube and record liver weight. 350 µL ethanolic KOH (30% KOH: ethanol; 1:3). The tube was incubated at 55°C overnight and vortex to aid in digestion. By morning, tissue should be digested with no oil layer visible. If oil layer presents, longer digestion

or more ethanolic KOH is required. The volume was added up to 1000µL with 50% ethanol and spin at 13200rpm for 5 minutes. The supernatant was moved to a new tube. Brought the volume of the supernatant to 1200µL, vortex and moved 200µL to a new tube. 215µL 1mol/L $MgCl_2$ was added, followed by 10-minute incubation on ice.

The prepared samples were centrifuged and diluted 1:2 in water. Add 10µL of the diluted sampl to 96-well plate. 150µL Free glycerol reagent was then added to the well and incubated at room temperature for 15 minutes. The absorbance at 540nm was measured. The line of best fit for standards was generated the sample triglyceride were calculated. The sample was 415 times diluted and the triglyceride was expressed as per gram tissue.

4 Measurement of plasma triglyceride content

10µL plasma was put into 1.5 mL tube with 20 µL ethanolic KOH (30% KOH: ethanol; 1:3). The tube was incubated at 55°C overnight and vortex to aid in digestion. 20 µL 50% ethanol was added and centrifuged at 13200rpm for 5 minutes. 20µL of the sample was moved to a new tube. 25µL 1mol/L $MgCl_2$ was added, followed by 10-minute incubation on ice.

30µL of the diluted sample was added to 96-well plate. 150µL Free glycerol reagent was then added to the well and incubated at room temperature for 15 minutes. The absorbance at 540nm was measured. The line of best fit for standards was generated the sample triglyceride were calculated. The sample was 200 times diluted.

5 Cell culture and treatment

All cells are cultured in the incubator with a condition set of 37°C and 5% CO2.

5.1 RAW264.7 cell line cultivation and treatment

The mouse leukemic monocyte-macrophage Raw264.7 cell line and the 293T cell line were purchased from ATCC. RAW264.7 cells were cultured in high-glucose DMEM (Sigma-Aldrich) with10% FBS and P/S, in 12-well plates with approximately 1×106 cells in each well. M1-like differentiation was induced by differentiation medium (DMEM, 1%FBS, P/S, LPS 100ng/mL and IFNγ 2.5ng/mL). Curcumin was added to cell differentiation medium at different concentrations (2.5, 5, 10, 20 µmol/L), or FGF21 at different concentrations (0.25, 0.5, 1, 2 nmol/L) to study the effect on macrophages differentiation.

The FBS concentration of the primary adipocyte culturing medium was adjusted to 1% and RAW264.7 cells were cultured in this medium for 10 hours. Cells were harvested for further experiments.

5.2 Rat primary adipocytes isolation and treatment

Epididymal adipose tissues from male wild-type Wistar rats fed a normal diet were excised, minced in digestion medium (high glucose DMEM, 3% fatty acid free BSA, 0.5% FBS, 1% P/S) with 4mg/ml collagenase Type I (Worthingtion Chemical Corporation, 195Unit/mg) at 37 ℃, on 80rpm shaker for 1 hour. The digested adipose tissue was filtered through a mesh (200um) and centrifuged at 400rpm for 1 min to separate and wash twice all adipocytes floating on the top with maintenance medium (high glucose DMEM, 1% fatty acid free BSA, 0.5% FBS, 1% Penicillin/Streptomycin (P/S)). Split the adipocytes to plates and cells were maintained at 37℃ with maintenance medium for 16 hours. The culture medium was collected for other cell treatment. Curcumin was added at a concentration of 10uM for 6 hours. After treatment, adipocytes were then harvested for further experiments.

5.3 Brown adipocytes cell culture and treatment

The mouse brown adipocyte cell line (mBAT) was the gift of Shingo Kajimura[131]. Cells were cultured with grown medium (high glucose DMEM with 10%FBS and 1% P/S) until 95-100% confluence. The culture medium was then changed to introduction medium (high glucose DMEM, 10%FBS, 1% P/S, 5μg/mL insulin, 1nmol/L T3, 0.123mmol/L Indomethacin (Sigma I-7378), 2μg/mL dexamethasone (Sigma D-1756) and 0.1mmol/L IBMX (Sigma I-5879)) for two days. Then the medium was changed to maintenance medium (high glucose DMEM, 10%FBS, 1% P/S, 5μg/mL insulin, 1nmol/L T3). After 36-48 hours, the medium was changed to growth medium until cells fully differentiated. Curcumin was added at 10umol/L as a treatment throughout differentiation period. Curcumin was added at the concentration of 4μmol/L for 10 hours after differentiation.

5.4 293T cell culture, transfection and treatment

293T cells were cultured in DMEM (Sigma-Aldrich) with10% FBS and P/S, in 12-well plates with approximately 1×106 cells in each well. The construction of the fusion gene constructs in which the expression of the luciferase (LUC) reporter is driven by mouse UCP1 promoter. For

each well, 1µg UCP1 promoter-luciferase plasmid was mixed with 2µg polyethyleneimine (PEI) in 100µl Opti-MEM® (Gibco, 31985-070), standing at room temperature for 10 minutes. The volume was then added up to 1mL with DMEM and cells were incubated with the plasmid for 24 hours. The next day, the medium was changed to no FBS DMEM with a 10-hour indicated treatment: curcumin, 10µmol/L; WY14643, 10µmol/L; GW6471, 2µmol/L; Rosiglitazone, 2µmol/L, GW9662, 5µmol/L. After a 10-hour treatment, the cells were harvested with the harvest buffer (50mmol/L TRIS/MES, 1mmol/L DTT and 0,1% Triton X-100).

5.5 Mice peritoneal macrophage isolation and treatment

Thioglycollate (4%) was injected 1mL per mouse into the peritoneal cavity. Four days later, the mice were sacrificed with CO2. 5mL cold PBS with 2% FBS was then injected into the peritoneal cavity. After 5 minutes, the fluid was aspirated. The macrophages were centrifuged and washed twice. After washing the cell pellet, the cells were seeded in 12-well plates, 106 cells per well. Curcumin was added to the wells at the concentration of

6 Luciferase assay

The 50µl cell lysate was mixed well with 50 µl D-luciferin (3mg/mL D-luciferin, 5mmol/L KH2PO4) and 7.5 µl ATP cocktail (100mM Tris pH 7.8, 5mM MgOAc, 150µM ATP) and the light intensity was measured immediately. The data were normalized with protein concentration in each cell lysate sample.

7 MTT assay

cells in 100 µL media per well were plated in a 96-well plate while leaving 8 wells empty for blank controls, 5,000-10,000 cells each well. Cells were incubated overnight and curcumin was added at the concentration of 2.5, 5, 10, 20 µmol/L on the next day for a 10-hour treatment. MTT (Thiazolyl Blue Tetrazolium Bromide) was dissolved in PBS at 5mg/mL. Fresh-made MTT solution is required. 20µL MTT solution was added to each well and the plate was placed on a shaking table, shaking at 150rpm for 5 minutes to mix the MTT into the media. The cells were then incubated for 1-5 hours to allow the MTT to be metabolized. Discard the media and dry the place on paper towels after incubation. The formazan (MTT metabolic product) will be resuspended in 100µL DMSO, shaking at 150rpm for 5 minutes. The optical density at 560nm

and subtract background at 670nm were measured. The optical density should be directly correlated with the cell quantity.

8 Histology and immunohistochemistry

Mouse brown adipose tissue, epididymal adipose tissue and inguinal adipose tissue was fixed in 4% paraformaldehyde for 24 hours. The next day, tissues were embedded in paraffin. Tissue sections were stained with hematoxylin and eosin or immune-stained with the UCP1 antibody, with the assistance from Toronto Centre of Phenogenomics (TCP). Adipocyte cell size was measured using image pro plus software.

9 Processing of immune cells from epididymal adipose tissue and spleen.

Epididymal adipose tissue immune cells were isolated as described[132]. The cell culture medium was collected for the Luminex® multiplex assay, conducted by the Service Laboratories of University Health Network.

10 Flow cytometry

Immune cells were stained for 30 minutes with fluorophore-conjugated antibodies to CD206, CD11c, CD86, and CD 80, using recommended dilutions. Cells were identified using Fortessa cell analyzer™ (BD Biosciences, Mountain View, CA). Data acquired on the flow cytometer was analyzed with FlowJo software (Tree Star).

11 Western blotting analysis

Tissues and cells were lysed in lysis buffer containing a protease inhibitor cocktail. Total protein lysates were diluted with 5xSDS and heated at 95°C for 10 minutes. 10% SDS-PAGE was followed and samples were transferred to a polyvinylidene difluoride membrane after electrophoresis. The membrane was cut and incubated with iNOS (Cell Signaling 13120S), UCP1 (Cell Signaling 14670S), and β-actin (Cell Signaling 3700S) antibody for overnight. Next day, the membranes were incubated with anti-mouse (Cell Signaling 7076S) or anti-rabbit (Cell Signaling 7074S) secondary antibodies. Development was carried out using enhanced chemiluminescence. Densitometric quantification was performed by the ImageJ software.

12 Quantitative real-time reverse transcriptase polymerase chain reaction.

Tissues and cells were lysed with TRI® Reagent (Sigma–Aldrich T9424). Chloroform was added and the aqueous phase was separated. Isopropanol was then added to the aqueous phase and RNA pellets were obtained after 15 minutes 10000xg centrifuge. RNA was dissolved in DEPC water and converted to cDNA using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, ref 4368814). Specified cDNA (listed below) copy number was determined using Real-time PCR (SensiFAST SYBR Hi-ROX Kit, Cat. No. BIO-92020). Primers are listed in Table 2

13 Statistical analysis

All data were expressed as the mean ± SEM. Comparisons between groups were performed using one-way ANOVA. $P<0.05$ was considered significant. * or #, $p<0.05$; ** or ##, $p<0.01$; and *** or ###, $p<0.005$

Table 2. Sequences of primers utilized in this study.

Primers Sequence

	Oligonucleotide		
	Forward	Reverse	Product size (bp)
Mouse PGC-1a	5'-AATGCAGCGGTCTTAGCACT-3'	5'-TTTCTGTGGGTTTGGTGTGA-3'	207
Mouse IL-4	5'-CAAACGTCCTCACAGCAACG-3'	5'-AGGCATCGAAAAGCCCGAAA-3'	166
Mouse IL-6	5'-GATGGATGCTACCAAACTGGA-3'	5'-CTGAAGGACTCTGGCTTTGTC-3'	141
Mouse IL-10	5'-CTATGCTGCCTGCTCTTACTGA-3'	5'-AGGAGTCGGTTAGCAGTATGTTG-3'	190
Mouse Arg1	5'-GTCCACCCTGACCTATGTGTC-3'	5'-TCAGGAGAAAGGACACAGGTTG-3'	109
Mouse iNOS/NOS2	5'-CAAGCACCTTGGAAGAGGAG-3'	5'-AAGGCCAAACACAGCATACC-3'	149
Mouse MCP-1/Ccl2	5'-TCAGCCAGATGCAGTTAACG-3'	5'-GTCAGCACAGACCTCTCTCT-3'	163
Mouse KLF4	5'-CCAAAGAGGGGAAGAAGGTC-3'	5'-CGTCCCAGTCACAGTGGTAA-3'	154
Mouse FIZZ1	5'-GGAACTTCTTGCCAATCCAGC-3'	5'-CTCCCAAGATCCACAGGCAA-3'	151
Mouse PPARα	5'-CGTCACGGAGCTCACAGAAT-3'	5'-ACTCGCGTGTGATAAAGCCA-3'	179
Mouse PPARγ	5'-CATAAAGTCCTTCCCGCTGA-3'	5'-ACCTCTTTGCTCTGCTCCTG-3'	165
Mouse UCP1	5'-CGTCCCCTGCCATTTACTGT-3'	5'-CCCTTTGAAAAAGGCCGTCG-3'	178
Rat TNFα	5'-CCTCTTCTCATTCCTGCTCG-3'	5'-GGGCTTGTCACTCGAGTTTT-3'	172
Rat MCP-1/Ccl2	5'-GGGCCTGTTGTTCACAGTTG-3'	5'-TTCTCCAGCCGACTCATTGG-3'	126
Rat IL-1β	5'-CTGTGACTCGTGGGATGATG-3'	5'-GGGATTTTGTCGTTGCTTGT-3'	210
Rat IL-4	5'-TCACCCTGTTCTGCTTTCTC-3'	5'-TCTGGTACAAACATCTCGGTG-3'	136
Rat IL-6	5'-CACTTCACAAGTCGGAGGCT-3'	5'-TCTGACAGTGCATCATCGCT-3'	114

Results

1 Dietary curcumin intervention attenuates HFD-induced metabolic defects

It has been reported that dietary curcumin intervention in HFD-fed mice reduces body weight gain and improved glucose disposal[58]. Here, I have set up an experiment to verify the metabolic beneficial effect of dietary curcumin intervention in mice with concomitant HFD feeding and curcumin intervention as illustrated in Figure 2. Forty-five 5-week-age C57BL/6J mice were randomly divided into three groups fed with different diets. The first group of mice was fed with regular chow diet (LFD). The second group of mice was fed with 60% high-fat diet (HFD). The third group of mice was fed with 60% high fat diet and 1% curcumin (Cur). In HFD, 60% of calorie was from the fat content. The experimental plan is illustrated in Figure 2. Moues body weight and food intake were measured weekly. The i.p.GTT, i.p.ITT and i.p.PTT was performed in the 10^{th}, 12^{th}, 14^{th} week respectively. In the 16^{th} week, mice fed with HFD or HFD plus Cur were put into metabolic cages for assessing the metabolic profile individually. The mice were scarificed after 18 weeks of the correspondent diet feeding. Plasma and tissues were then collected.

Similar to what has been reported previously[58,76-78], the body weight of mice received HFD feeding with and without dietary curcumin intervention showed the significant difference. The difference between HFD fed mice and Cur mice fed with HFD plus Cur started at the 5^{th} week (Figure 3A). However, the calorie intake was similar among the three groups of mice (Figure 3B). The adipose tissue weights from three major lipid deposits (Figure 3C) and the liver tissue weight (Figure 3D) was evidently decreased by curcumin administration. The glucose disposal was also improved in mice fed with Cur, assessed by i.p.GTT (Figure 4A). In addition, insulin sensitivity in the Cur group was higher (Figure 4B) along with improved i.p.PTT (Figure 4C). Plasma was obtained from mice in different conditions: fasting, 2-hour refeeding and random time. The fasting glucose level in the Cur group was significantly lower than that of the HFD group (Figure 5A). The plasma insulin level in Cur group was always lower than that of HFD mice (Figure 5B, C), indicating that the insulin resistance was improved by curcumin intervention.

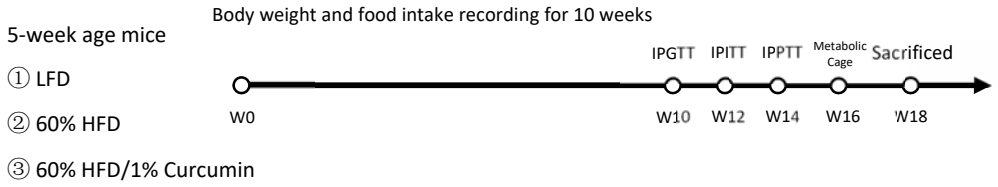

Figure 2. Illustration of the long-term curcumin intervention experimental procedure. Mice were fed with chow diet (LFD), or high fat diet (HFD), or HFD containing 1% curcumin (Cur). Body weight and food intake were measured every week. I.p.GTT, i.p.ITT and i.p.PTT were performed as indicated. Mice from HFD and Cur groups were put into metabolic cages individually in the 16th week. After 18 weeks of feeding, mice were sacrificed. Blood and tissue samples were collected for further experiments.

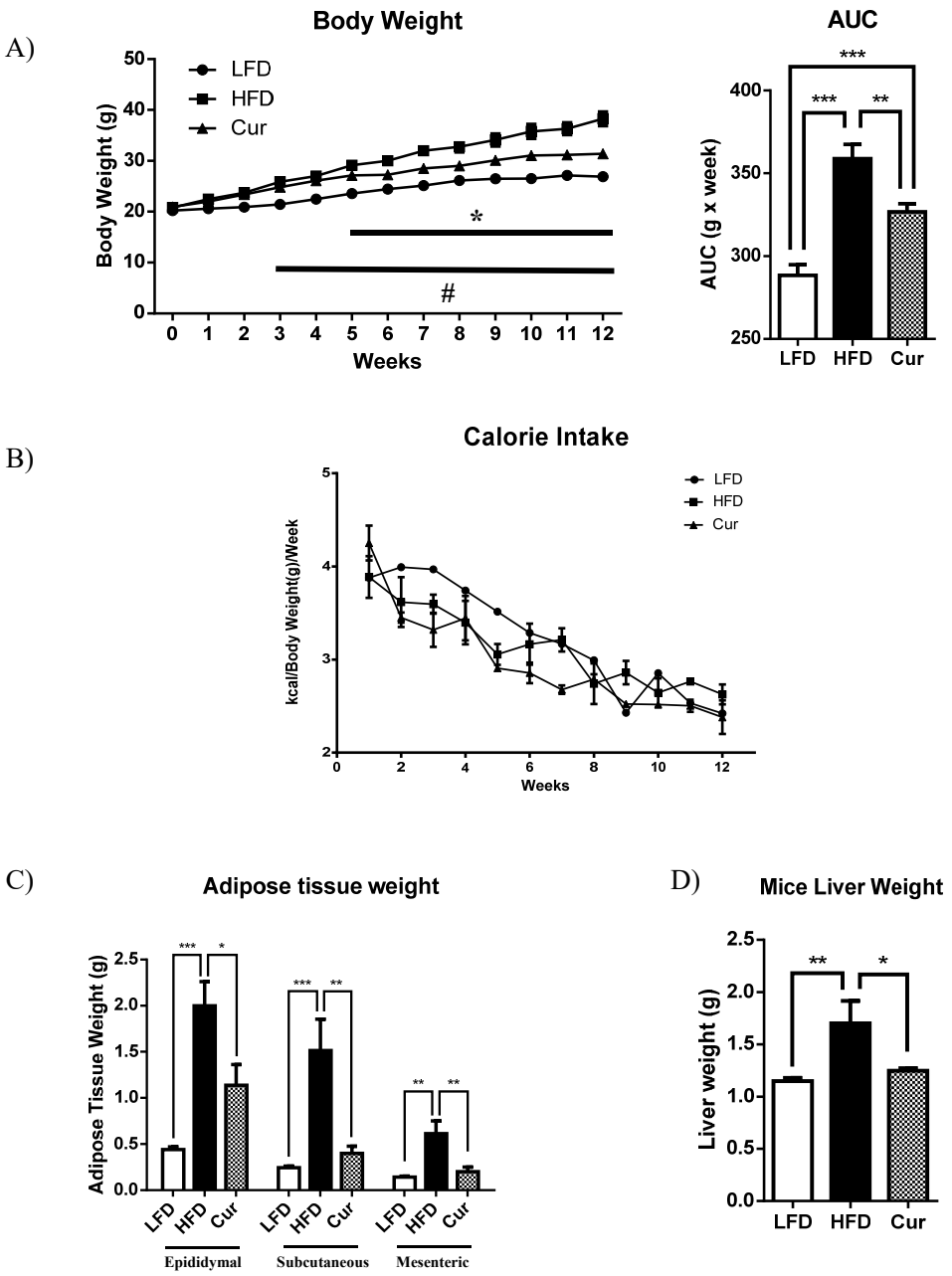

Figure 3. Mouse body weight, calorie intake and tissue weight. (A) The body weight recording during indicated diet feeding. The body weight became statistically different between HFD and Cur after the 5th week. *, HFD vs Cur, $p<0.05$; #, LFD vs HFD. The right panel shows area under curves (AUC). (B) Diet consumptions were normalized with body weight. (C) Mouse adipose and liver tissue weights n=15 for all panels. *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.

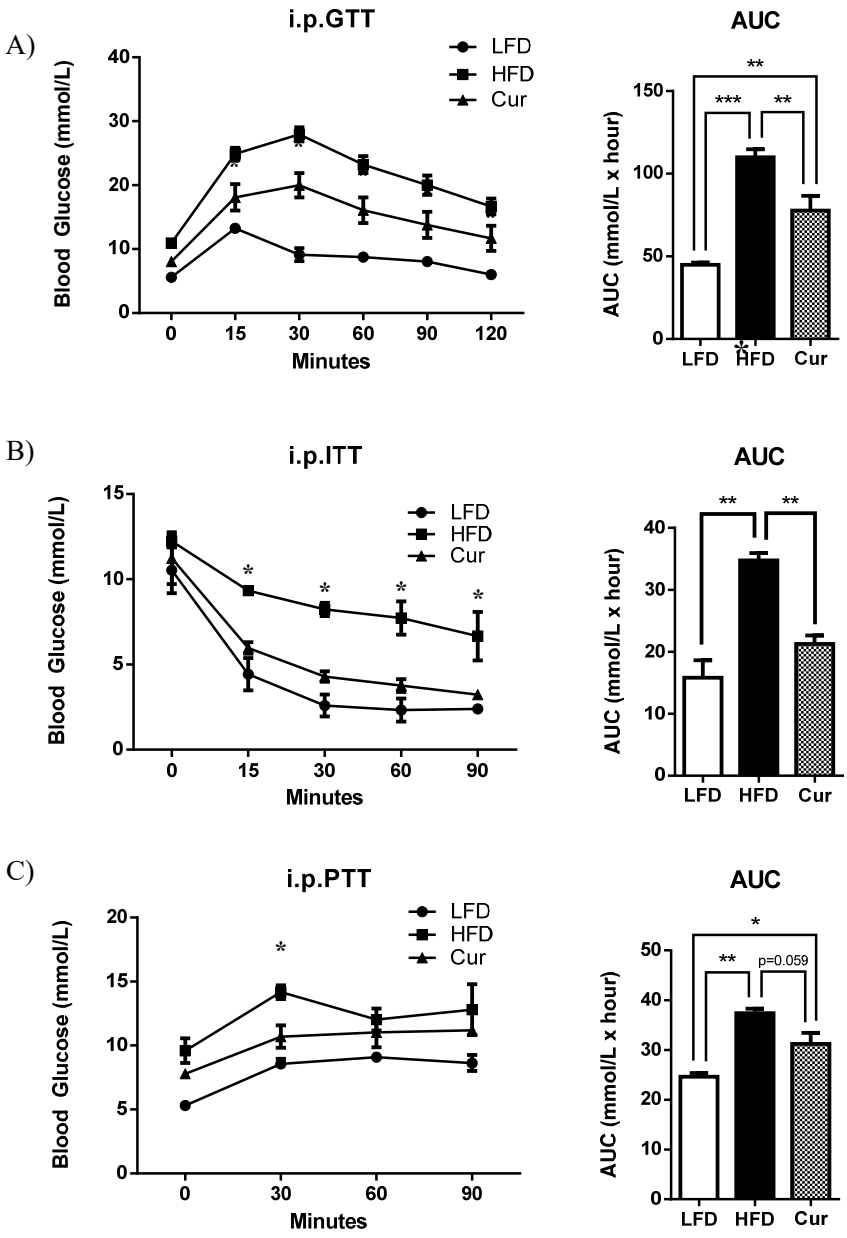

Figure 4. i.p.GTT , i.p.ITT and i.p.PTT were performed in mice fed with LFD, HFD, or curcumin containing HFD (Cur) at the time points indicated in Fig. 2. (A) i.p. GTT. (B) i,p. ITT. (C) i.p.PTT. n=6 for all panels. *, HFD vs CUR. Right panels are AUCs. *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.

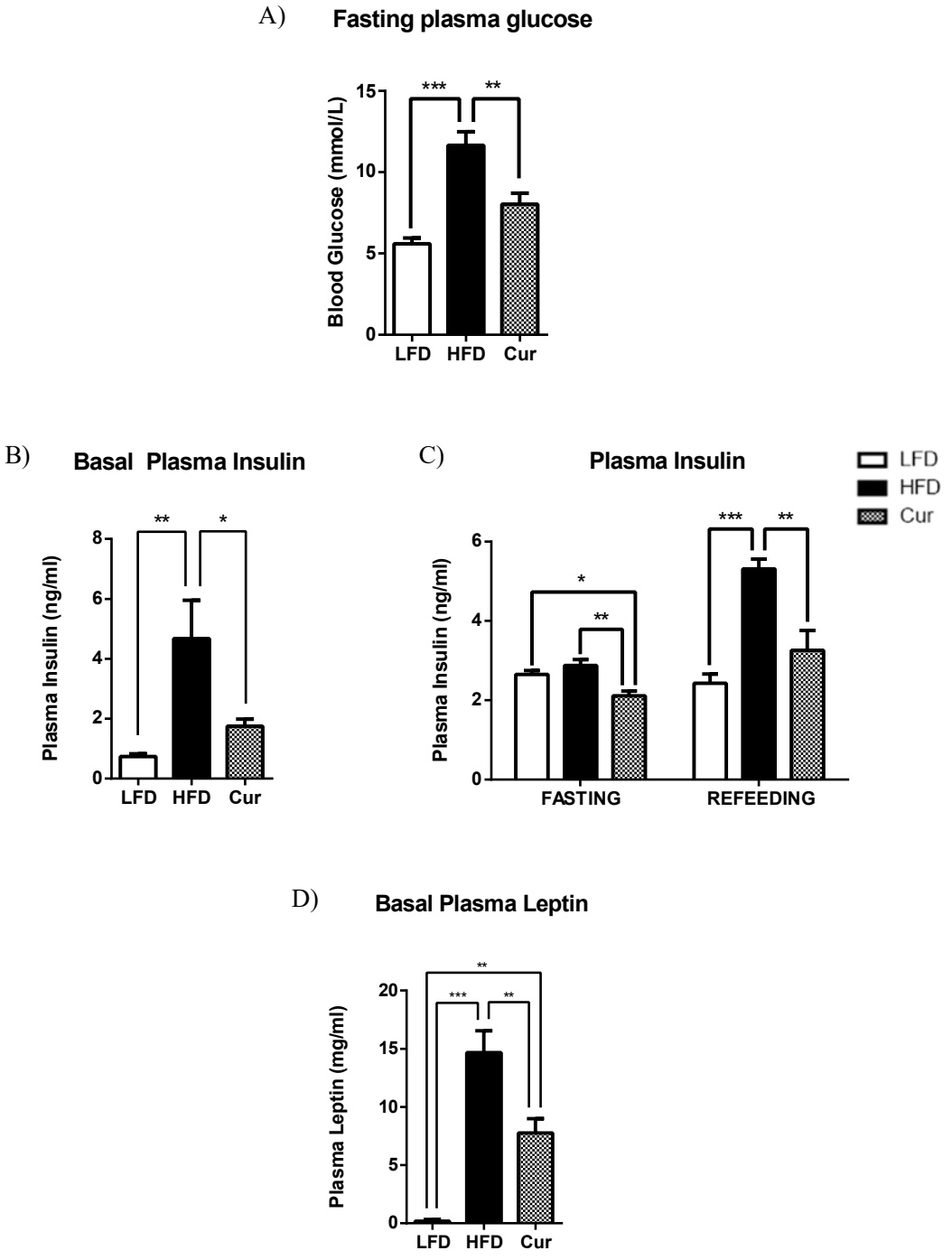

Figure 5. Mouse plasma glucose, insulin and leptin levels. (A) Fasting plasma glucose level. (B) Basal/fasting/refeeding plasma insulin levels. (C) Basal plasma leptin levels. n=5 for all panels. *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.

We found that leptin level in HFD group was 15-fold higher than that in the LFD group and dietary curcumin intervention reduced plasma leptin level (Figure 5D). Fibroblast growth factor 21 (FGF21) was shown to stimulate glucose uptake and sensitizes insulin signaling in adipose tissue[133]. Long-term high-fat diet feeding showed elevated plasma Fgf21 level (Figure 6A). The Fgf21 mRNA level was also increased in long-term HFD epididymal adipose tissue (Figure 6B). Dietary curcumin intervention reduced both plasma Fgf21 level and adipose tissue *Fgf21* mRNA expression (Figure 6A, B).

We also repeated previous observation[58] that increased plasma and liver triglyceride in response to HFD consumption was attenuated by dietary curcumin intervention (Figure 6C, D). Mice of the Cur group also showed decreased plasma free fatty acid level when compared with that in the HFD mice (Figure 6E).

To summarize, long-term dietary curcumin intervention reduced whole body, adipose tissue and liver tissue weight. It also improves glucose disposal and HFD-induced metabolic defects on lipid homeostasis.

2 Curcumin intervention reduces WAT M1-like/M2-like macrophage ratio and pro-inflammatory cytokine secretion in HFD-fed mice

Mouse epididymal tissue was dissected and the infiltrated immune cells were isolated as described[132]. The immune cells were cultured overnight and the culture medium was collected for Luminex® multiplex assay, conducted by the Service Laboratories of University Health Network. Our data showed that most of the pro-inflammatory cytokines produced by epididymal adipose tissue infiltrated immune cells were decreased in Cur administration mice. In addition, one of the key M2-like macrophage factors, IL-10[40], was shown to be increased (Table 3), suggesting that dietary curcumin intervention regulates macrophage polarization in adipose tissue.

The immune cells were stained for 30 minutes with fluorophore-conjugated antibodies to CD206, CD11c, CD86, and CD 80, with recommended dilutions. Cells were identified using a BD Fortessa™ cell analyzer. Data acquired on the flow cytometer was analyzed with FlowJo software. The dot plots implied that the total cell count and cell subtype are different (Figure 7 A, B).

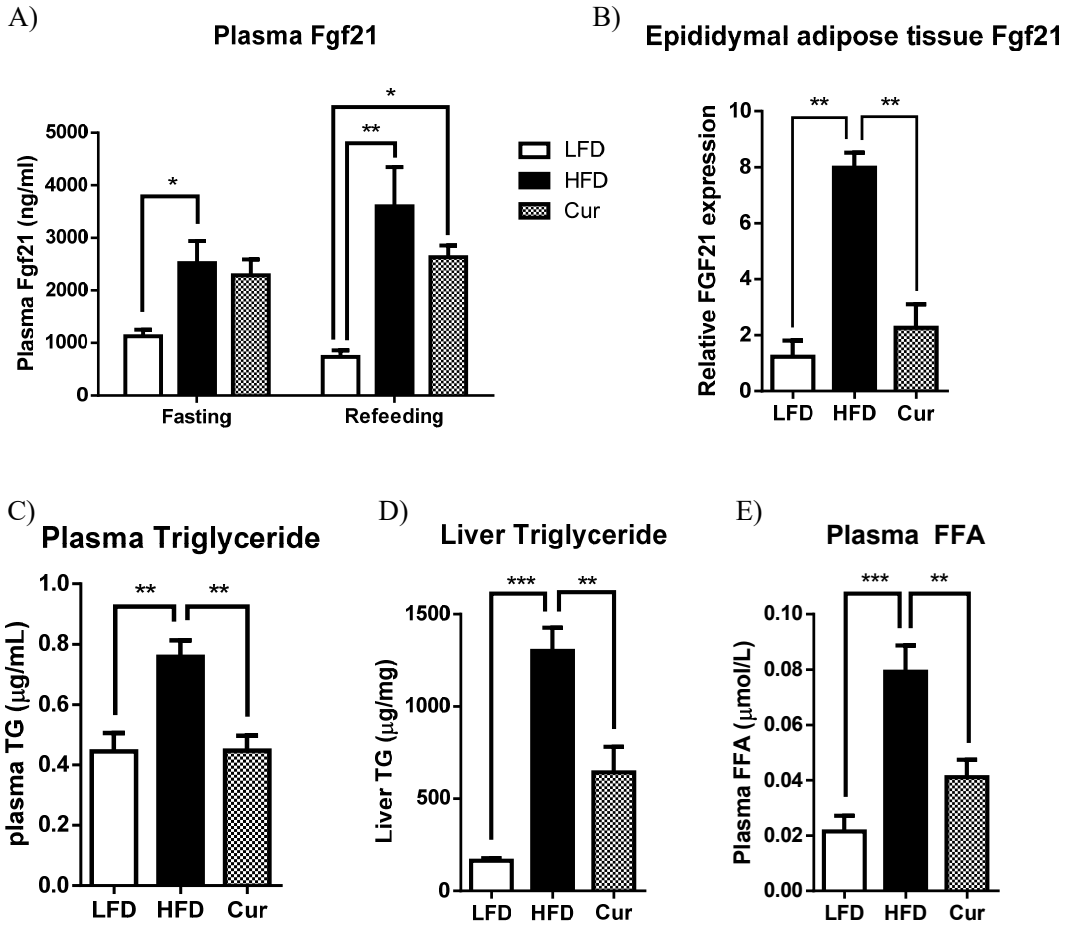

Figure 6. Mouse Fgf21, triglyceride and FFA levels in mice fed with indicated diet for 18 weeks. (A) Fasting and refeeding plasma Fgf21 levels (n=5). (B) Mouse epididymal adipose tissue *Fgf21* mRNA levels (n=3). (C-D) Mouse plasma and liver TG levels. (E) Mouse plasma FFA levels. (n=6). *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.

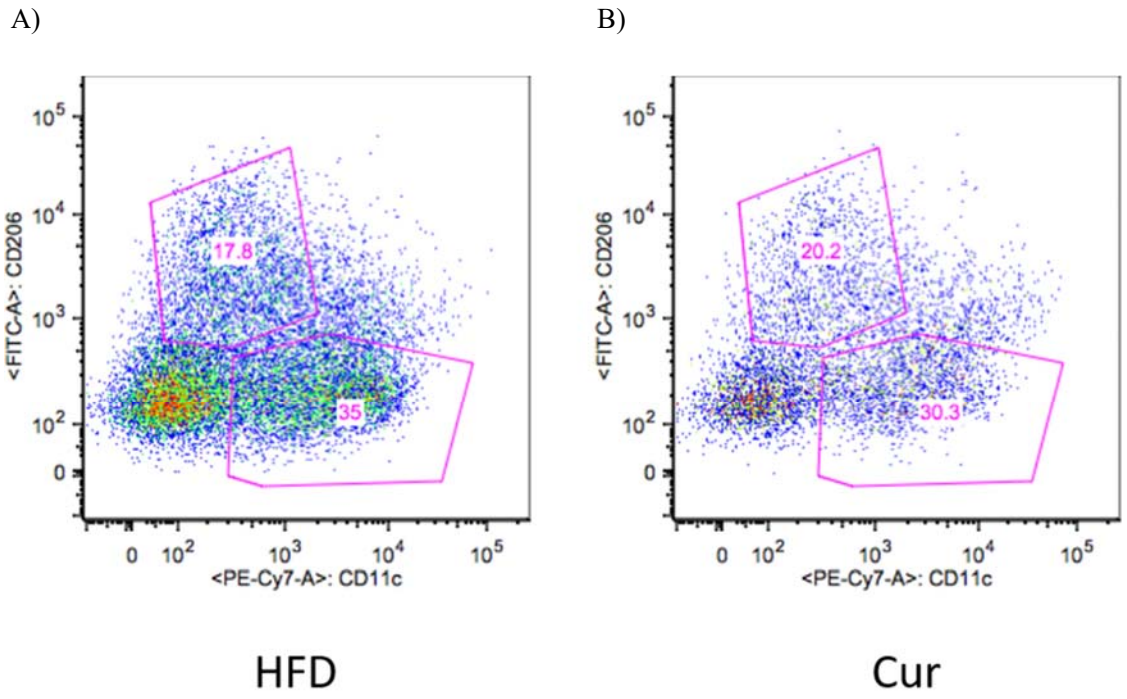

Figure 7. Dot plots of cell flowcytometry for infiltrated immune cells isolated form mice fed with indicated diet for 18 wks. Cells were identified using a BD Fortessa™ cell analyzer.

Table 3. Long-term special diet fed mice were sacrificed after 18 weeks of feeding. The epididymal adipose tissue infiltrated immune cells were extracted. After 24-hour culturing, the medium was sent to the Luminex® multiplex assays for immune cell cytokines. (*, p<0.05; **, p<0.01; ***, p<0.005).

Luminex® multiplex assays for immune cell cytokines

	HFD			Significance	Cur		
IL-1α	28.33833	±	3.081949	*	3.633333	±	1.478967
IL-1β	358.23	±	19.97456	*	83.24	±	8.302201
IL-2	7.406667	±	0.44464	N/A		OOR<	
IL-3	9.308333	±	1.10071	**	2.373333	±	0.280139
IL-4		OOR<		N/A		OOR<	
IL-5	1598.705	±	431.1624	*	45.53	±	2.05052
IL-6		OOR>		N/A	1477.053	±	130.1968
IL-9		OOR<		N/A		OOR<	
IL-10	44.24667	±	12.82369	***	187.19333	±	28.88765
IL-12(p40)	57.05333	±	3.525397	*	22.48	±	4.516868
IL-12(p70)	84.64167	±	7.885371	*	27.99	±	2.452393
IL-13	296.6633	±	6.593959	ns	247.7433	±	21.02634
IL-17	26.845	±	9.642319	N/A		OOR<	
Eotaxin	452.095	±	133.0914	N/A		OOR<	
G-CSF	35136.61	±	4813.975	**	1102.503	±	556.6802
GM-CSF	384.285	±	121.4681	N/A		OOR<	
IFN-γ	6.795	±	1.041277	N/A		OOR<	
KC	22473.01	±	3837.754	ns	19687.42	±	1230.979
MCP-1		OOR>		N/A	6338.84	±	1519.185
MIP-1α	5832.663	±	980.4733	**	178.5233	±	47.066
MIP-1β	2084.3	±	380.2988	***	51.14667	±	9.034167
RANTES	5716.025	±	1598.435	*	74.58333	±	27.12339
TNF-α	172.775	±	9.916168	**	119.4033	±	5.524269
unit pg/ml							

OOR = Out of Range; OOR> = Out of Range Above; OOR< = Out of Range Below

Our data show that dietary curcumin intervention not only reduced WAT macrophage volume but also reduced the number of M1-like macrophages and increased the number of M2-like macrophages (Figure 8A-C). The mean fluorescence intensity of CD86 from antigen-presenting cells was also decreased (Figure 8D, E), supporting the notion that dietary curcumin intervention inhibits immune system pro-inflammatory activity in epididymal adipose tissue. However, such effect of curcumin on macrophages was not observed in the spleen samples (Figure 8F-H).

We hence suggest that long-term dietary curcumin intervention not only reduces epididymal adipose tissue macrophage infiltration in response to HFD consumption but also exerts the opposite effects on the differentiation and function of M1-like and M2-like macrophages.

3 *In vitro* curcumin treatment attenuates Raw264.7 cell differentiation towards the M1-like subtype

We then assessed the direct effect of curcumin treatment *in vitro* in the mouse leukemic monocyte-macrophage Raw264.7 cell model, with the dosages of curcumin that generated no appreciable inhibition on cell viability determined by the MTT assay (Figure 9A). Here we classified M1-like macrophages using M1 cell specific markers including iNOS, MCP-1 and IL-6. The M2-like macrophage markers assessed in this study include KLF4, ARG1, PPARγ and IL-4. Curcumin treatment dose-dependently attenuated the stimulatory effect of LPS/IFNγ treatment on iNOS expression (Figure 9B). The LPS/IFNγ induced iNOS, MCP-1 and Il-6 mRNA expression can also be dose-dependently inhibited by curcumin treatment (Figure 10A-C). The stimulation of the differentiation markers of the M2-like subtype (KLF4, PPARγ and ARG1) as well as the anti-inflammation cytokine gene IL-4, however, was observed only when the dosage of curcumin reached 20 μM (Figure 11A-D). The mouse peritoneal macrophages were then isolated and treated with curcumin to mimic *in vivo*. Our real-time RT-PCR results show that the M2-like differentiation-promoting effect of curcumin was limited (Figure 11E).

Acute curcumin treatment stimulates hepatic Fgf21 expression[60]. Here we show that *in vitro* curcumin treatment also stimulated Fgf21 mRNA expression in primary adipocytes (Figure 12A). *In vitro* FGF21 treatment, however, did not reduce the expression of the differentiation markers of the M1-like subtype in the Raw264.7 cell model (Figure 12B-E), suggesting that curcumin regulated adipose tissue macrophage subtype-switch may not be directly regulated by Fgf21 on repressing the M1-subtype differentiation.

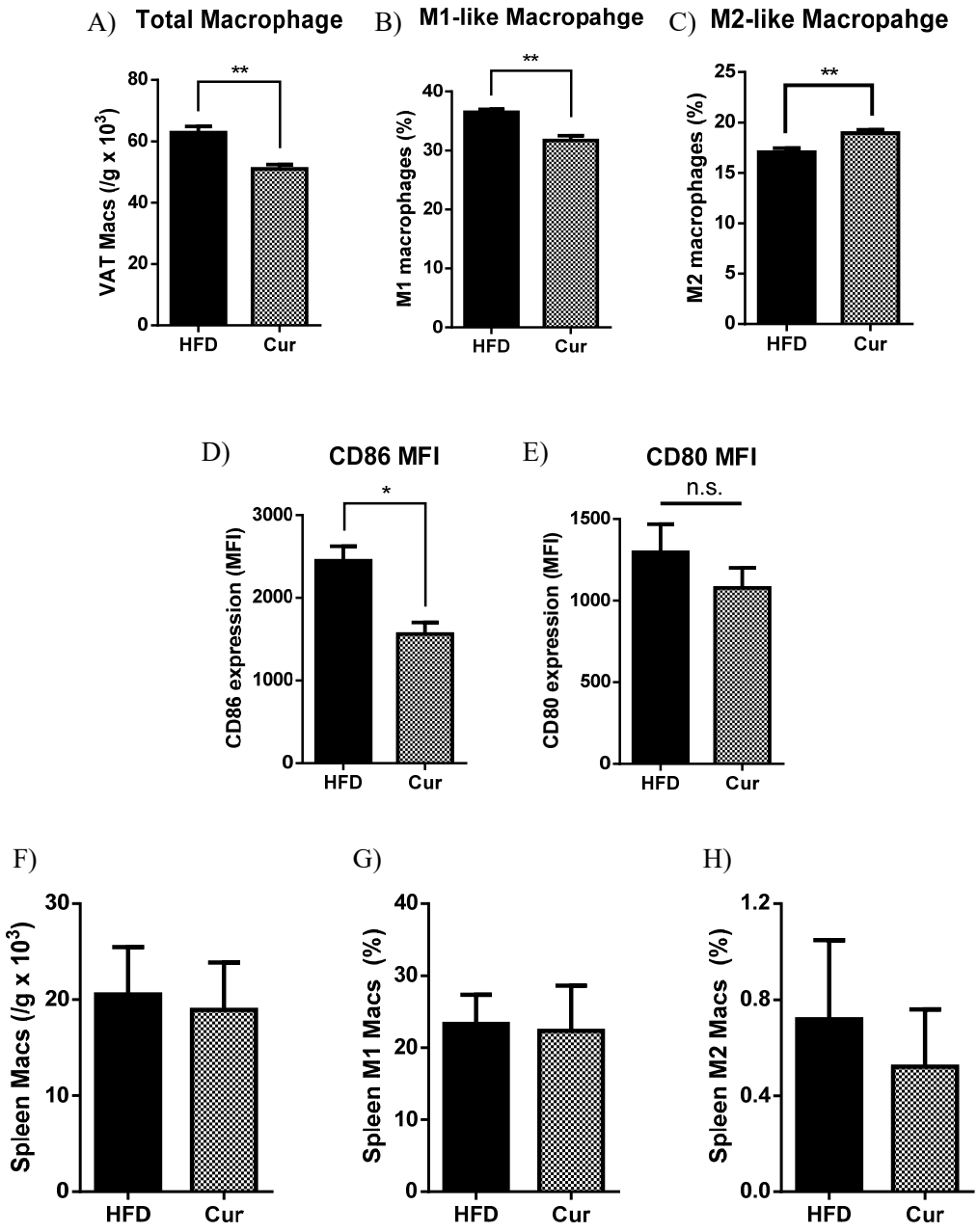

Figure 8. Total cell counts, relative ratios, as well as the mean fluorescence intensities (MFI) of indicated immune cells from epididymal adipose tissues of mice fed with indicated diets identification. (A) Total macrophage counts. (B) Ratios of M1-like macrophages. (C) M2-like macrophage ratios. (D-E) MFI of CD86 and CD80. Panels F to H show indicated measurements on macrophages isolated from spleen samples. *, $p<0.05$; **, $p<0.01$; n.s., non-significant.

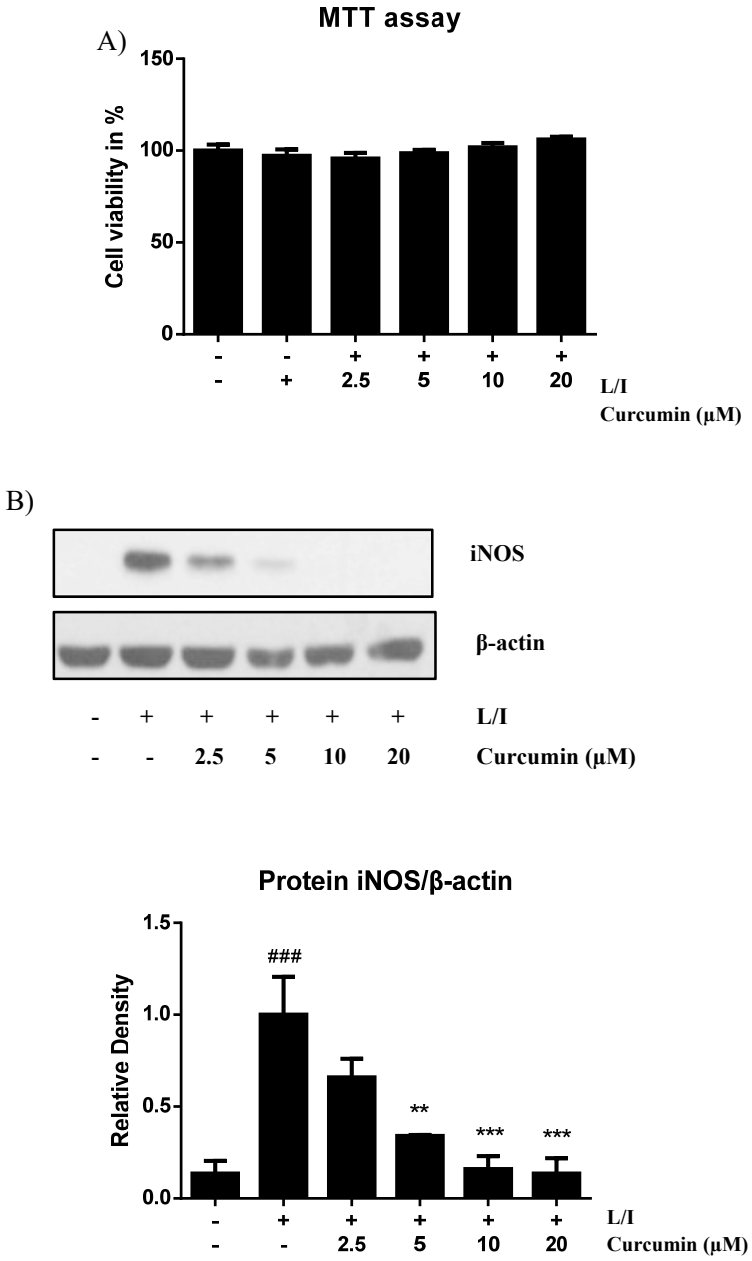

Figure 9. Assessment of the effect of curcumin treatment on attenuating iNOS expression induced by LPS and IFNγ (L/I) treatment in RAW264.7 cells. (A) MTT assay shows the viability of cells treated with indicated dosages of curcumin for 10 hours (n=12). (B) Western blotting shows iNOS protein expression in cells received indicated treatment for 10 hours. Lower panel shows results of densitometry analyses (n=3). *, **, or *** L/I vs curcumin; ###, L/I vs control.

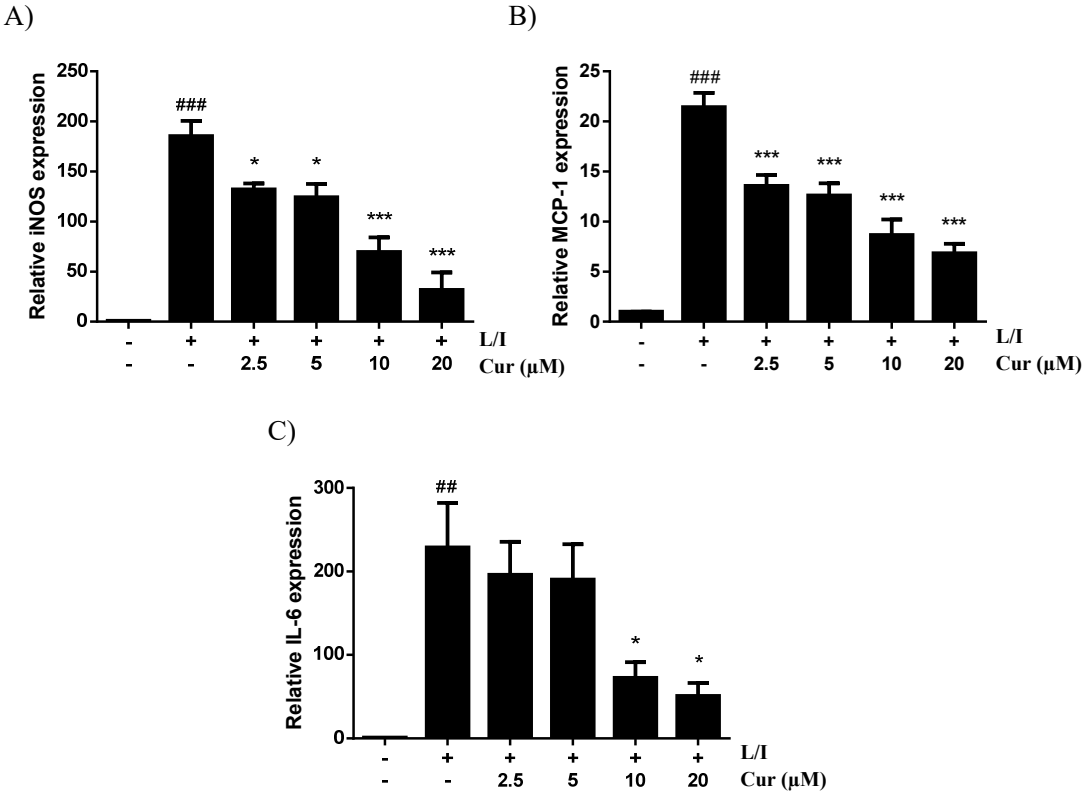

Figure 10. M1-like macrophage marker expression in Raw264.7 cells received indicated treatment for 10 hours. Panel A to C shows the mRNA expression. (A) *iNOS*. (B) *MCP-1*. (C) *IL-6.* Total RNAs were isolated from treated cells for qRT-PCR. The results were normalized against β-actin. n=3 for all panels. *, **, ***, L/I vs curcumin. #, ##, ###, L/I vs control.

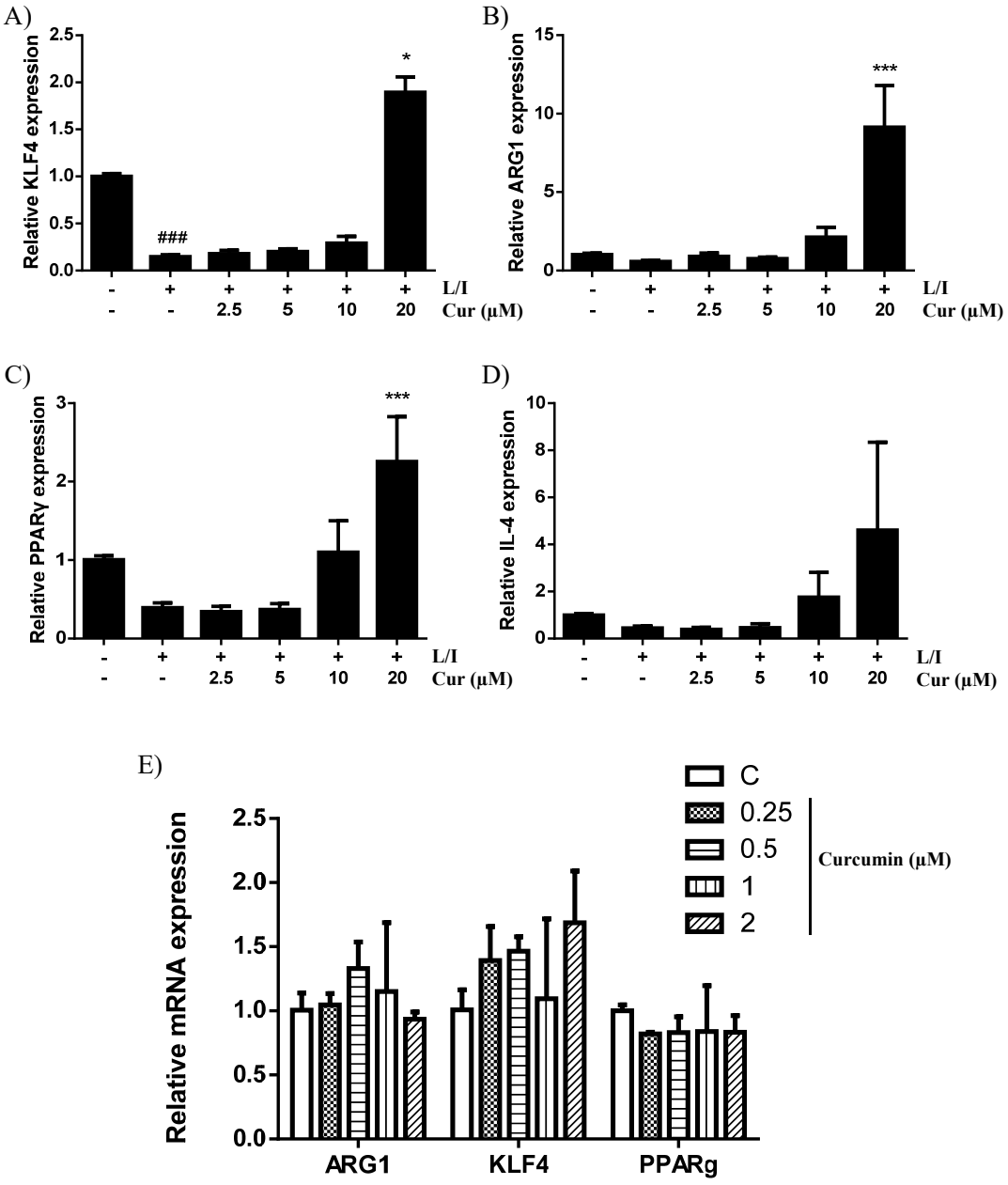

Figure 11. M2-like macrophage markers expression in macrophages received indicated treatment for 10 hours. Panels A to D show Raw264.7 cells treatment. Panel A to D shows the mRNA expression. (A) *KLF4.* (B)*ARG1.* (C)*PPARγ.* (D) *IL-4.* (E) Mouse peritoneal macrophage M2-like cell markers expression after curcumin treatment. Total RNAs were isolated from treated cells for qRT-PCR. The results were normalized against β-actin. n=4 for all panels. *, **, ***, L/I vs curcumin. #, ##, ###, L/I vs control.

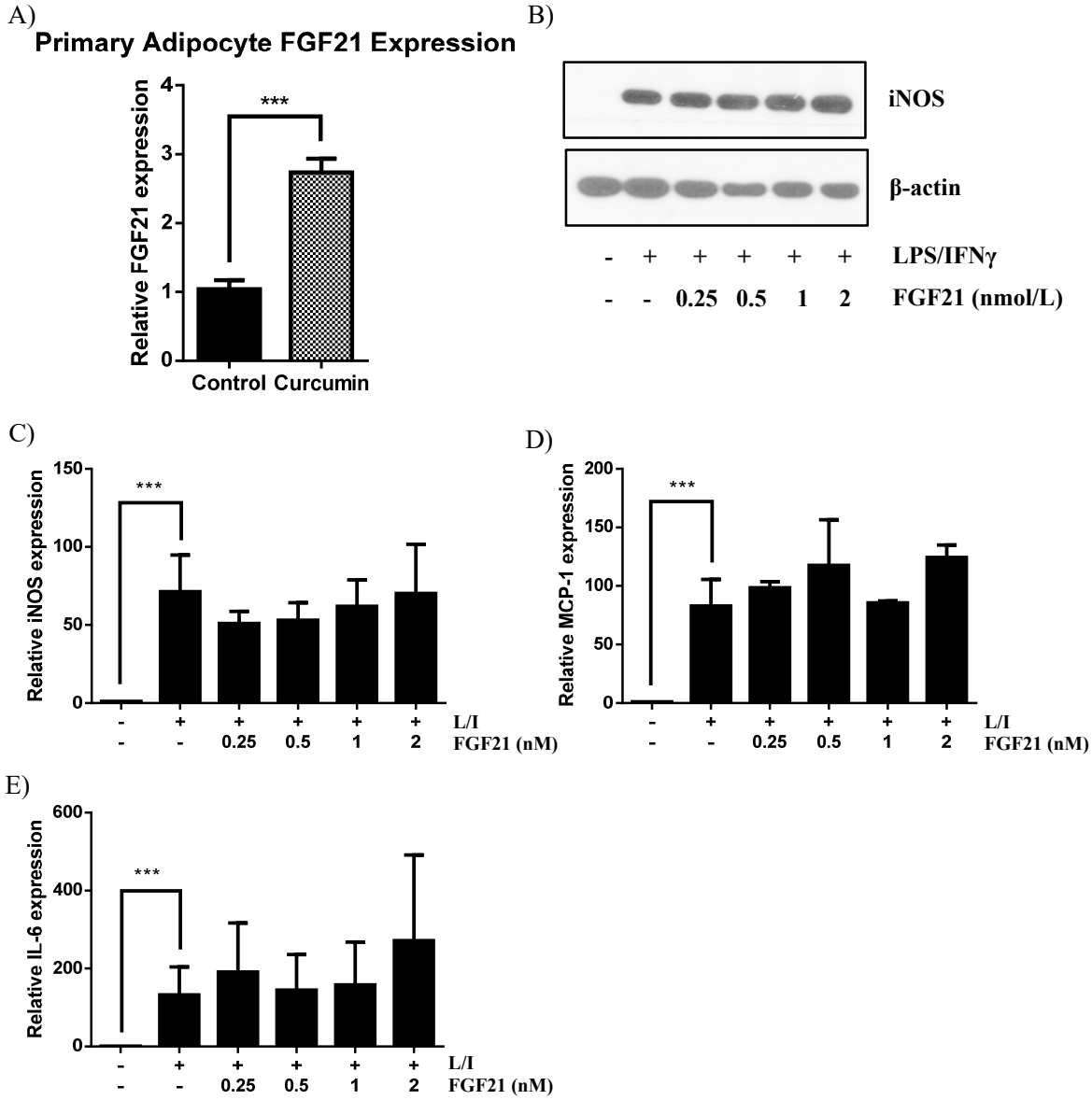

Figure 12. M2-like macrophage marker expression in Raw264.7 cells received FGF21 treatment for 10 hours. (A) Rat primary adipocyte *Fgf21* mRNA expression after curcumin treatment. (B) iNOS protein expression in RAW264.7 cells after FGF21 treatment. (C-E) M1-like macrophage marker expression in Raw264.7 cells after FGF21 treatment. (C) *iNOS*. (D) *MCP-1*. (E) *IL-6*. n=3 for all panels. *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.

4 Curcumin treatment inhibits adipose tissue mediated M1-like differentiation

To further explore the effect of curcumin on regulating macrophage differentiation, we asked whether curcumin represses M1-subtype differentiation via interacting with the adipocytes. For this purpose, we have first assessed the effect of adipocyte conditioned medium on M1-sybtype differentiation. The adipocyte conditioned medium treatment experiment was shown in Figure 13. Rat primary adipocytes were cultured overnight after the isolation. The next day, the medium was collected. Raw264.7 cells were cultured with the conditioned medium. At the meantime, the adipocytes were cultured with 2μmol/L or 10μmol/L curcumin for 10 hours. The Raw264.7 cells received adipocyte conditioned medium treatment showed significantly M1-like differentiation marker expression (Figure 14A-C). On the other hand, curcumin treated primary adipocyte showed decreased pro-inflammatory (or pro-M1-like differentiation) cytokines mRNA expression (Figure 14D-G). The anti-inflammatory cytokine, IL-4, which is also an M2-like cell inducer was increased in the primary adipocytes after 10μmol/L curcumin treatment (Figure 14H).

Thus, this *in vitro* Raw264.7 cell model allowed us to reveal the effect of curcumin in attenuating the expression of the pro-inflammation cytokines by both macrophages and mature adipocytes. Increased expression of the anti-inflammatory cytokine IL-4 gene in mature adipocytes but not in Raw264-7 cells was observed with curcumin at 2-10 μmol/L dosages.

5 Dietary curcumin intervention increases energy expenditure

Mice received HFD feeding without or with curcumin at the 16th week (Figure 2) were subject to metabolic cage analysis and the data were normalized to the body weight. Curcumin increased CO_2 production (Figure 15A), O_2 consumption (Figure 15B) and energy expenditure (Figure 15C). During the whole 30-hour metabolic cage analysis period, there was a trend of increase ($p=0.0502$) on overall respiration exchange ratio (RER) in mice received HFD with curcumin diet (Figure 15D, E). When we analyzed the data from the 12-hour dark period, the significant increase on RER was found in mice received HFD with curcumin ($p=0.0004$) (Figure 15D, F). These findings, along with the lack of effect on food intake or physical activities (Figure 16A, B), made us suggest that dietary curcumin intervention increases thermogenesis and facilitates the utilization of fat as the source of energy.

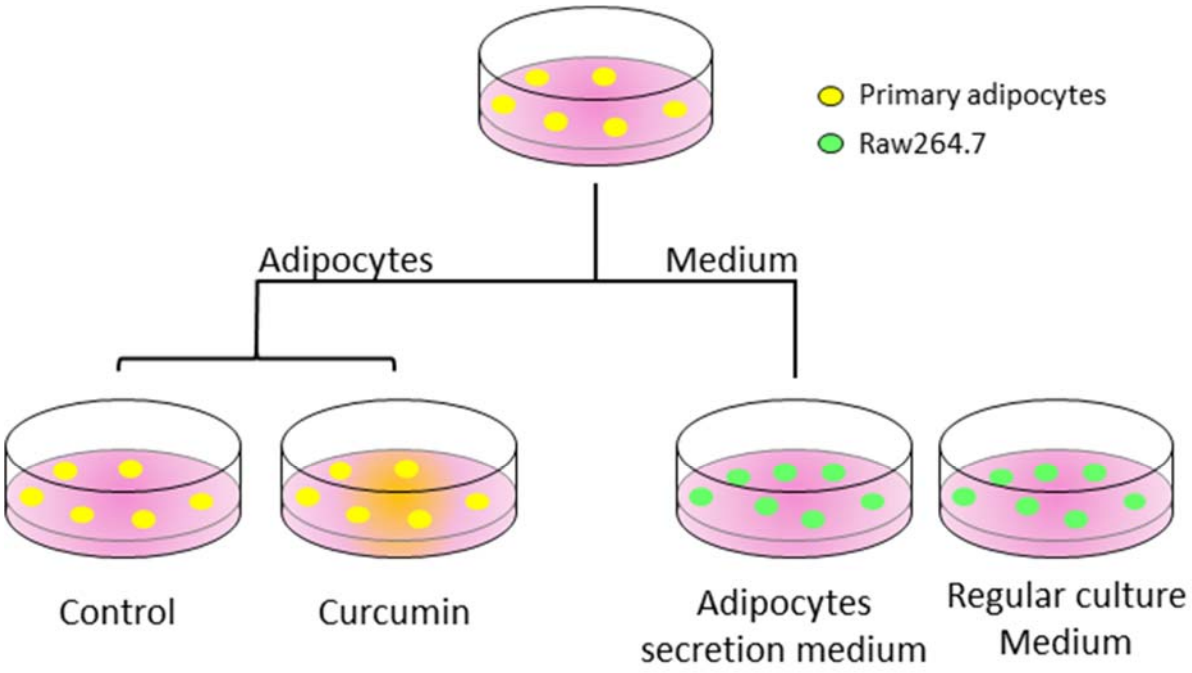

Figure 13. Illustration of strategies for assessing interactions between adipocytes and macrophages. The left panel shows that rat adipocytes were cultured with the DMSO vehicle (control) or curcumin for 10 hours, followed by RNA isolation and qRT-PCR (see Figure 14 for results). The right panel shows that Raw264.7 cells were treated with either the regular culture medium or the rat adipocyte conditioned medium, followed by RNA isolation and qRT-PCR (see Figure 14 for results).

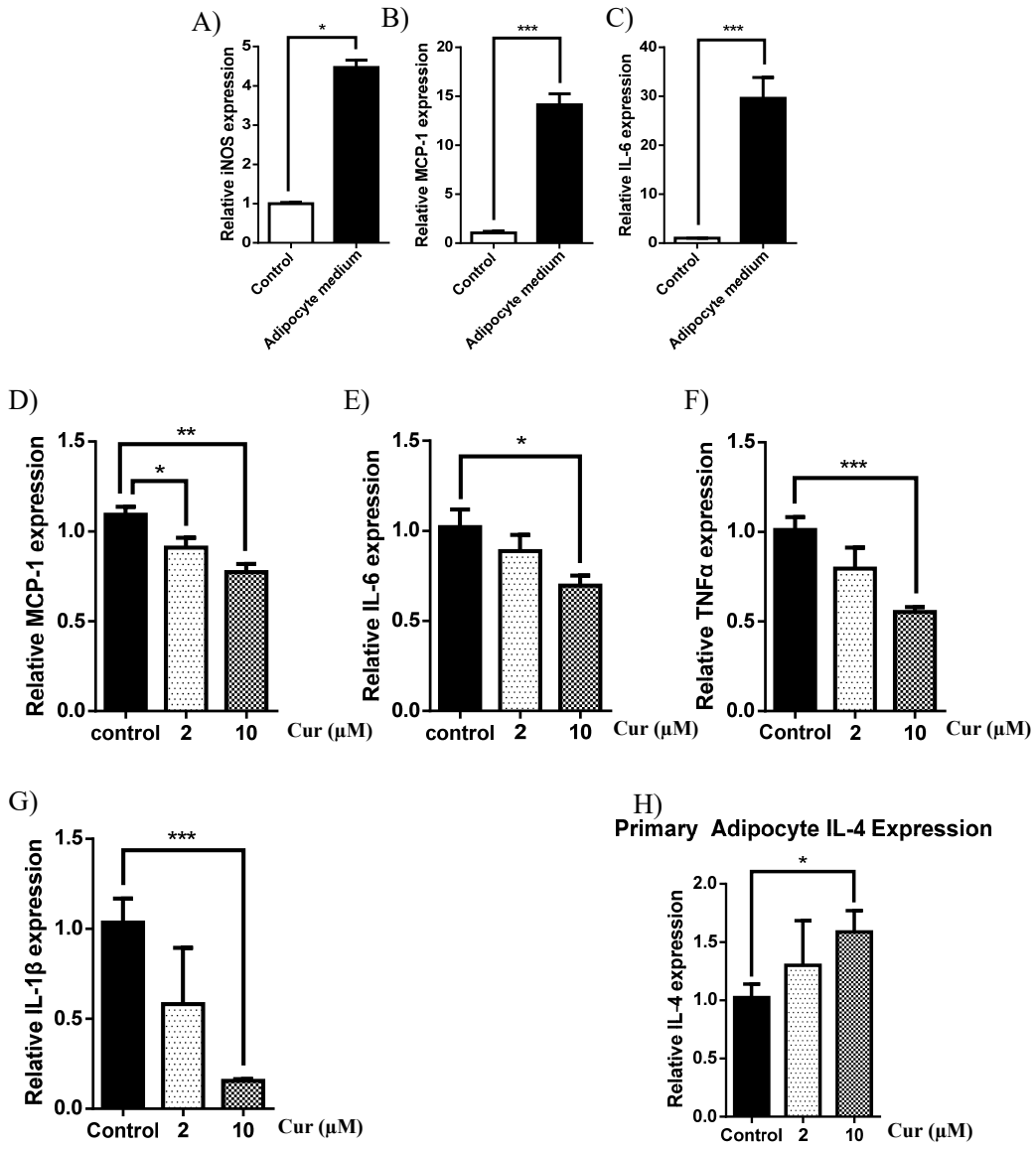

Figure 14. M1-like macrophage marker expression in Raw264.7 cells after adipocyte conditioned medium treatment and cytokine expression in adipocytes after curcumin treatment. (A-C) M1-like macrophage marker expression in raw264.7 cells received adipocyte conditioned medium treatment for 10 hours. (A) *iNOS*. (B) *MCP-1*. (C) *IL-6* (n=4). (D-G) Pro-inflammatory cytokine expression in primary adipocytes after curcumin treatment (D) *MCP-1.* (E) *IL-6.* (F) *TNFα.* (G) *IL-1β.* (H) Anti-inflammatory *IL-4* cytokine expression in primary adipocytes after curcumin treatment. n=3 for panels D to H. *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.

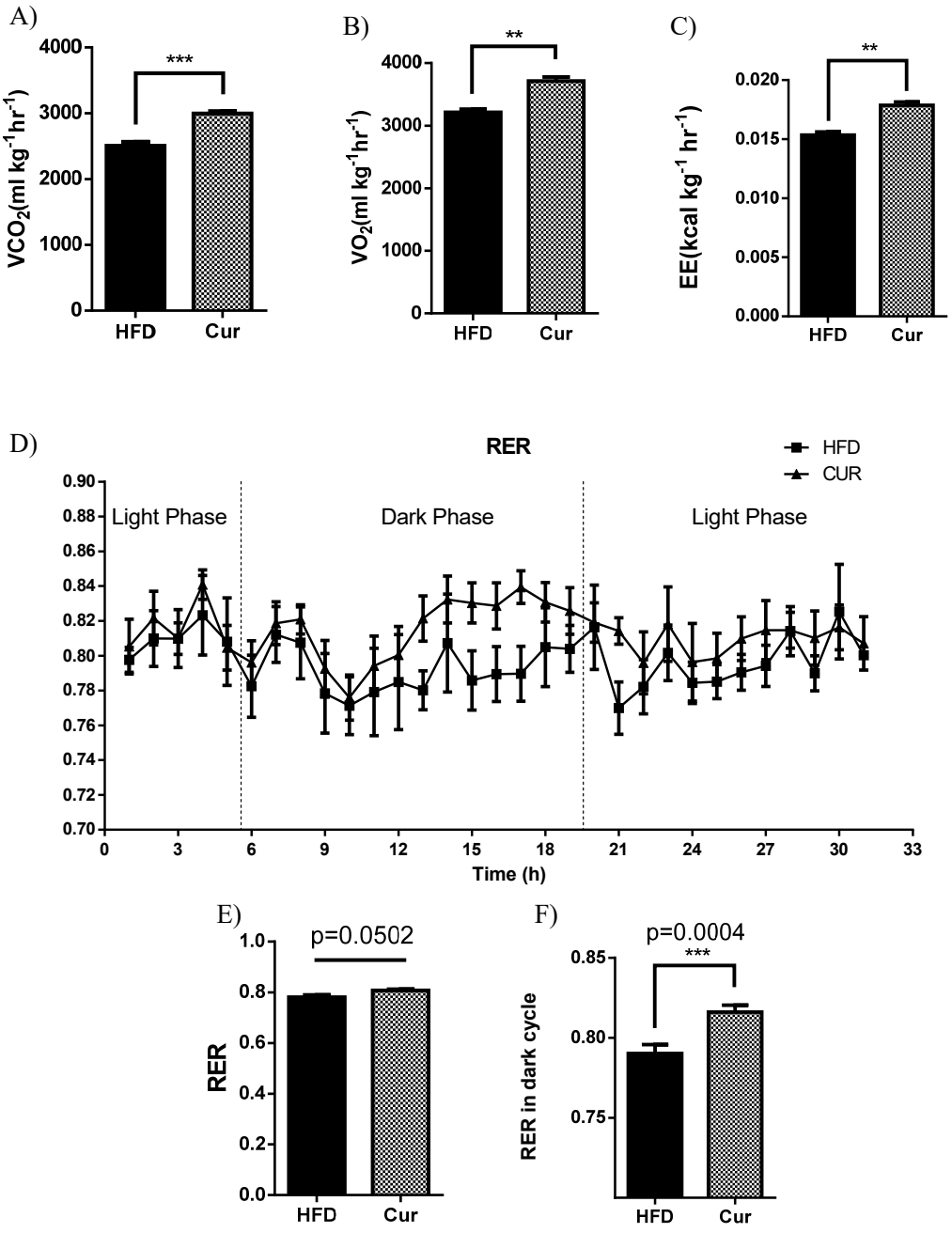

Figure 15. Metabolic cage data analyses. (A) Carbon dioxide production rate. (B) Oxygen consuming rate. (C) Energy expenditure (EE). (D) Diagram of respiratory exchange rate (RER) generated by metabolic cage analyses. (E) Overall RER. (F) RER during the dark cycle only. n=4 for all panels. *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.

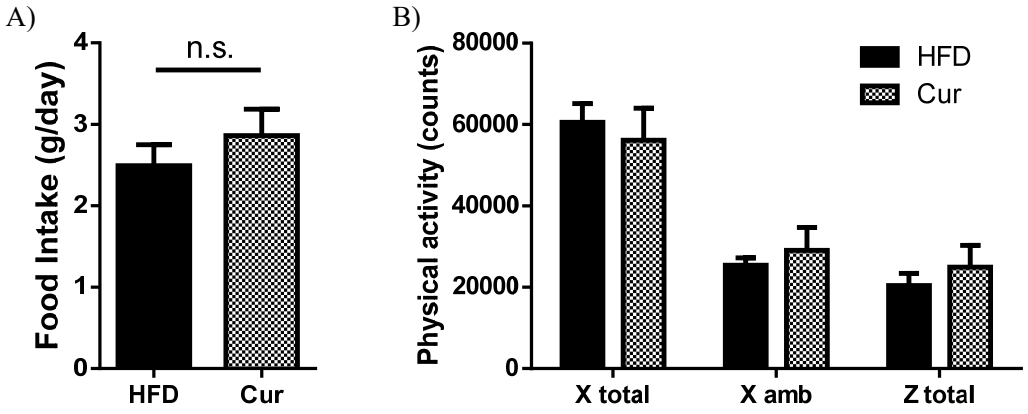

Figure 16. Food intake and physical activity measurement in metabolic cages. (A) Mouse food intake in metabolic cages. (B) Mice physical activities in metabolic cages. n=4 for all panels. n.s., non-significant.

6 Dietary curcumin intervention enhances thermogenic capacity in response to cold exposure

To further verify that curcumin intervention increases thermogenesis, the 2nd set of mouse experiment with dietary curcumin intervention was designed (Figure 17). The cold challenge on mice received indicated diet feeding for 2 and 4 weeks was performed for 48-period (Figure 18A) while cold challenge on mice received indicated diet feeding for 6 weeks was performed for 72-hour period. The experiment started in the afternoon. During the cold challenge, mice rectal temperature was recorded every 4 hours. In the 2nd week, significant differences in mouse temperature were observed among the three groups of mice and the differences mainly happened during the dark cycle (from 8:00 p.m. to 4:00 a.m.). The area under the curve showed the overall body temperature elevation effect of curcumin intervention. The body thermogenic capacity changes appeared ahead of the body weight difference (Figure 3A). A similar result was obtained from the 4-week curcumin-fed mice (Figure 18B). After 6 weeks of dietary curcumin intervention when body weight difference had shown, the body weight difference became more obvious that most of the temperature recorded showed the significant increase in HFD fed mice with curcumin intervention (Figure 18C).

Our data also showed that at the 2nd week and the 4th week, HFD feeding also increased mouse thermogenesis, when compared with mice received LFD feeding, in agreement with a recent report[44].

The thermogenesis improvement effects of curcumin, however, were not observed in mice housed at room temperature (Figure 19A-C), suggesting that either our experimental approach is not sensitive enough in revealing the difference or a “second hit”, such as β-adrenergic agonist, is required for the activation of UCP1.

7 Curcumin intervention increases BAT UCP1 expression

As dietary curcumin intervention increased thermogenic potential ahead of its body weight lowering effect, we asked whether it affects UCP1 expression and subcutaneous adipose tissue browning.

Mouse brown adipose tissue, epididymal adipose tissue and inguinal adipose tissue from the three groups of mice were fixed with 4% paraformaldehyde for 24 hours. Tissues were

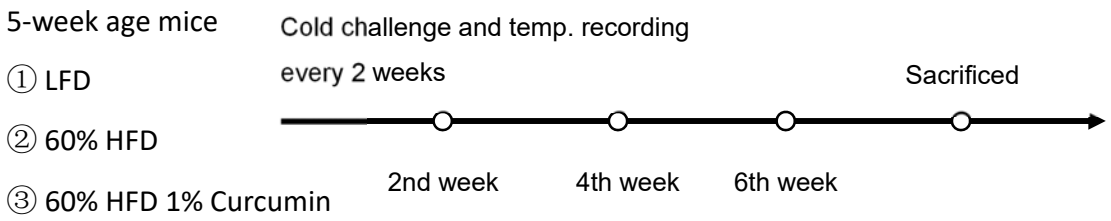

Figure 17. Illustration of rectal temperature recording experiment procedure. Mice were fed with indicated diets for 8 weeks. At the 2nd, 4th and 6th week, mice received cold challenge (4℃) for 48 hours (72 hours on the 6th week). During the cold challenge, the rectal temperature was recorded every 4 hours.

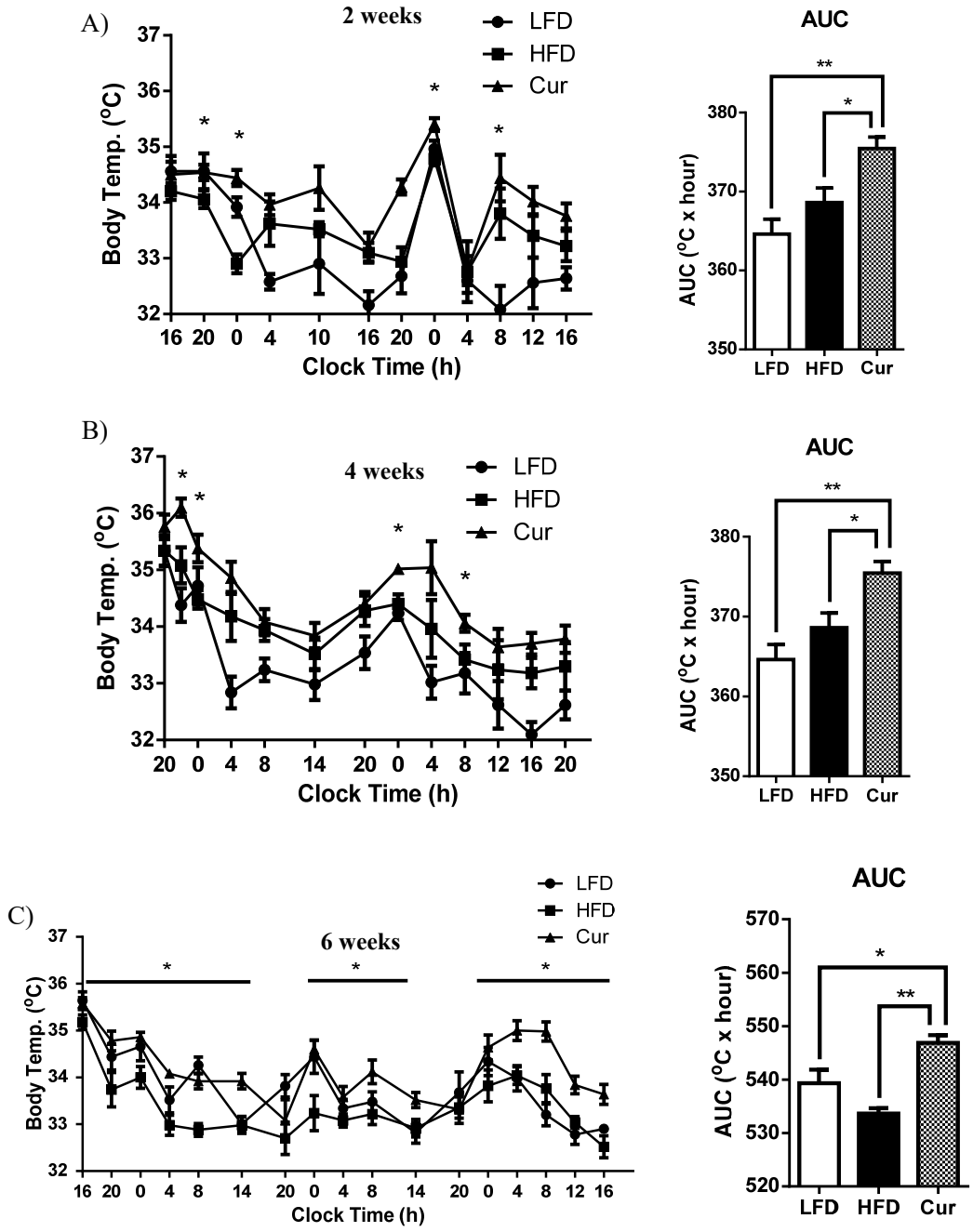

Figure 18. Rectal temperature records of the mice receiving cold challenge (4°C). (A) Two-week feeding mice temperature. (B) Four-week feeding mice temperature. (C) Six-week feeding mice temperature. Right panels show area under curves. n=5 for all panels. *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.

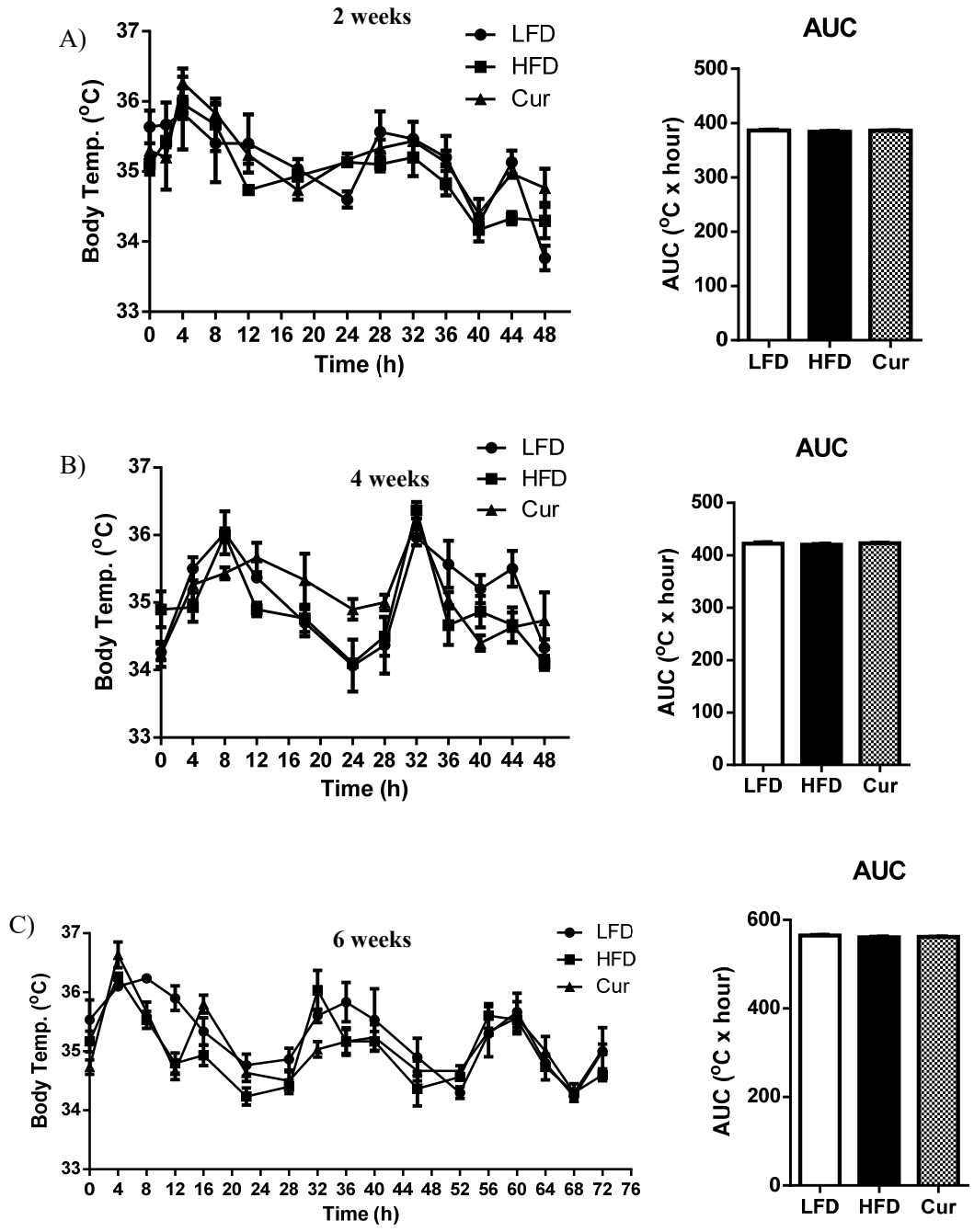

Figure 19. Rectal temperature records of the mice at room temperature (20°C). (A) Two-week feeding mice temperature. (B) Four-week feeding mice temperature. (C) Six-week feeding mice temperature. Right panels show area under curves. n=3 for all panels. *, $p<0.05$.

embedded in paraffin the next day. Tissue sections were immune-stained with the UCP1 antibody. Adipocyte cell size was measured using Image Pro Plus software.

The effect of dietary curcumin intervention on reducing the size of BAT adipocyte was highly appreciable (Figure 20A). The cell lipid droplets size was nearly 10-fold decreased after curcumin intervention. In addition, in both epididymal adipose tissue and inguinal adipose tissue, curcumin-fed mice also showed smaller adipocyte cell size (Figure 20B, C). The UCP1, which is the key thermogenic factor, was found to be increased significantly in curcumin-fed mice BAT at both protein and mRNA level (Figure 21A, Figure 22A). Notably, unlike a recent report on mice fed with LFD and curcumin[77], although there are a few UCP1 positive cell clusters in the subcutaneous adipose tissue of our long-term dietary curcumin intervention mice (Figure 20C). we did not see any changes in *UCP1* mRNA expression in subcutaneous adipose tissue after dietary curcumin intervention (Figure 21B, C). This is likely due to variable experimental conditions between the two studies, including the animal models, the dosages, the way of curcumin that was administrated, and the length of the experiments.

In BAT, increased UCP1 expression in response to dietary curcumin intervention was associated with increased PPARα, but not PPARγ or PGC-1α (Figure 22A), while in rat primary white adipocytes, direct curcumin treatment also increased UCP1 level, associated with PPARα and PGC-1α elevation (Figure 22B), indicating that curcumin may stimulate UCP1 expression, involving PPARα activation. We then tested the effect of curcumin treatment on UCP1 expression in a mouse brown adipocyte cell line (mBAT)[131]. After cell differentiation shown by Oil-Red-O staining in Figure 22C, 5μmol/L of curcumin was added to the maintenance medium for a 10-hour treatment. After the differentiation process, curcumin treatment also increased UCP1 protein expression (Figure 22D). The mRNA expression of UCP1, PPARα, PPARγ and PGC-1α was all significantly increased (Figure 22E).

We have also transfected UCP1/Luciferase fusion gene plasmid into the 293T naïve cell system, followed by curcumin treatment in the absence and presence of PPARα or PPARγ antagonist. Data shows that curcumin or the PPARα agonist WY14643 could both stimulate UCP1 promoter activity. Even the luciferin intensity was inhibited by PPARα antagonist GW6471, the stimulatory effect of curcumin cannot be blocked by the PPARα antagonist (Figure 23A).

The PPARγ agonist/antagonist experiment demonstrated a similar result. The stimulatory effect of rosiglitazone was appreciable, while the induction of UCP1 promoter activity by curcumin treatment cannot be blocked by the PPARγ antagonist GW9662 (Figure 23B).

Together, our observations suggest that curcumin stimulates UCP1 expression via both PPAR dependent and independent mechanisms.

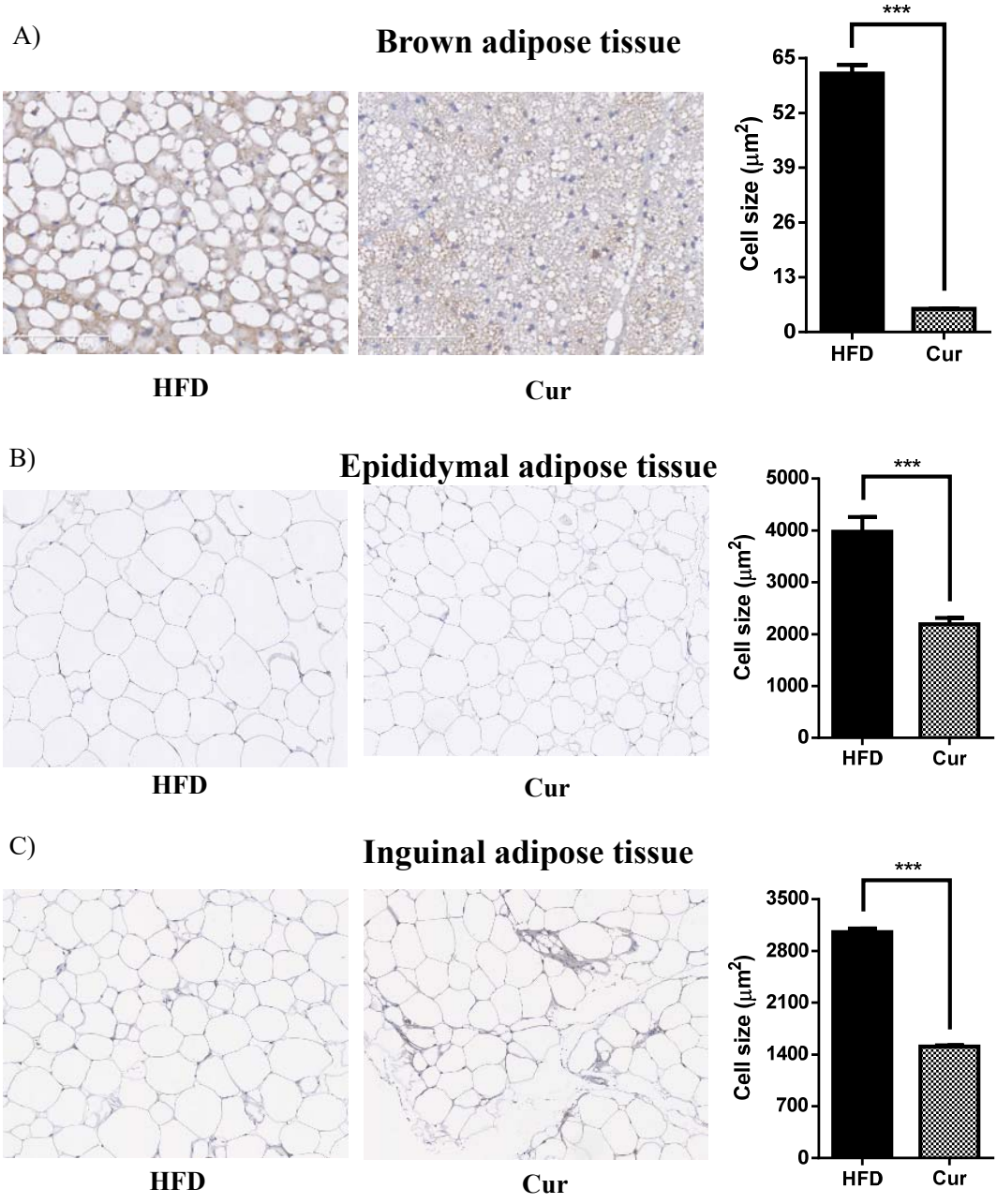

Figure 20. Adipose tissues UCP1 immunostaining and cell size measurement from mice received curcumin intervention. (A) Brown adipose tissue (cell count HFD vs Cur: 6423 vs 59987). (B) Epididymal adipose tissue (cell count HFD vs Cur: 1065 vs 1678). (C) Inguinal adipose tissue (cell count HFD vs Cur: 3158 vs 7775). Right panel shows the adipocyte cell size measurement. *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.

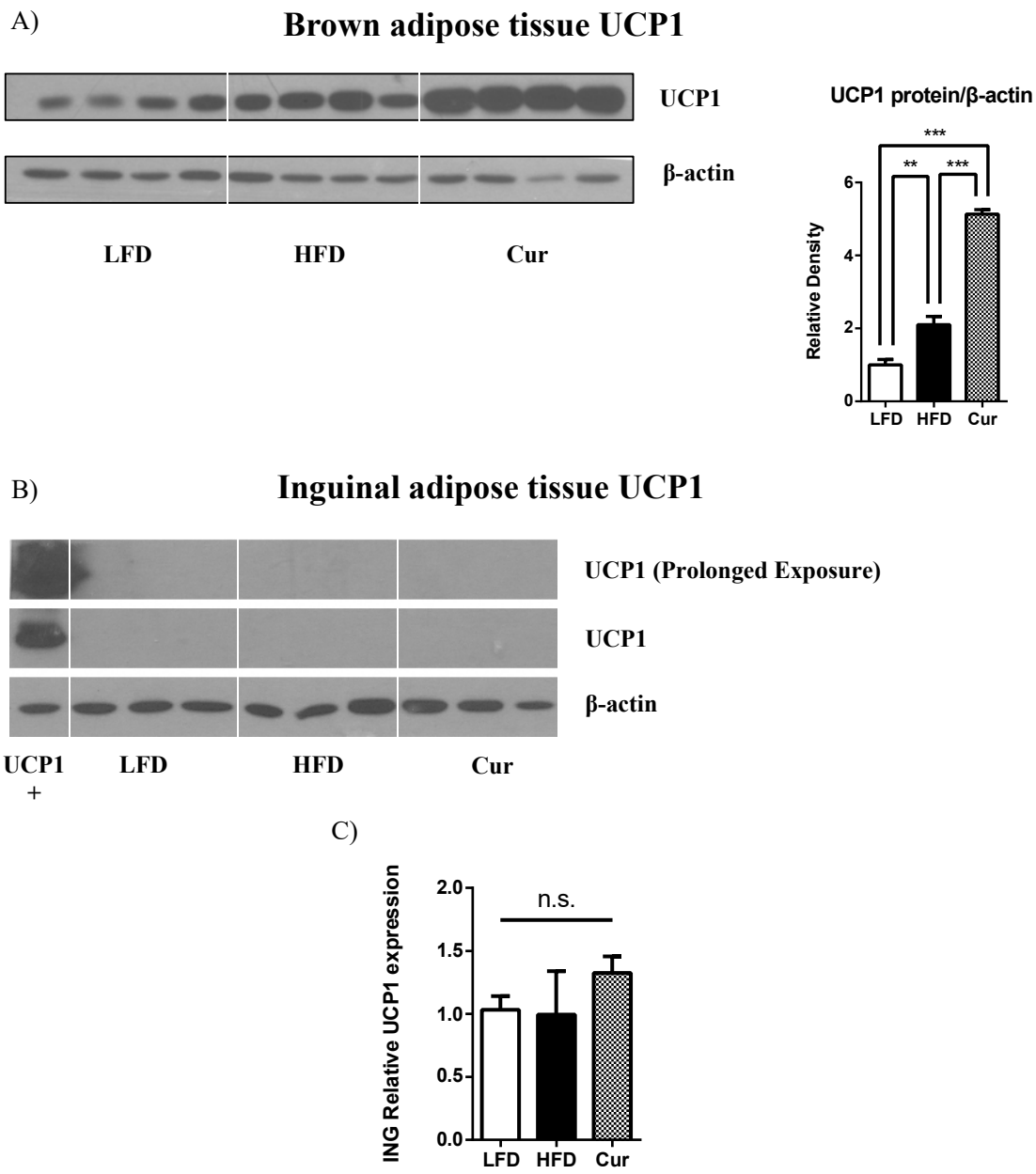

Figure 21. UCP1 protein level in BAT and inguinal adipose tissue from mice received curcumin intervention. (A) Western blotting shows the UCP1 protein expression in BAT from the long-term curcumin intervention mice. Right panel shows the results of densitometry analyses (n=4) (B) Western blotting shows the UCP1 protein expression in inguinal adipose tissue (n=3). (C) Real-time PCR shows *UCP1* mRNA expression in inguinal adipose tissue (n=3). *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.

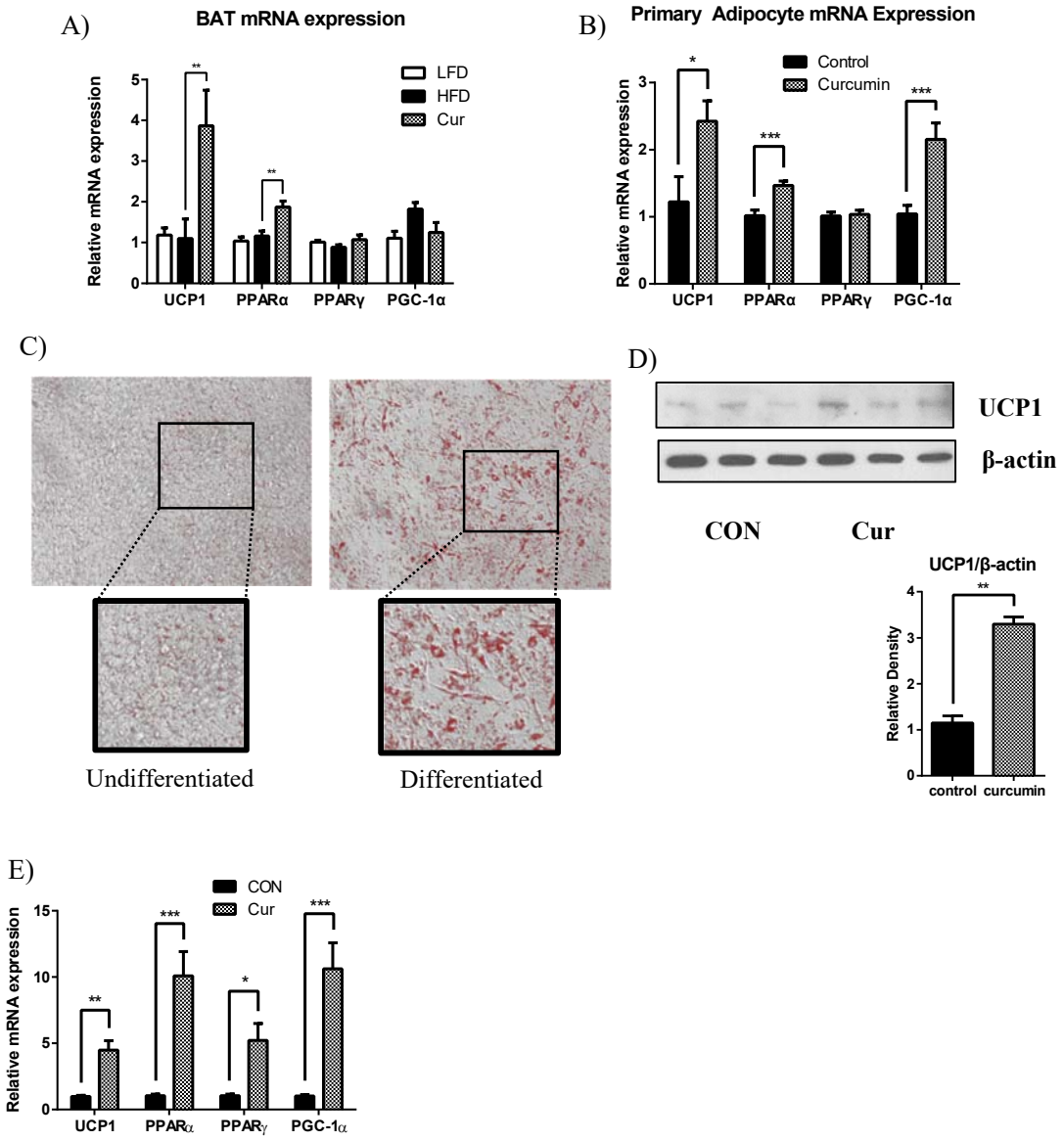

Figure 22. Thermogenic gene mRNA expression in BAT and adipocytes after curcumin treatment. (A) The mRNA expression of *UCP1, PPARα, PPARγ* and *PGC-1α* in BAT (n=5) (B) The mRNA expression of *UCP1, PPARα, PPARγ* and *PGC-1α* in rat primary adipocyte with or without curcumin treatment. (C) Oil-red-O staining for mBAT cells after differentiation. (D) UCP1 protein level in differentiated mBAT cells after curcumin treatment. Lower panel is the densitometry analysis. (E) The mRNA expression of *UCP1, PPARα, PPARγ* and *PGC-1α* in mBAT. n=3 for panels B to E. *, $p<0.05$; **, $p<0.01$; n.s., non-significant.

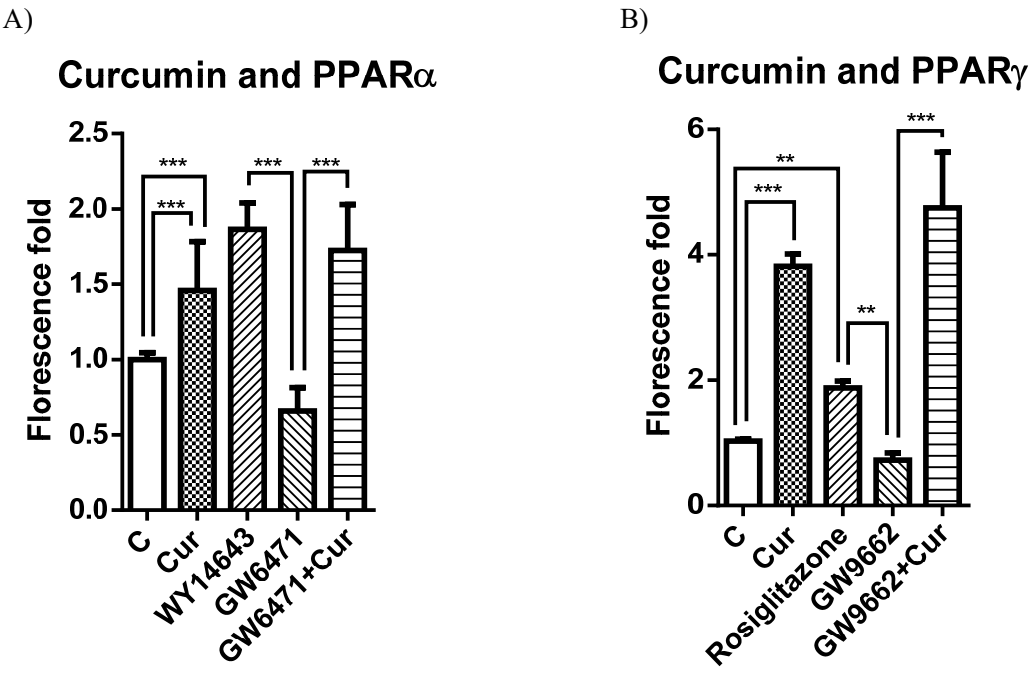

Figure 23. UCP1-LUC reporter gene analyses in 293T cells. UCP1-LUC reporter plasmid was transfected into 293T cells. (A) After transfection, the cells were treated with curcumin, WY14643 (PPARα agonist) and GW6471 (PPARα antagonist) as indicated. (B) After transfection, the cells were treated with curcumin, Rosiglitazone (PPARγ agonist) and GW9662 (PPARγ antagonist) as indicated. The luciferin florescence intensity was measured referring to UCP1 promoter activity after treatment. n=5 for all panels. *, $p<0.05$; **, $p<0.01$; ***, $p<0.005$.

Discussion

Overweight and obesity have been associated with several metabolic diseases, causing enormous economic burdens with a rapidly growing population globally. Obesity is often diagnosed using body mass index (BMI), which is body weight (kg) divided by the square of height (m). According to the WHO's standard, The BMI between 25 and 30 kg/m^2 is defined as overweight and the BMI over 30 kg/m^2 is considered as Obesity[134]. It was defined as a disease by the American Medical Association in the year of 2013.

Obesity is one of the most concerned public health problems as a consequence of its prevalence[135]. The population of obese people is rising rapidly. In the year of 2014, there were 1.9 billion of adult (39% of the global population) who were overweight and over 600 million of them were obese. Moreover, 42 million children under 5-yrear-old are suffering from overweight or obesity[136].

The major causative factors of obesity are excessive energy intake, the lack of exercise, genetic defects, endocrine disorders and drug affections[134]. Although reduced energy expenditure caused adipose tissue expansion and body weight increase[137,138], studies revealed that the basal metabolic rate in obese individuals is relatively higher than non-obese people[139].

The risk of cardiovascular disease, T2D, sleep apnea, cancer and osteoarthritis can be substantially increased with obesity onset[140].

Thus, it is urgent to develop effective methods for the treatment and prevention of obesity.

1 The connection of obesity to this study

Increased WAT inflammation and reduced energy expenditure are among the key causative factors of obesity and its related metabolic disorders, tightly associated with urbanization and sedentary lifestyles[141,142]. Therapeutic and prevention tools that target these two key pathological processes simultaneously are desired and our current study suggests that this can be achieved via dietary polyphenol interventions.

2 Summary of the major findings

We show here that dietary curcumin intervention inhibits WAT inflammation induced by HFD consumption and activates BAT UCP1 production and function. As illustrated in Figure 24, the inhibition on WAT inflammation by dietary curcumin intervention is achieved not only by the reduction of macrophage infiltration but also by the regulation of M1-like macrophage and M2-like macrophage differentiation and function. Importantly, we demonstrated the role of adipocyte-produced pro-inflammatory cytokines on stimulating M1-like macrophage differentiation and the inhibitory effect of curcumin on the production of pro-inflammatory cytokines by both infiltrated macrophages and mature adipocytes. The stimulation of anti-inflammatory cytokine production by curcumin, however, may mainly occur in adipocytes. Furthermore, stimulations of BAT UCP1 production and function by dietary curcumin intervention likely occurs ahead of its body weight lowering effect and this is likely mediated by both PPAR dependent and independent mechanisms; as the *in vitro* stimulation cannot be blocked by either PPARα or PPARγ antagonists.

3 The exact active molecule of curcumin is still unknown and there is no known receptor for curcumin and other polyphenols

Curcumin, anthocyanin and resveratrol are the most studied dietary polyphenols for their therapeutic potential or applications on various diseases including T2D and other metabolic disorders[61]. They share the common feature in targeting multiple organs without a defined membrane bound or nuclear receptor. This feature also applies to the widely-utilized diabetes drug metformin and the potential repurposed diabetes drug artemisinin; both are plant derived chemicals[143,144]. Another common feature for most, if not all, dietary polyphenols is their low bio-availabilities due to the low gut absorption rate[90]. These characteristics have generated certain difficulties in dissecting the mechanistic insights of their functions and may have created some astray for drug hunters, as suggested very recently[145,146]. Scientists, however, are gradually approaching the function of curcumin and other plant polyphenols, including the recognition of the stimulatory effect of curcumin[60], resveratrol[147] and anthocyanin (unpublished observations by Dr. Jin's lab) on hepatic hormone Fgf21 production. Zeng *et al.* in our laboratory has also demonstrated that dietary curcumin intervention restored HFD feeding induced Fgf21 resistance[60]. Moreover, dietary polyphenol study is among the driving forces for scientists to pay

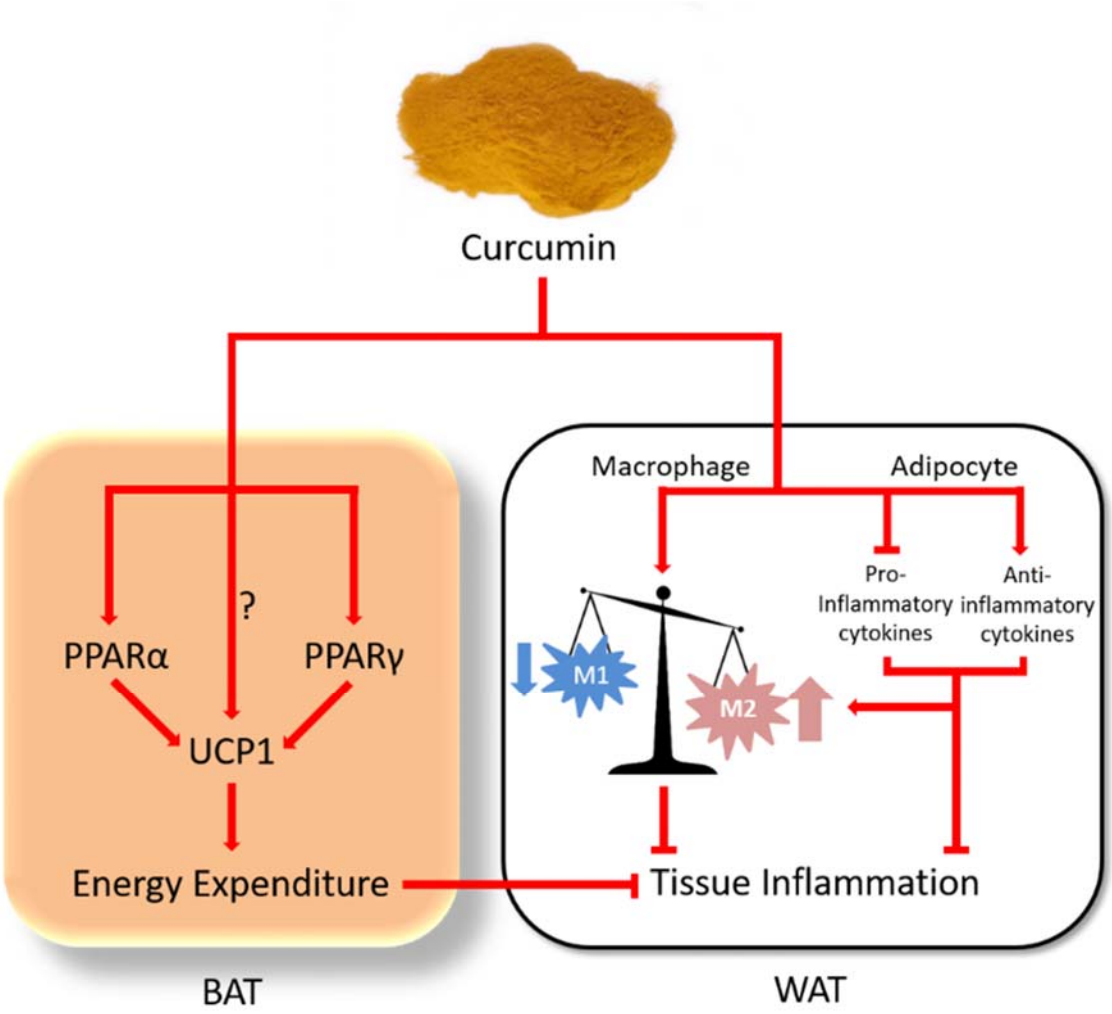

Figure 24. Summary diagram of the major findings of this study. Curcumin intervention reduces WAT inflammation, achieved by reducing pro-inflammatory cytokine expression in both macrophages and mature adipocytes, and increasing the anti-inflammatory cytokine expression in mature adipocytes. These effects collectively lead to reduce the ratio of M1-like/M2-like macrophages. Curcumin intervention also increases energy expenditure via both PPAR dependent and independent mechanisms in stimulating UCP1 expression in BAT.

more close attention to the gut microbiota. We have now generally accepted that at least some of the biological functions of dietary polyphenols are exerted via their interactions with the gut, including the stimulation of certain duodenum signaling cascade, the production of gut microbiota metabolites of polyphenols, and the generation of gut microbiota products that regulate metabolic homeostasis via several means[148-153].

Referring to the current study, we cannot eliminate the possibility that the effective molecules in exerting the *in vivo* effects of dietary curcumin intervention are not the same curcumin molecules utilized in our *in vitro* assays.

4 Curcumin stimulates UCP1 expression in both PPAR dependent and independent manner

In exploring the activation of hepatic Fgf21 expression by *in vitro* curcumin treatment, Zeng *et al.* in our laboratory have also noticed its stimulation of the nuclear receptor (NR) PPARα. Curcumin, however, is unlikely a direct ligand of PPARα[60]. Zeng *et al.* conducted a GAL4-NR luciferase (GAL4-NR-LUC) reporter assay in HEK293 cells. In this assay, curcumin did not activate exogenously introduced PPARα, although vitamin D receptor was slightly activated by 10 μmol/L curcumin[60]. Furthermore, conditioned medium of primary hepatocytes treated with curcumin also cannot stimulate the GAL4-NR-LUC reporter, suggesting that curcumin may increase the intracellular PPARα ligand level or the ligand activity. In the current study, PPARα and PPARγ expression were stimulated by curcumin treatment *in vitro* in mBAT (Figure 22E). In conducting another line of research, Tian *et al.* in our laboratory has also noticed the stimulatory effect of curcumin on PPAR activation[59]. Unexpectedly, the direct stimulatory effect of curcumin on *UCP1* promoter cannot be blocked by PPARα or PPARγ antagonist, although they are known trans-activators of UCP1[154-156]. Further investigations are needed to clarify how curcumin activates UCP1 transcription via triggering both PPAR dependent and independent pathways. These, along with observations in intensive studies on curcumin and other plant polyphenols prompted us to suggest that a revolution in our receptor theory is needed. The current theory cannot always explain physiology or endocrinology observations; even for certain peptide hormones such as GLP-1 with defined receptors[157].

5 Curcumin increases Fgf21 mRNA expression and energy expenditure apart from its anti-inflammatory property

A recent study by Lynch *et al.* show that adipose tissue Fgf21 production can be stimulated by invariant natural killer T cells (iNKT) in response to α-galactosylceramide or GLP-1 based drugs[158]. This signaling cascade appears to activate thermogenesis and subcutaneous adipocyte browning[158]. Zeng *et al.* in our laboratory demonstrated that hepatic Fgf21 expression was increased after curcumin treatment *in vitro* or short-term gavage *in vivo*[60]. I found that Fgf21 treatment did not repress LPS/IFNγ induced Raw264.7 cell towards M1-like macrophage differentiation, suggesting that should Fgf21 possess the anti-inflammation property, it is not mediated directly via inhibiting the M1-like macrophage differentiation. Considering that curcumin treatment can stimulate Fgf21 production in the liver[60] and in mature adipocytes (Figure 12A), why we did not see the beiging effect in our long-term dietary curcumin intervention animals? Our explanation is that although short-term curcumin gavage in mice fed with chow diet increased hepatic Fgf21 production and the plasma Fgf21 level, in HFD-fed mice, concomitant dietary curcumin intervention attenuated HFD stimulated Fgf21 production and improved Fgf21 sensitivity[60]. Indeed, we found in the current study that HFD feeding also increased WAT Fgf21 mRNA expression and the increase was significantly attenuated by 18-week dietary curcumin intervention (Figure 6A, B).

We were unable to demonstrate the “browning” effect in subcutaneous adipose tissue via curcumin intervention, reported by another group in mice without involving HFD feeding[77]. This is likely due to various experiment differences in these two studies, including the animal models, the dosages and the sources of curcumin, the way to administer curcumin and the length of the experiments. Together with our body temperature results, the subcutaneous adipose tissue browning potential may be improved by curcumin intervention; however, a second hit, such as cold-challenge or β-adrenergic receptor agonist, is required. On the other hand, as we have discussed in above, the “browning” effect might be attributed mainly to the increases in circulating and adipose tissue Fgf21 levels, which could be elevated in mice fed with chow diet and curcumin but not in HFD-fed mice with curcumin intervention[60].

6 Curcumin primarily inhibits M1-like macrophage differentiation

The Raw264.7 cell line was utilized in this study as the *in vitro* cell model to assess the effect of curcumin on M1-like and M2-like macrophage differentiation. It appeared that curcumin repressed of the M1-like macrophage marker expression but not stimulated the M2-like macrophage marker expression. Thus, dietary curcumin intervention may directly inhibit M1-like macrophage differentiation. Its stimulatory effect on M2-like macrophage differentiation could be secondary, as the results of reduced M1-like macrophage differentiation in combining with the regulation of pro-inflammatory and anti-inflammatory cytokine expression in mature adipocytes. This notion is supported by the lack of stimulation of IL-4 and I-13 expression in infiltrated WAT immune cells received 18 weeks of dietary curcumin intervention (Table 3).

In conclusion, our current study advanced our knowledge on the metabolic beneficial effects of the curry compound curcumin. Dietary intervention with this plant polyphenol plays a dual modulatory effect in preventing obesity and its related metabolic disorders: the attenuation of WAT inflammation and the increase of energy expenditure via targeting BAT UCP1.

7 Long-term dietary polyphenol intervention and lifestyle modification

The population of obese people is growing fast as a public health problem[134]. Diseases such as atherosclerosis, T2D and cancer can be caused by the accumulated plasma fatty acid and cytokines that are produced by adipose tissue from obese individuals[140]. Treatment and agents that prevent or cure obesity are desired unprecedented. The lifestyle modification is one of the most recommended ways to fight against obesity and overweight. Studies have revealed that dietary intervention with plant polyphenols would not only reduce body weight gain but also prevent organ damages from pro-inflammatory cytokines produced by adipose tissues[58,59,61]. The metabolic beneficial effect of dietary polyphenols prompted scientists to study them for proper utilization and enhanced function.

The three most studied dietary polyphenols, anthocyanin, resveratrol and curcumin, have been proved to be health beneficial[57-64,66,69,70], which was attributed to their anti-inflammatory and anti-oxidant properties. Studies showed that plasma pro-inflammatory cytokines were

significantly reduced after dietary polyphenol interventions[58,61,73]. We hypothesized that the polyphenols have multiple targets or organs to interact with to exert the metabolic beneficial function beyond the anti-inflammatory properties.

One previous study in our laboratory demonstrated that hepatic Fgf21 production was increased in LFD fed mice by short term curcumin gavage intervention[59]. Fgf21 production *in vitro* in hepatocytes with curcumin treatment was increased as well[60]. The mechanistic exploration suggested that the stimulation of Fgf21 production involved the activation of PPARα which is the key stimulator for *Fgf21* gene transcription. In this study, the primary adipocyte also showed increased *Fgf21* mRNA expression after curcumin treatment[155]. Our long-term dietary curcumin intervention mice showed decreased plasma Fgf21 level. It was discussed in the previous publications from our laboratory that long-term HFD feeding could result in a Fgf21 resistance condition. The dietary curcumin intervention, however, sensitizes Fgf21 resistancereceptors[60].

I showed that the anti-inflammatory function of dietary curcumin intervention in adipose tissue was dependent on both macrophages and adipocytes. Curcumin intervention reduced the population of pro-inflammatory macrophages and the expressions of pro-inflammatory cytokines in both tissue-infiltrated immune cells and adipocytes.

WAT beiging was not observed. In addition, UCP1 mRNA or protein was found to be increased in mouse inguinal adipose tissue. Increased body thermogenic capacity and energy expenditure were mostly contributed by BAT. Dietary curcumin intervention increased BAT UCP1 expression. The smaller lipid droplet sizes in association with increased RER implied the existence of increased carbonhydrate metabolism in mice with dietary curcumin intervention. Mechanistic study suggested that curcumin could stimulate UCP1 expression in both PPARα and PPARγ dependent and independent manners.

Although there is no known receptor for natural polyphenols such as curcumin, it has been shown that dietary polyphenol intervention reduced body weight, blood glucose and tissue inflammation. They also inhibit and prevent the progression of certain diseases such as obesity, T2D and cancer[57,61]. In this study, mechanistic understanding on the function of dietary polyphenols has been expanded. In addition to its anti-inflammatory function, we have demonstrated that curcumin intervention increases Fgf21 production, regulates macrophage functional polarity and cytokine production, inhibits adipocyte pro-inflammatory cytokine

expression, increases energy expenditure and lipid metabolism. The function of curcumin from certain aspects may not be remarkable, however, as the whole, the daily dietary curcumin intervention significantly improves metabolism and prevents subjects from metabolic disorders.

8 Future directions

My study has provided new insights into the metabolic beneficial effect of curcumin. While we have examined the effect of curcumin intervention on regulating WAT inflammation and BAT thermogenesis, a number of key questions remained to be future investigated.

8.1 Whether other dietary polyphenols possess the same or similar effects as curcumin on WAT and BAT

Studies have identified the metabolic beneficial and anti-inflammatory effect of dietary polyphenols such as anthocyanin and resveratrol. However, the study on the mechanism of the anti-inflammation and body weight lowering properties of these two polyphenols was limited. Examinations on other dietary polyphenols would be necessary to investigate the metabolic beneficial effect of dietary polyphenols in general. The generality among those studied polyphenols (anthocyanin, resveratrol and curcumin) on macrophage functional polarity, inflammatory cytokine expression in WAT and UCP1 expression in BAT would provide new insights into the function of dietary polyphenols.

8.2 Whether the stimulation on white adipocyte beiging is a transient event that can be observed with the modification of the experimental procedures.

Wang *et al.* reported that curcumin promotes white adipocyte beiging in mice received daily curcumin gavage for 50 days in the absence of HFD consumption[77]. However, I observe no significant beiging effect in my study with concomitant curcumin intervention and HFD consumption. This could be due to reduced Fgf21 level in plasma in long-term dietary curcumin intervention. Our lab has shown that short-term curcumin gavage increased Fgf21 level[59,60] in mouse plasma. Whether beige adipocytes can only be found when Fgf21 expression is high is required to be investigated. Such further investigations will clarify the relationship among curcumin, Fgf21 and WAT beiging.

WAT beiging can be induced by cold environment, administration of catecholamine agonists (like β3-adrenergic agonist)[46,77,159]. In our experiment, curcumin-fed mice did not show higher

body temperature compared with the mice received LFD or HFD at room temperature, indicating that the cold challenge was required for curcumin fed mice to exert increased thermogenesis. Additional treatment, compound or drugs which enhance the beiging effect of curcumin would allow us to optimize the use of curcumin with other agents to gain better metabolic benefits.

8.3 Whether curcumin interacts with intestinal microbiota to exert the metabolic beneficial effects.

Intestinal microbiota has profound impacts on the host metabolism. Animal studies and human clinical trials have indicated that obesity was associated with intestinal microbiota alteration[160]. In addition, the host metabolism disorders, such as obesity, insulin resistance and fatty liver could be affected by intestinal environmental modification and gut microbiota alteration[160,161]. Dietary curcumin intervention could have a great impact on intestinal microbiota, which regulates host metabolism afterward. In turn, the intestinal microbiota would modify curcumin molecule that has yet to be defined. The understanding of the interaction between curcumin (or other dietary polyphenols) and intestinal microbiota would further broaden our knowledge on the metabolic beneficial effects of dietary polyphenols beyond their anti-inflammation and anti-oxidant properties.

8.4 Whether dietary curcumin intervention inhibits WAT adipogenesis via Wnt signaling pathway stimulation

Further expanded understanding on mechanisms underlying adipocyte differentiation may lead to the development of novel therapeutic tools for the treatment of obesity and its related disorders. Wnt signaling pathway activation was reported to inhibit adipogenesis[162]. Tian and colleagues in our laboratory have revealed recently that curcumin repressed 3T3-L1 cell adipogenic differentiation by inhibiting the expression of miR-17-5p and stimulating Wnt signaling pathway *in vitro*[79]. It is necessary to study whether in *in vivo* settings, curcumin intervention represses unnecessary adipogenic differentiation, involving the regulation of miR17-5p and other microRNAs.

9 Conclusion

The current study advanced our knowledge on the metabolic beneficial effects of the curry compound curcumin. Dietary intervention with this plant polyphenol is capable of playing a

dual-modulatory effect in preventing obesity and its related metabolic disorders: the attenuation of WAT inflammation and the promotion of energy expenditure via targeting BAT UCP1. Observations made in this study, along with the discoveries that curcumin and other plant polyphenols (anthocyanin and resveratrol) regulate Fgf21 production and function[60,147], bring us a novel overview on dietary polyphenol research.

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