Interleukin-4 Regulates Lipid Metabolism by Inhibiting Adipogenesis and Promoting Lipolysis

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Type of manuscript: Original article  
Word count: Abstract: 197; Main Text: 4364  
Running title: Anti-adipogenesis and pro-lipolysis capacity of IL-4

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Abstract

Long-term cytokine-mediated inflammation is a risk factor for obesity and type 2 diabetes mellitus (T2DM). Our previous studies reveal significant associations between promoter single nucleotide polymorphisms (SNPs) of interleukin-4 (IL-4) and T2DM, as well as between SNPs in genes encoding IL-4/IL-4 receptor alpha chain and high-density lipoproteins. Our animal study uncovers that IL-4 regulates glucose/lipid metabolism by promoting glucose tolerance and inhibiting lipid deposits. The above results strongly suggest the involvement of IL-4 in energy homeostasis. In the present study, we focused on examining the regulatory mechanism of IL-4 to lipid metabolism. Our results show that IL-4 inhibits adipogenesis by down-regulating the expression of peroxisome proliferator-activated receptor-γ and CCAAT/enhancer-binding protein-α. Additionally, IL-4 promotes lipolysis by enhancing the activity and translocation of hormone sensitive lipase (HSL) in mature adipocytes, which suggests that IL-4 plays a pro-lipolytic role in lipid metabolism by boosting HSL activity. Our results demonstrate that IL-4 harbors pro-lipolytic capacity by inhibiting adipocytes differentiation and lipid accumulation as well as promoting lipolysis in mature adipocytes to decrease lipid deposits. The above findings uncover the novel roles of IL-4 in lipid metabolism and provide new insights in the interaction among cytokines/immune responses, insulin sensitivity and metabolism.

Key words: interleukin-4, adipogenesis, lipolysis, peroxisome proliferator-activated receptor-γ, CCAAT/enhancer-binding protein-α, hormone sensitive lipase, perilipin
**Abbreviations**

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<tr>
<th>Abbreviation</th>
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<tr>
<td>ATGL</td>
<td>Adipocyte triglyceride lipase</td>
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<td>C/EBPs</td>
<td>CCAAT/enhancer binding protein</td>
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<td>Dex</td>
<td>Dexamethasone</td>
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<td>IBMX</td>
<td>3-isobuty-methylxanthine</td>
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<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
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<td>ERK1/2</td>
<td>Extracellular-signal regulated kinase 1/2</td>
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<td>aP2</td>
<td>Fatty acid binding protein 4</td>
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<td>GLUT4</td>
<td>Glucose transporter 4</td>
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<td>GSK3β</td>
<td>Glycogen synthase kinase 3β</td>
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<td>High-density lipoprotein-cholesterol</td>
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<td>Hormone sensitive lipase</td>
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<td>LDs</td>
<td>Lipid droplets</td>
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<td>IL-</td>
<td>Interleukin</td>
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<td>IL-4Rα</td>
<td>IL-4 receptor alpha chain</td>
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<td>MCE</td>
<td>Mitotic clonal expansion</td>
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<tr>
<td>PPARγ</td>
<td>Peroxisome proliferator-activated receptor-γ</td>
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<tr>
<td>PKA</td>
<td>Protein kinase A</td>
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<tr>
<td>STAT6</td>
<td>Signal transducer and activator of transcription 6</td>
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<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
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<tr>
<td>TZD</td>
<td>Thiazolidinedione</td>
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<td>T2DM</td>
<td>Type 2 diabetes mellitus</td>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
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<td>WAT</td>
<td>White adipose tissue</td>
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Introduction

Obesity is characterized by an expansion of white adipose tissue (WAT) mass resulting from increased adipocyte number and/or size (1). It is a key risk factor leading to type 2 diabetes mellitus (T2DM), hyperlipidemia, and has become a pan-endemic health problem with rapid-growing global incidence (2,3).

Obesity is associated with systemic chronic inflammation characterized by altered cytokine production and activation of inflammatory signaling (4,5). Abundant studies have linked the increased production of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6), and certain adipokines to the inflammatory process of obesity, as well as to the development of insulin resistance (6-8). However, the effect of anti-inflammatory cytokine, such as IL-4, in the development of insulin resistance or obesity is less understood.

IL-4, mainly secreted by activated Th2 lymphocytes, executes pleiotropic functions such as induction of Th2 differentiation, immunoglobulin class switching, and B cell proliferation (9). The production of IL-4 by splenic lymphocytes from diet-induced obese mice is increased (10) while level of serum IL-4 is decreased in Sprague-Dawley rat after receiving visceral fat remove surgery (11). IL-4 secreted from adipocytes and hepatocytes shows the capacity of modulating local immune response and insulin sensitivity (12,13). These results suggest IL-4 may participate in the processes leading to diet-induce obesity and metabolism. In support of the abovementioned studies, significant associations between IL-4 genotypes and T2DM, as well as between IL-4 genotypes and circulatory levels of high-density lipoprotein-cholesterol (HDL-C), are identified in our
previous study (14). Our most recent report also demonstrates that genetic polymorphisms of IL-4 receptor alpha chain (IL-4Rα) are significantly associated with HDL-C (15). In addition, results from our animal study reveal that IL-4 improves insulin sensitivity and glucose tolerance while inhibits lipid accumulation in fat tissues, which leads to decreased weight gain and fat mass (16). Taken these studies together, it suggests that IL-4 may participate in lipid metabolism and diabetic susceptibility.

To further explore the roles of IL-4 in lipid metabolism and pathogenesis of obesity and T2DM, the present study aimed at elucidating the effects of IL-4 on adipogenesis and lipid metabolism by using adipocytes as study model. Our data show that IL-4 not only inhibits adipogenesis at the early phase of adipocyte differentiation through signal transducer and activator of transcription 6 (STAT6) signaling pathway, but also promotes lipolysis by up-regulating the activity of hormone sensitive lipase (HSL), the important enzyme for triacylglyceride degradation.
Materials and Methods

Reagents

Mouse recombinant IL-4 was purchased from Millipore (Temecula, CA, USA). Tyrphostin AG 490 (AG490) and ECL reagent were purchased from Calbiochem (Merck Millipore, Billerica, MA, USA). 3-isobutyl-methylxanthine (IBMX), dexamethasone (Dex), insulin, isoproterenol, thiazolidinedione (TZD) and free glycerol determination kit were purchased from Sigma (St. Louis, MO, USA). Protein A/G beads, Trizol Reagent and Applied Biosystems SYBR Green Realtime PCR Master Mix were purchased from Life Technology (Carlsbad, CA, USA). Small interfering RNA (siRNA) and DharmaFECT 1 reagent were purchased from Dharmacon (Lafayette, CO, USA).

Cell culture, adipogenesis of 3T3-L1 cells and cell counting

3T3-L1 preadipocytes were maintained in DMEM containing 10% calf serum (Hyclone Laboratories, South Logan, Utah, USA). 2-day postconfluent 3T3-L1 cells (designated day 0) were induced to differentiate by addition of a standard cocktail (MDI) composed of 0.5 mM IBMX, 1 μM Dex, and 10 μg/ml insulin in 10% FBS for 2 days. The cells were then cultured in DMEM supplemented with 10% FBS and 5 μg/ml insulin. The medium was replaced by fresh medium every two days. For AG490 experiments, cells were pre-incubated with 10 μM AG490 for 1 h and then treated with 10 ng/ml IL-4. For cell counting, cells were harvested and stained with trypan blue (0.5%; Biological Industries, Kibbutz Beit Haemek, Israel), and viable cells were counted using hemocytometer (Lauda-Königshofen, Marienfeld-Superior, Germany).
**Oil red O staining**

Oil-Red O staining was performed as previously described (17). For quantification, the dye was eluted by adding 100% isopropanol and the extracts were determined by measuring the absorbance at 490 nm.

**Western blot analysis, immunodepletion, and immunoprecipitation assay**

Cell lysates were prepared in RIPA buffer containing protease inhibitors as described previously (18). Sixty μg of cell extracts were subjected to SDS-PAGE, transferred to PVDF membrane and blotted with specific primary antibodies. Antibodies against C/EBPβ, C/EBPδ, C/EBPα, PPARγ, aP2 (fatty acid binding protein 4), HSL, phospho-Ser⁵⁶³ HSL, perilipin, phospho-(Ser/Thr) protein kinase A (PKA) substrate, adipocyte triglyceride lipase (ATGL) and β-actin were purchased from Cell Signaling Technology (Danvers, MA, USA). Anti-p27 Kip1 was purchased from GenTex, Inc. (Irvine, CA, USA). Anti-STAT6/anti-phospho-STAT6 (Y641) and anti-IL-4Rα was purchased from BD Biosciences (San Jose, CA, USA) and R&D system (Minneapolis, MN, USA), respectively. For detection, membranes were incubated with secondary antibodies (Merck Millipore, Billerica, MA, USA) for 1 h, and results were visualized with ECL regent and exposed to X-films. The blot was quantified by Labscan software. For immunodepletion assay, 250 μg cell lysates were incubated with 1 μg anti-perilipin antibody overnight and captured by protein A/G agarose for 2 h. Supernatant was harvested and subjected to Western blotting. For immunoprecipitation assay, 500 μg cell lysates were incubated with 1 μg anti-phospho-PKA substrate antibody overnight and captured by protein A/G agarose.
agarose for 2 h. After washing with RIPA buffer, immunoprecipitates were eluted in sample buffer, denatured, and subjected to Western blotting as described.

**Real-time quantitative PCR**

Total RNA was isolated by TRIzol reagent according to the manufacturer’s instructions. About 2 µg of total RNA was reversed transcribed with Improm-II Reverse Transcription Kit (Promega, Madison, WI, USA), and real-time PCR was performed using the Applied Biosystems SYBR Green Realtime PCR Master Mix and StepOnePlus™ Real-Time PCR System. The following primers were used for detection of the gene expression: C/EBPβ (forward, 5’-AAGCTGAGCGACGAGTACAAGA-3’; reverse, 5’-GTCAGCTCCAGCACCTTGTG), C/EBPδ (forward, 5’-TCAGCGCCTACATTGACTC-3’; reverse, 5’-GCTTTTGTTGCTGTGTGAAG-3’), PPARγ2 (forward, 5’-GATGCAGCAGAGCTGAG-3’; reverse-5’-AGAGGTCCGCTCAGCTTGTTG-3’), C/EBPα (forward, 5’-AGCAACCGAGTACCAGCAG-3’; reverse, 5’-TGCTTTTGCTTTTATCTCGGCTC-3’), aP2 (FABP4, forward, 5’-CACCGCAGACGAGCAG-3’; reverse, 5’-GCACCTGACCGGAG-3’), GLUT4 (forward, 5’-ACTCATCTTTGGACGCTCTC-3’; reverse, 5’-CAACCCGAAGATGGAGTG-3’), and 18s rRNA (forward, 5’-CGGCTACCACATCAAGGAA-3’; reverse, 5’-GCTGGAATTACCGCAGGCT). All real-time PCR reactions were carried out with following conditions: 10 min at 95°C, followed by 40 cycles of 15s at 95°C, and 30s at 60°C. For semi-quantification RT-PCR, PCRs were optimized to determine the linear phase of amplification. Primer sequences used for amplification were: FAS (forward, 5’-TGCTCCAGCTGCAGGC; reverse, 5’-GCCCGTAGCTCTGGGTGA), LPL
(forward, 5’-ATGGAGAGCAAGCCCTGC; reverse, 5’-AGTCCTCTCTCTGCAATCAC), resistin (forward, 5’-AGACTGCTGTGCCTTCTGGG; reverse, 5’-CCCTCCTTTTCTTTTCTTCTTG) and adiponectin (forward, 5’-TCCTGGAGAGAAGGGAGAAAG; reverse, 5’-TCAGCTCCTGTCATTCCAACAT). The quantitative results were presented as the mean of three independent experiments.

**Small interfering RNA (siRNA)**

For siRNA transfection, the 2-day postconfluent cells were transfected with 100 nM STAT6 siRNA (Cat. L-040690-01) or control siRNA (Cat. D-001810-10-05) by DharmaFECT 1 reagent according to the manufacturer’s instruction. The cells were incubated in medium containing the siRNA-DharmaFECT1 complex for 16 h. The medium was then replaced with DMEM supplemented with 10% calf serum, and the cells were incubated for another 24 h and induced into differentiation.

**Electrophoretic mobility shift assay (EMSA)**

EMSA was performed according to the manufacturer’s instruction (LightShift Chemiluminescent EMSA Kit, Thermo Scientific, Rockford, IL, USA). The post-confluent cells were induced by MDI for 24 h or 48 h and then nuclear extracts were collected using nuclear protein extraction kit for EMSA experiments (Thermo Scientific, Rockford, IL, USA). The DNA sequence used for EMSA was 5’-GTATTTCCCAGAAAAGGAAC-3’. Probes were prepared by annealing oligonucleotides at their 3′-end with biotin.
Measurement of lipolysis

3T3-L1 cells were incubated in serum free DMEM in the presence or absence of IL-4 and/or isoproterenol. Concentrations of glycerol and non-esterified free fatty acids in the culture medium were then analyzed, respectively, with the free glycerol determination kit and free fatty acid kit (Biovision, Milpitas, CA, USA).

Confocal microscopy

Cells were fixed with 3.7% formaldehyde for 15 min and then permeabilized with 0.5% triton X-100 for 15 min at room temperature. The cells were then blocked with 5% BSA for 30 min at room temperature, followed by incubation with the primary antibody against phospho-Ser$^{563}$ HSL overnight at 4°C. Goat anti-rabbit IgG conjugated DyLight™594 (Jackson Immunoresearch Laboratories, PA, USA) was added together with BODIPY (Molecular Probes, Eugene, USA) and incubated for 1 h at room temperature. Cells were then mounted with GEL/MOUNT containing DAPI (Molecular Probes, Eugene, USA). The images were taken by ZEISS LSM 700 confocal fluorescence microscope system using 63X objective len.

Statistical analysis

All values were presented as mean ± SEM. For statistical analysis, the $P$ value was calculated using a two-tailed unpaired Student’s $t$-test with $P<0.05$ considered as statistically significant.
Results

**IL-4 inhibits adipogenesis**

To evaluate the putative effects of IL-4 on adipogenesis, 3T3-L1 pre-adipocytes were allowed to differentiate into mature adipocytes (19) in the presence or absence of IL-4 during the entire differentiation period. The extent of the differentiation was evaluated by Oil-Red O (ORO) staining. In addition, the expression of important genes mediating adipogenesis, such as CCAAT/enhancer binding protein-α (C/EBPα) and peroxisome proliferator-activated receptor-γ (PPARγ), and makers of mature adipocytes, including fatty acid binding protein 4 (aP2) and glucose transporter 4 (GLUT4), were analyzed. Results of ORO staining showed that lipid accumulation in differentiated cells was inhibited by ~30% in the presence of IL-4 treatment (Fig. 1, A&B). The expression of 2 important regulators for terminal adipogenesis, PPARγ and C/EBPα, were subsequently examined for exploring the underlying inhibitory mechanism of IL-4 to adipogenesis. Fig. 1C showed that IL-4 not only caused reduced levels but also an expression lag of PPARγ and C/EBPα during the differentiation process (Fig 1 C&D). Meanwhile, expression of aP2, an adipogenic gene encoding a fatty acid-binding protein in mature adipocytes, was also significantly down-regulated (Fig. 1, C&D). The mRNA expression of *Pparg* and *Cebpa* was further analyzed for probing the regulatory mechanism of IL-4 treatment to adipogenesis. Consistent with the previous observations, *Pparg* and *Cebpa* mRNA expression was also decreased under IL-4 treatment during the entire differentiation process (Fig. 1E). The above observations indicate that IL-4
harbors inhibitory capacity to adipogenesis by suppressing the expression of important transcription factors driving adipocyte differentiation. Moreover, mRNA expression levels of adipokines, such as Adipoq and Retn, were temporally and significantly inhibited by IL-4 (Fig. 1F). The IL-4 inhibitory effects were also observed on genes involved in glucose uptake and lipid metabolism, such as Glut4 (Fig. 1E), Fasn and Lpl (Fig. 1F).

IL-4 inhibits adipogenesis by affecting cell proliferation at mitotic clonal expansion phase

Thiazolidinedione (TZD) is an adipogenic compound that activates multiple downstream genes in adipocytes through binding to PPARγ (20). To further verify the inhibitory effects of IL-4 on adipogenesis, we examined whether TZD treatment could rescue the IL-4-suppressed adipocytes differentiation. Our results demonstrated that, in the presence of TZD treatment, the degree of lipid accumulation in IL-4-treated cells were comparable with that in control counterparts (Fig. 2, A&B). It suggests that the inhibitory effect of IL-4 on adipogenesis is targeted at early stage of differentiation.

The entire adipogenesis process can be divided into the initial mitotic clonal expansion (MCE) and terminal differentiation phase. When cells are induced to differentiate, they first undergo several rounds of cell cycle to proliferate during the MCE phase which lasts about 2 days, followed by the differentiation phase through which they acquire the characteristics of mature adipocytes (17, 21). For dissecting the IL-4 inhibitory effect on adipogenesis, cells were induced to differentiate under various schemes of IL-4 treatment as shown in Fig. 2C, then the extents of adipocyte differentiation were examined. The
results showed that while lipid accumulation was significantly suppressed by exposing the cells to IL-4 during the entire differentiation process (MDI/IL-4), short-term IL-4 exposure at MCE stage (MDI/IL-4-MCE) was sufficient to inhibit adipogenesis (Fig 2, C&D). Parallel to the ORO staining results (Fig. 2C&D), the protein expression levels of C/EBPα and PPARγ were decreased in MDI/IL-4-MCE and MDI/IL-4 cells (Fig. 2E). The results support our previous observation that IL-4 exerts inhibitory capacity to adipogenesis at the early MCE phase.

C/EBPβ and C/EBPδ are important transcription factors which are up-regulated at MCE phase and subsequently activate PPARγ and C/EBPα to trigger adipogenesis. Thus, the putative regulation of IL-4 to the expression of C/EBPβ and C/EBPδ as well as cell proliferation capacity was subsequently analyzed by qPCR and trypan blue exclusion, respectively. The results showed that mRNA expression patterns of Cebpb and Cebpd were not affected (Fig. 3A). However, cell numbers in MCE phase was significantly decreased about 20% under IL-4 treatment (Fig. 3B), which was parallel to the degree of IL-4 reduced lipids accumulation (Fig. 1A&1B). Expression of p27 Kip, the key regulator of cell cycle in MCE stage, was subsequently examined to verify the above results (22). While p27 Kip expression was declined in MDI and MDI/IL-4 once the cells were induced to differentiate, its level in MDI/IL-4 cells was significantly higher than in MDI cells at 22 hr post induction (Fig. 3C). These data suggest that IL-4 inhibits adipocyte differentiation by regulating cell proliferation at MCE phase.

**IL-4 inhibits adipogenesis through STAT6 pathway**

We next investigated the signaling pathways responsible for the inhibition of
adipogenesis by IL-4. As shown in Fig 4A, while phosphorylation of glycogen synthase kinase 3 beta (GSK3β) was not altered by IL-4, activation of extracellular-signal-regulated kinases 1/2 (ERK1/2) was partially suppressed.

STAT6, an important downstream molecule in IL-4 signaling, is abundantly expressed in 3T3-L1 cells (23-24). Therefore, we would like to examine whether IL-4 inhibits MCE phase through STAT6. Our data indicated that IL-4 significantly induced STAT6 phosphorylation, which persisted to 48 h after induction (Fig 4A). Notably, IL-4 induced the binding of STAT6 to its target sequences although IL-4Rα expression was rapidly down-regulated after induction (Fig. 4 A&B). These data demonstrate that IL-4 may inhibit adipogenesis through STAT6-dependent signaling bypassing IL-4R at early MCE phase of adipogenesis.

For verifying the above observation, cells were treated with JAK inhibitor AG490 in the presence or absence of IL-4 during differentiation, and degrees of adipogenesis were determined. The results indicated that the inhibitory effects of IL-4 on adipocyte differentiation were blocked by AG490 (Fig 4C). We next investigated if the IL-4-mediated inhibition of lipid accumulation could be sequestered when STAT6 expression was silenced. As shown in Fig 4D, STAT6 siRNA specifically counteracted the IL-4 inhibited effects on adipogenesis. Moreover, the expression levels of PPARγ and C/EBPα were recovered in STAT6 siRNA-treated MDI/IL-4 cells (Fig 4E). These data strongly suggest that IL-4 inhibits the expression of PPARγ and C/EBPα through STAT6 pathway, which results in the inhibition of adipogenesis.

_IL-4 enhances basal and isoproterenol-induced lipolysis in mature adipocytes_
Our previous study showed that IL-4-treated mice have higher serum non-esterified fatty acid (NEFA) levels and fewer fat pads (16). We speculated that IL-4-induced NEFA elevation may result from its capacity to inhibit lipid accumulation while promote lipolysis in adipose tissues. In this context, we examined the effect of IL-4 on lipolysis in mature adipocytes to verify the above hypothesis. As anticipated, glycerol levels were significantly increased by IL-4, which suggests IL-4 has pro-lipolytic activity (Fig 5A).

Subsequently, whether IL-4 induced lipolysis by up-regulating the expression and/or activities of ATGL and HSL, the key enzymes for triacylglycerol hydrolysis and NEFA release, were analyzed (25). Our results showed that neither the expression of ATGL and HSL (Fig. 5A) nor the mRNA levels of CGI-58 (data not shown), the regulator of ATGL lipolytic activity (26), was altered by IL-4. We further investigated if IL-4 had synergistic effect with the lipolysis-inducing agent, isoproterenol, to enhance lipolysis. Levels of both glycerol and NEFA were significantly increased by isoproterenol and IL-4 combined treatment, compared to the counterparts in the environment containing either isoproterenol or IL-4 (Fig. 5 B&C). The results suggest that IL-4 harbors the capacity to promote lipolysis both at the basal level and isoproterenol-stimulated status.

The HSL lipolytic activity is primarily controlled by its phosphorylation status at Ser\(^{563}\) (27). Therefore, the amounts of Ser\(^{563}\)-phosphorylated HSL (Ser\(^{563}\)-pHSL) were examined in the presence of IL-4 and/or isoproterenol treatment. Our results showed that, while Ser\(^{563}\)-pHSL level was slightly elevated (~1.5 to 2 fold) by IL-4, it was synergistically increased under isoproterenol-induced lipolysis in the presence of IL-4 (Fig 5D). Whereas, neither IL-4 nor combined treatment affected HSL protein levels. In
addition to being phosphorylated, the translocation of HSL from cytosol to the surface of lipid droplets is required for its getting access to the lipid core and exerting lipolytic activity. Accordingly, the localization of pHSL under IL-4 or IL-4/isoproterenol treatment was analyzed. Ser\textsuperscript{563}-pHSL was ubiquitously detected in cytosol under IL-4 treatment, whereas, IL-4 synergistically enhanced the translocation of Ser\textsuperscript{563}-pHSL from cytosol to lipid droplets (LDs) surface under isoproterenol-stimulated lipolysis (Fig. 5E). Collectively, these data suggest that IL-4 alone stimulates lipolysis by increasing HSL activity. In addition, IL-4 shows synergistic effects on promoting HSL phosphorylation and translocation with pro-lipolytic signals such as isoproterenol.

**IL-4 promotes lipolysis through PKA pathway in mature adipocytes**

Protein kinase A (PKA) is an upstream regulator to promote HSL activity by enhancing HSL phosphorylation at Ser\textsuperscript{563} (28). Accordingly, it was tempting to us to assess if IL-4 induced lipolysis by up-regulating HSL activity through PKA. As shown in Fig. 5D & 6A, the level of phosphorylated PKA substrate was increased both in IL-4 and IL-4/isoproterenol treatment, which supported our speculation that IL-4 enhanced HSL activity through PKA pathway. Notably and interestingly, a prominent, isoproterenol-dependent 62 kDa phospho-PKA substrate was detected (Fig 6A). In addition, phosphorylated perilipin levels were also increased by IL-4 (Fig 6A). We suspected that perilipin was a potential candidate for the detected 62 kDa phospho-PKA substrate as it is the major phosphorylated protein in adipocytes responding to increased cAMP concentration (29,30).

To test the above speculation, perilipin was first immunoprecipitated from cell lysates
and the perilipin-depleted supernatant was blotted with the phospho-PKA substrate antibody. Fig 6B showed that the 62 kDa protein was no longer detected in perilipin-depleted supernatant, supporting the assumption that it was perilipin. In addition, the immunoprecipitation-immunoblotting results also demonstrated that IL-4 enhances perilipin phosphorylation by PKA (Fig. 6C). We further analyzed the amounts of phosphorylated perilipin levels by treating PKA inhibitor H-89 in adipocytes exposed to either IL-4 or IL-4/isoproterenol to verify whether IL-4-stimulated lipolysis was mediated by activating cAMP-dependent PKA. Our results showed that H-89 abrogated IL-4-induced PKA-dependent perilipin phosphorylation (Fig. 6D). These data support our speculation that IL-4 promotes lipolysis by enhancing phosphorylation of HSL and perilipin through PKA pathway.
**Discussion**

Pro-inflammatory cytokines have been shown to participate in T2DM etiology. However, little is known about the role of other cytokines in diabetic pathogenesis (31). In our previous studies, significant association between IL-4 promoter polymorphisms and T2DM is identified (14), as well as that between IL-4Rα and HDL-C (15). We further demonstrate that IL-4 regulates glucose and lipid metabolism by promoting insulin sensitivity, glucose tolerance and inhibiting lipid deposits (16). In the present study, our results reveal that, in addition to inhibiting adipocytes differentiation through STAT6 pathway, IL-4 also enhances lipolysis by up-regulating perilipin phosphorylation and HSL activity/translocation through PKA pathway. These observations are consistent with our previous findings and reveal that IL-4 inhibits lipid deposits. At the best of our knowledge, this is the study first demonstrates the novel roles of IL-4 in regulating lipid metabolism through inhibiting adipogenesis and promoting lipolysis.

The adipogenic programming is finely orchestrated and driven by the sequential activation of transcription factors, such as C/EBPβ, C/EBPδ, PPARγ, and C/EBPα, which trigger the cells enter the terminal differentiation by controlling the coordinate expression of a number of key adipocyte genes. Our results reveal that while IL-4 down-regulates the expression of C/EBPα and PPARγ, C/EBPβ and C/EBPδ are not affected (Fig 1B&3A). In addition, IL-4 inhibits adipogenesis by targeting at MCE phase (Fig 3C). IL-4 is known to inhibit cell cycle progression through STAT6 which alters expression of several key regulators such as p21(Waf1) and p27(Kip) (32-36). We speculate that the net anti-adipogenic effect of IL-4 might result from its capacity to either elevate the
expression of p27/p21 or protect these proteins from proteasome degradation through STAT6 pathway, and thus inhibit MCE during adipogenesis. The results that expression of p27 in IL-4-treated cells is higher than that in control counterparts (Fig. 3C) further support the above speculation.

The rapidly elevated C/EBP-β/δ at early stage of adipogenesis remains inactive until being phosphorylated (20,21). Only after being phosphorylated can C/EBPβ acquire DNA-binding and trans-activating activity to make preadipocytes traverse the G1-S checkpoint at MCE phase (37,38). C/EBPβ is phosphorylated sequentially, first by MAPK and then much later by GSK3β (37,38). MAPK-mediated phosphorylation of C/EBPβ Thr^{188} occurs ~4 h after induction of differentiation and thus is required for MCE, C/EBPβ DNA binding activity and terminal differentiation (37,38). Our results show that activation of MAPK p44/42 (ERK1/2) is partially suppressed by IL-4 (Fig 4A), suggesting that Thr^{188} phosphorylation and, therefore, C/EBPβ DNA binding activity might also be partially inhibited. However, this speculation awaits further investigation.

Role of STATs in adipocytes differentiation has been extensively studied, including STAT1, 3 and 5 (39-44). However, little is known about the role of STAT6 in adipogenesis. We demonstrate that IL-4 inhibits adipogenesis by inducing STAT6 activation in 3T3-L1 cells although IL-4Rα is rapidly downregulated at the early phase of differentiation (Fig 4A). The results that STAT6 knockdown rescues IL-4-inhibited adipogenesis further verify the above observation. Interestingly, IL-4 transgenic mice show a considerable reduced adipocyte layer in dermis (45), suggesting IL-4 inhibits lipid storage and adipocyte differentiation. Our previous animal study also indicates that
IL-4 administration leads to lower fat mass and reduced lipid contents in adipocytes (16). Our observations echo the recent finding from Ricardo-Gonzalez et al. that STAT6 null mice gain significantly less weight and have smaller WAT deposits when challenged with high fat diet (46). Taken together, these evidences support that IL-4-inhibited adipogenesis is an STAT6-dependent effect.

Nevertheless, the finding that IL-4 suppresses adipocyte differentiation by mediating PPARγ expression is contradictory to another study (47). We suggest that this discrepancy may result from different experimental conditions: In our study, pre-adipocytes were maintained in medium containing calf serum, which was replaced by FBS when cells were induced to differentiate. In addition, the MDI cocktail in the induction medium was removed after 48 hours and replaced by insulin-containing fresh medium. Whereas, study from Hua et al. used FBS-containing medium both in pre-adipocytes maintenance and entire adipocytes differentiation process, and the cells were exposed to MDI cocktail for 72 hours. Therefore, the possibility that differential gene expression patterns of 3T3-L1 cells responding to different experimental conditions cannot be ruled out.

The cAMP-dependent PKA pathway is the major route leading to lipolysis, through which adenyl cyclase is activated by the stimulated Gs-coupled receptors (48). The subsequently increased intracellular cAMP levels then result in PKA activation, followed by HSL phosphorylation, translocation, and access to LDs for mediating triacylglycerol hydrolysis (48). In the present study, our results show that IL-4 enhances the major phosphorylation site of HSL, Ser563, by PKA (Fig 5D). Moreover, IL-4 increases the level
of PKA-dependent perilipin phosphorylation, which is attenuated by PKA-specific inhibitor H-89 (Fig. 6C). These results strongly suggest that IL-4 participates in lipid metabolism by evoking a conventional lipolytic pathway in adipocytes. Unlike TNF-α and IL-6, which are known to stimulate lipolysis by activating ERK1/2 pathway and suppress the expression of multiple genes involved in preventing lipolysis (49,50), IL-4 does not alter the expression of HSL, perilipin, ATGL (Fig. 5A), and CGI-58 nor activate ERK signaling (data not shown). How IL-4 transverses its anti-adipogenesis and pro-lipolysis signaling needs further investigation because expression of IL-4Rα is rapidly declined when the cells enter differentiation (Fig. 4A).

In addition to inhibiting adipocytes differentiation, our results also show that IL-4 modulates the expression of adiponectin and resistin (Fig. 1F). Resistin is an adipocyte-specific hormone which has been indicated in promoting lipolysis, glucose intolerance and insulin resistance (51). The down-regulated resistin in IL-4-treated cells support our previous conclusion that IL-4 is involved in lipoid metabolism by inhibiting lipid accumulation in fat tissues, which lead to decreased weight gain and fat mass in IL-4 treated mice (16). On the contrary, discrepancy in adiponectin levels was observed in the present study. Adiponectin is known to have anti-lipolytic ability (52). We hypothesize that the increased adiponectin levels in IL-4-treated mice should reflect the integrative effects of IL-4 on metabolism in the complicated interplay/network among IL-4 and other intrinsic physiological factor(s) (16). Hence, we suggest that regulating adipokine secretion may be one of the potential mechanisms by which IL-4 modulates energy metabolism. Nevertheless, as lipodystrophic state in mice would perhaps lead to ectopic lipid deposition and
insulin resistance (53), whether reduced adipogenesis and fat storage resulted from IL-4 administration would lead to the above metabolic abnormality in certain organs needs further investigation.

Obesity is accompanied by an increased infiltration of adipose tissue macrophages (ATMs), possibly recruited by the signals released from damaged or dead hypertrophic adipocytes (54). While the ATMs in lean animals correspond to M2 anti-inflammatory phenotype, the ATMs from obese animals are predominately M1 pro-inflammatory phenotype (55). It is known that adipocytes produce IL-4 and IL-13 cytokines locally, establishing a reciprocal functional crosstalk between adipocytes and the resident M2 ATMs which leads to suppression of M1 macrophages as well as the improvement of lipid metabolism and insulin sensitivity (12,13). Treatment of adipocytes with conditioned medium from M2 macrophages also induces phosphorylation of perilipin and HSL (56). Therefore, except for the results demonstrating IL-4 directly inhibits adipogenesis and promotes lipolysis through STAT6 and PKA pathway respectively, we infer that the microenvironmental IL-4 may promote M2 macrophages polarization, which plays positive roles in lipid metabolism by reducing inflammation in adipose tissues and therefore preventing the incidence of insulin resistance.

Combined the evidences from the present study and our previous reports altogether (14-16), the novel role of IL-4 in regulating lipid metabolism is uncovered by the following findings. First of all, significant association between IL-4 genotypes and T2DM (14) as well as that between the genotypes of IL-4 and IL-4Rα with HDL-C are observed (15). Secondly, IL-4-injected mice show decreased fat mass and increased serum NFFA level,
revealing the inhibitory capacity of IL-4 to lipid accumulation in fat tissue and the subsequent elevated circulatory NFFA levels (16). Thirdly, consistent to our animal study results, the present study indicates that IL-4 harbors anti-lipogenic ability by suppressing adipocyte differentiation and promoting lipolysis in mature adipocytes. Hopefully, the above findings not only provide new insights in the interaction among cytokines, lipid metabolism and obesity, but also the clues to the underlying mechanism which leads to the ultimate T2DM metabolic tragedy.
Acknowledgements

This work was supported by grants from National Science Council (NSC 100-2320-B-241-003 and NSC 101-2320-B-010-052-MY3) and Ministry of Education (Aiming for the Top University Plan, 101AC-P653 and 102AC-P686), Taipei, Taiwan. We thank the confocal microscopy technical services provided by Imaging Core Facility of Nanotechnology, UST-NYMU.
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Figure Legends

Figure 1. Effects of IL-4 on adipogenesis of 3T3-L1 cells.

Lipid accumulation and expression levels of adipocyte-specific genes during adipogenesis under IL-4 treatment were analyzed. (A) 3T3-L1 cells were induced into mature adipocytes in the absence or presence of 10 ng/ml of IL-4 and subjected to Oil-red O staining. (B) Quantification of the Oil-red O staining results by measuring the corresponding absorbance of isopropanol-extracted dye at 490 nm. (C) Protein expression of aP2, C/EBPα, C/EBPβ, and PPARγ in 3T3-L1 was analyzed at the indicated time. β-actin was served as loading control. (D) Quantification of Western blotting by densitometer. The data were presented as the mean ± SEM (n=7; *: P <0.05 and **: P <0.01, compared with MDI). (E and F) mRNA expression of the aP2, C/EBPα (Cebpa), PPARγ (Pparg), GLUT4 (Glut4) and adipocytes-specific genes (adiponectin: Adipoq; resistin: Retn; fatty acid synthase: Fasn; lipoprotein lipase: Lpl) in 3T3-L1 was analyzed by real-time PCR (E) and RT-PCR (F), respectively, at the indicated time. All results were normalized to 18s rRNA. The data were presented as the mean ± SEM (n=3), and statistically analyzed by two-tailed unpaired Student’s t-test (*P <0.05, compared with MDI).

Figure 2. IL-4 inhibits adipogenesis at early mitotic clonal expansion stage.

Effect of IL-4 inhibitory effect on differentiation under various schemes of IL-4 treatment was analyzed. (A and B) PPARγ agonist thiazolidinedione (TZD) rescued adipogenesis under IL-4 treatment. 3T3-L1 cells were treated with 10 μM TZD on day 4, and lipid
accumulation was examined by Oil-red O staining on day 8. (B) The Oil-red O staining in cells were extracted with isopropanol and quantitated by OD 490 nm (*: \(P<0.05\), DMSO v.s. TZD; **: \(P<0.01\), MDI v.s. MDI/IL-4). Data were presented as the mean ± SEM (n=3). (C-D) Schematic illustrator indicated the different IL-4 exposure periods of adipocytes during differentiation (PRE: IL-4 treatment from day -2 to 0; MCE: IL-4 treatment from day 0 to 2; POST: IL-4 treatment from day 2 to 8). (C) Intracellular lipid contents were stained by Oil red-O on day 8. Size bar = 10 µm (D) The corresponding lipid contents of staining results were measured by spectrophotometer. Data were presented as the mean ± SEM (n=3, ***: \(P<0.001\), compared with MDI). (E) Expression of C/EBPγ and PPARγ in adipocytes with differential IL-4 treatment as indicated in (C) was analyzed. Briefly, cells were treated with IL-4 as indicated, then cell lysates were harvested on day 8 and subjected into Western blotting with β-actin as loading control.

Figure 3. IL-4 partially inhibits cell cycle progression at mitotic clonal expansion stage.

Expression levels of transcription factors and cell proliferation activity during MCE stage under IL-4 treatment were analyzed. (A) mRNA expression of C/EBP- β (Cebpb) and δ (Cebpd) in 3T3-L1 cells was analyzed by real-time PCR at the indicated time in the absence or presence of 10 ng/ml IL-4. (B) 3T3-L1 cells were induced into differentiation in the absence or presence of IL-4, and then cells were trypsinized, stained with trypan blue and counted at the indicated time. Data are presented as the mean ± SEM (n=9, *: \(P<0.05\), MDI/IL-4 v.s. MDI). (C) Expression of p27 Kip was analyzed at the
indicated time in the absence or presence of IL-4 treatment by Western blotting with β-actin as loading control. Quantitative results were presented as the mean ± SEM (n=3, *: P<0.05, compared with MDI).

Figure 4. IL-4 induces STAT6 signaling pathway during adipogenesis.

Expression and phosphorylation levels of molecules in the signaling pathway at MCE stage under IL-4 treatment were analyzed. (A) 3T3-L1 cells were induced to differentiate with MDI cocktail in the absence or presence of 10 ng/ml IL-4. Cell lysates were harvested at the indicated times and then subjected to Western blotting analysis using antibodies against STAT6, phospho-STAT6 (Tyr641), phospho-ERK1/2, total ERK1/2, pGSK3β and total GSK3β. (B) Cells were allowed to differentiate in the absence or presence of IL-4. The nuclear extracts were harvested at the indicated times and then subjected to EMSA experiments. The upper and lower arrow indicated STAT6-probe complex and unbound free probe, respectively. (C) Pretreatment with AG490 (10 μM) rescued IL-4-inhibited adipogenesis. 3T3-L1 cells were pre-treated with 10 μM AG490 and then induced to differentiate in the absence or presence of IL-4. Lipid accumulation was observed by Oil-red O staining and quantified (Size bar = 20 μm, ***: P<0.001). (D) 3T3-L1 cells were transfected with 100 nM STAT6 siRNA for 24 h and then subjected to differentiation in the absence or presence of IL-4. Lipid accumulation was observed by Oil-red O staining on day 8 and quantified (Size bar = 10 μm, ***: P<0.001). (E) Expression of PPARγ and C/EBPα in 3T3-L1 cells transfected with 100 nM nonspecific siRNA or STAT6 siRNA. Data were presented as the mean ± SEM (n=9).
Figure 5. IL-4 enhances basal and isoproterenol-stimulated lipolysis in mature adipocytes.

Effects of IL-4 on lipolysis and HSL expression/activity in 3T3-L1 mature adipocytes were analyzed. (A) **Left panel:** Cells were treated with 10 ng/ml IL-4 for 24 h. Culture medium were collected and contents of glycerol were determined. Data were presented as the mean ± SEM (n=8). **: $P<0.01$, compared with untreated control. **Right panel:** HSL, ATGL and perilipin protein levels in cells after 24 h of 10 ng/ml IL-4 treatment were analyzed by Western blotting with β-actin as loading control. (B&C) Cells were treated with 10 ng/ml IL-4 for 3 h, and followed by 10 µM isoproterenol (ISO) for 1 h. Culture medium were collected and contents of glycerol and NEFA were determined. Data were presented as the mean ± SEM (n=8, *: $P<0.01$). (D) **Upper panel:** Cells were treated with IL-4 in the absence or presence of isoproterenol. Cell lysates were harvested and subjected to Western blotting using antibodies against phospho-PKA substrate, Ser$^{563}$-pHSL and total HSL with β-actin as loading control. **Lower panel:** Quantitative results were presented as mean ± SEM (n=3, **: $P<0.01$ and ***: $P<0.001$). (E) Ser$^{563}$-pHSL was recruited to LDs surface (arrow). Cells were treated with vehicle (DMSO), 10 ng/ml IL-4, 10 µM ISO, or 10 µM ISO with 10 ng/ml IL-4 for 1 h prior to fixation. Cells were immunolabeled with Ser$^{563}$-pHSL (red), lipid droplets (BODIPY, green) and DAPI (blue). Size bar = 20 µm. Mean fluorescence intensity (MFI) of the cells and LDs was analyzed by using ZEN 2009 software. Data were presented as mean ± SEM of three separate experiments (*: $P<0.05$ and **: $P<0.01$).
Figure 6. IL-4 promotes perilipin phosphorylation in mature adipocytes via PKA pathway.

Expression and phosphorylation level of perilipin in 3T3-L1 mature adipocytes under IL-4 treatment was analyzed. (A) Cells were treated either with 10 ng/ml IL-4 or combined with 10 μM isoproterenol (ISO) treatment for 1 h. Cell lysates were harvested and subjected to Western blotting using antibodies against phospho-PKA substrate and perilipin with β-actin as loading control. Data were presented as mean ± SEM of three separate experiments (*: P<0.05). (B-C) The 62 kDa phospho-PAK substrate was identified as perilipin. (B) Cells were treated with 10 ng/ml IL-4 for 1 h. Perilipin in the cell extracts was first depleted by anti-perilipin antibodies, then the perilipin-cleared supernatant was harvested and subjected to Western blot analysis. (C) Cell lysates after IL-4 treatment were immunoprecipitated with anti-phospho-PKA substrate antibodies and subjected to Western blotting. (D) Cells were pretreated with 20 μM PAK inhibitor H-89 for 1 h and followed by IL-4 treatment in the absence or presence of 10 μM ISO for 1 h. Cell lysates were harvested and subjected to Western blotting analysis using antibodies against phospho-PKA substrate with β-actin as loading control.
Fig 1

E

Relative mRNA Expression
Fad7/Fas

Days after Induction

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F

Relative mRNA Expression
Gadd45β / 18s

Relative mRNA Expression
Glut4 / 18s

Relative mRNA Expression
Pparγ / 18s

Relative mRNA Expression
Panhx / 18s
Fig 2

A

MDI

MDI/IL-4

DMSO

TZD

B

Oil-Red O Staining (%)

**

*
FIG 3

A

B

C

Relative mRNA Expression

Cell Proliferation (%)

p27 Kip

Actin

MDI

MDI/IL-4

0 16 20 22

0 16 20 22

0 16 20 22

0 16 20 22

0 16 20 22

0 16 20 22

0 16 20 22

0 16 20 22

0 16 20 22

0 16 20 22

0 16 20 22

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0 16 20 22

0 16 20 22

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0 16 20 22

0 16 20 22

0 16 20 22

0 16 20 22

0 16 20 22
FIG 4

C

DMSO

AG490

MDI

MDI/IL-4

Oil-Red O Staining (%)
FIG 4

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- mIL4Rα
- STAT6
- PPARγ
- C/EBPα
- Actin
Fig 5

D

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Relative expression level of phospho-HSL563/HSL

ISO (10 μM) + + + +
IL-4 (ng/ml) − 1 10 50
Figure 6

A

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![Graph showing p-PKA substrate/Actin](image)

B

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![Graph showing Phospho-PKA substrate](image)
Fig 6

C

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D

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