Mechanism of the Fungal-like Particles in the Inhibition of Adipogenesis in 3t3-l1 Adipocytes

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Abstract

The dynamic ability of adipocytes in adipose tissue to store lipid in response to changes in the nutritional input and inflammatory elicitors has a major impact on human health. Previously, we established laminarin-coated beads or LCB as an inflammatory elicitor for adipocytes. However, it was not clear whether LCB inhibits lipid accumulation in adipocytes. Here, we show that LCB acts in the early stage of adipogenesis through both IRAK and SYK pathways, resulting in the activation of the AMPK and NF-kB complexes, which subsequently cause cell cycle arrest, suppression of C/EBPb, PPARg, C/EBPa, FAS, FABP4, and ACC proteins, downregulation of other transcription factors and enzymes, such as *Pparg*, *C/ebpa*, *Srebp-1*, *Lpl*, and *Fas* gene expression, inhibition of adipogenesis, and stimulation of an inflammatory response. Unlike the inhibition of adipogenesis, LCB could stimulate an inflammatory response at any stage of differentiation. In addition, we find that *Tlr2* and *Clec7a/Dectin-1* but not *Tlr4* and *Cd36* gene expression are upregulated upon the treatment with LCB, suggesting that TLR2 and CLEC7A/Dectin-1 might be the b-glucan receptors for the cells. Together, we present the mechanism of LCB, as fungal-like particles, that elicit an inflammatory response and inhibit adipogenesis at the early stage of differentiation.

Introduction

Obesity, an independent risk factor for several health issues such as heart disease, hypertension, osteoarthritis, and type 2 diabetes, has become a major health problem all over the world [1]. Obesity, characterized by excessive fat accumulation in the body that might cause health problem, is mainly caused by over nutrition. Although obesity could cause a serious health problem, it is preventable.

The major site for fat accumulation is adipose tissue, a complex immune organ that contains many other cell types, such as adipocytes, fibroblasts, preadipocytes, endothelial cells, and immune cells. Not only does this tissue function as a fat storage, but it also secretes adipokines which are hormones, cytokines, and small molecules that regulates the whole-body energy homeostasis. In response to changes in excessive nutrients, adipose tissue undergoes a dynamic expansion by either increasing the number of cells (hyperplasia) or the size of the cells (hypertrophy) [2,3]. Hyperplasia is considered a healthy event in which it recruits new precursor cells that undergo adipogenesis. Adipose tissue hyperplasia contains small adipocytes and does not express inflammatory cytokines. In contrast, hypertrophic adipose tissue has large adipocytes that produces inflammatory cytokines, which associate with obesity, and metabolic syndromes. Therefore, understanding the mechanism of dynamic changes in fat stored in adipose tissue is a necessary step to understand and prevent obesity.

Adipocytes or fat cells are derived from the precursor cells (preadipocytes) that undergo the differentiation process called adipogenesis or lipogenesis. The mechanism of adipogenesis is understood in detail. The in vitro differentiation of preadipocytes (e.g., 3T3-L1 cell line) is initiated by an addition of the hormonal inducers, which include insulin, IBMX (1-methyl-3-isobutylxanthine), and dexamethasone [4]. IBMX, a synthetic glucocorticoid, stimulates the glucocorticoid pathways and activates

CCAAT/enhancer binding protein beta (*C/ebpb*) gene expression, whereas dexamethasone/DEX upregulates the *C/ebpd* gene expression [5]. The expression of both C/EBPb and C/EBPd marks the early stage of adipogenesis. During the 2nd day of differentiation, C/EBPb activates the expression of peroxisome proliferator-activated receptor gamma (*Pparg*), which in turn, stimulates the expression of *C/ebpa*[6,7], which signifies the intermediate stage of differentiation. During the intermediate stage, some lipid accumulation in the cells can be observed. The collaboration of both PPARg and C/EBPa is critical for the expression of enzymes involved in triglyceride synthesis in the late stage or terminal differentiation, such as the fatty-acid synthase (FAS), lipoprotein lipase (LPL), sterol regulatory element-binding protein 1 (SREBP-1), and fatty acid binding protein 4 (FABP4) [5].

To combat against obesity, many research investigators have identified molecules or compounds that inhibit adipogenesis. Among those, b-glucan, a polymer of b-glucoses that link through b-(1®3) glycosidic bonds and found in the cell walls of yeast, fungi and some seaweeds, has been shown to inhibit adipogenesis [8–10]. Noticeably, a previous study demonstrated that yeast-derived insoluble β-1,3-glucan strongly inhibited adipogenesis better than soluble β-1,3-glucan [11]. Furthermore, another study demonstrated that zymosan, a yeast-derived insoluble b-glucan, inhibited the expression of *Pparg* gene in adipose tissue of animals [12], suggesting that zymosan could also inhibit adipogenesis in tissue culture. These data also suggest that there must be a specific b-glcuan receptor on the surface of the cells.

b-glucan receptors and their functions have been known mostly from studies done in the context of immune cells. For example, the C-type lectin domain family 7 member A (CLEC7A) or Dectin-1, which contains an immunoreceptor tyrosine-based activation (ITAM)-like motif in its intracellular domain, is a b-glucan receptor for many immune cells, especially in macrophages [13]. CLEC7A/Dectin-1 has been shown to work in concert with toll-like receptor 2 (TLR2) receptor, a member of the toll-like family receptors, in an inflammatory response by macrophages [14]. Interestingly, TLR2 has been suggested as a receptor candidate for b-glucan in 3T3-L1 adipocytes due to its upregulation in the cells upon the treatment with lipopolysaccharide or LPS [15,16]. In addition to TLR2, a recent study suggested the CD36, a scavenger receptor, to be a b-glucan receptor on both macrophages and 3T3-L1 adipocytes [17]. Therefore, while the structure and function of b-glucan receptors in the immune cells have been well characterized, it remains unclear for what the b-glucan receptor is on the 3T3-L1 adipocytes.

We have recently reported that laminarin-coated beads, namely LCB, which mimics fungal particles, is an inflammatory stimulator for both differentiating and differentiated 3T3-L1 adipocytes [18]. Thus, it is mostly likely that LCB could inhibit adipogenesis. Therefore, we carried out a series of experiments to test whether LCB inhibits adipogenesis and investigate the potential b-glucan receptor on the 3T3-L1 adipocytes and. The mechanism of how adipocytes receive the signal from LCB is analyzed and discussed in this study.

Materials And Methods

Preparation of laminarin-coated beads (LCB)

The preparation of LCB was performed using 1,1'-carbonyldiimidazole (CDI)-mediated conjugation as previously described [18].

Cell culture and differentiation of 3T3-L1 adipocytes

Mouse 3T3-L1 fibroblasts (ATCC) were cultured in the growth media or Dulbecco's modified Eagle's medium-high glucose (DMEM, Sigma-Aldrich) supplemented with 1% Pen-Strep (100 U/mL Penicillin and 100 µg/mL Streptomycin) and 10% fetal bovine serum (FBS) at 37 °C and 5% CO_2. 3T3-L1 adipocyte differentiation was performed using the differentiation/activation medium (D/A) as described earlier [18]. In brief, the differentiation was started by incubation of two-day post-confluent cells (defined as Day 0) with the D/A medium containing the hormonal inducers (10 µg/mL insulin, 0.5 mM IBMX (1-methyl-3-isobutylxanthine), and 1.0 µM dexamethasone). On Day 2, the D/A medium was changed to the maintenance medium or D/M (DMEM-high glucose, 10% FBS, 1% Pen-Strep, and 10 µg/mL insulin) and incubated for another 2 days (from Day 3-4). Subsequently, the medium was switched to the growth medium and refresh every 2 days until it was fully differentiated to adipocyte (Day 8). The level of differentiation was quantified using the Oil Red O staining and the triglyceride assays.

Oil Red O staining

To determine lipid accumulation, cells were washed with 1xPBS prior to fixing with 10% formalin solution for 1 hour at room temperature. The fixed cells were washed with 60% isopropanol and let dry completely. 0.3% (w/v) Oil Red O solution was added and incubated for 10 minutes at room temperature. After incubation, cells were washed 3 times with distilled water to remove the excess dye. Images of the cells were collected using an inverted microscope (CKX53, Olympus). Then, isopropanol was added to dissolve the dye and the absorbance was measured at 520 nm.

Triglyceride assay

The assay was modified from the previous study [56]. Briefly, after washing with 1xPBS, the cells were extracted using lysis solution (0.05% Triton X-100 in 1xPBS). The whole cell lysates were then heated up at 70 ºC for 10 min and gently vortex for 30 seconds or until the sample was completely homogenized. The protein content was measured according to the Bradford assay as previously described (Bradford 1976). For triglyceride (TG) detection, the assay was set up by mixing the homogenized sample with 250 unit/mL lipase (Sigma-Aldrich) in the ratio of 1:1. After incubation at 37 ºC for 60 minutes, the mixture was centrifuged at 12,000 rpm for 5 minutes, and the glycerol content in the sample was determined with a free glycerol reagent kit (Sigma-Aldrich) as recommended by the company. The concentration of TG in the sample was calculated by referring to the glycerol standard curve and the TG values were expressed as nmol of TG/mg of protein.

3T3-L1 differentiating adipocytes stimulation with LCB

To analyze the effect of LCB on adipocyte differentiation, LCB (1:150 cells:LCB) was added to the cells at different time intervals during the differentiation process as shown in Fig. 4A. The relative percentage of lipid accumulation was analyzed by Oil Red O staining on Day 8 of differentiation. To evaluate the molecular mechanisms of LCB on adipogenesis, a desired concentration of LCB (e.g. 1:150 cells:LCB) was added together with D/A medium to the 3T3-L1 fibroblast cells. Adipocyte differentiation was performed as described above. For gene expression analysis, cells were harvested at 3 and 48 hours after treatment for investigating inflammatory genes expression (e.g., IL-6) and adipogenic/cell cycle regulatory genes expression (e.g., *Pparγ* and *Cdc45l*), respectively. For determination of adipogenic protein expression, the incubation time (1–8 days) was performed before subjecting the cells to Western blotting assay. The expression of cyclin D1 was observed from 0-24 hours after the stimulation. The phosphorylation of AMPK and ACC was detected during the first 6 hours of adipogenesis, and the degradation of IκBα protein was examined at 30 minutes after the treatment with LCB.

Assay with inhibitors

After pre-treating the cells with the selective inhibitors; either SC-514 (50 μM, Sigma-Aldrich), IRAK 1/4 inhibitor (10 μM, Sigma-Aldrich), SYK inhibitor (10 μM, Abcam), or IRAK 1/4 plus SYK inhibitors (10 μM) in the growth medium for 30 minutes, the medium was removed. Then, a fresh D/A medium containing the same concentration of those inhibitors and LCB (1:150 cells:LCB) was added. Cells were harvested at the indicated time points for analyzing the molecular mechanisms of LCB on adipogenesis, as described above.

Quantitative PCR (qPCR)

Total RNA extraction and complementary DNA (cDNA) synthesis were performed as previously described [18]. Real-time PCR was conducted using a real-time PCR machine (Stratagene Mx3005P, Aligent Technologies) with EvaGreen fluorescence dye (Solis BioDyne). The primer sequences used for this study are listed in Table S1. All qPCR assays were performed with the annealing temperature at 60 °C for 40 cycles. Data were analyzed using the $2^{-\Delta\Delta Ct}$ method [57] and β-actin were used as an internal control.

Western Blot Analysis

Protein sample preparation was performed similarly as previously described [18], except that the lysates were prepared with PRO-PREP™ protein extraction solution (iNtRON Biotechnology). Samples were then resolved on 7.5%, 10%, or 15% SDS–polyacrylamide gel and transferred to a PVDF membrane (0.45 μm, Merck). Primary antibodies against IκBα (Abcam); total AMPKα (Santa Cruz), phospho-AMPKα (Thr 172) (Santa Cruz), phospho-Cdk (Thr14/Tyr15) (Santa Cruz); β-actin (Cell Signaling), C/EBPβ (Cell Signaling), C/EBPα, PPARγ (Cell Signaling), FABP4 (Cell Signaling), FAS (Cell Signaling), ACC (Cell Signaling), phospho-ACC (Ser79) (Cell Signaling), and cyclin D1 (Cell Signaling) were applied with 1:1,000-dilution, except for IκBα and β-actin which were used at the concentration of 1:10,000 and incubated for 12-16 hours at 4 °C. After incubating with 1:10,000-diluted horseradish peroxidase (HRP) conjugated secondary antibody (Cell Signaling) for 1 hour at room temperature, and enhanced chemiluminescence analysis

system (Wako Chemicals) was used to develop the signal. Images were captured using an ImageQuant™ LAS 4000 (GE Healthcare). All the signal intensities were quantified by ImageJ 1.49t software.

Measurement of double-stranded DNA (dsDNA)

To quantify the dsDNA concentration, 3T3-L1 cells were treated with the D/A medium in the presence or absence of LCB (1:150 cells:LCB) for 48 hours. The treated cells were washed twice with 1xPBS prior to lysing with SDS lysis buffer (10 mM Tris-HCl (pH 8), 1 mM EDTA, and 0.05 % SDS). After completing homogenization, the mixture was centrifuged at 13,000 rpm 4 °C for 5 minutes. Only the supernatant was subjected to the dsDNA assay, which was performed using Qubit® dsDNA BR Reagent (Thermo Fisher Scientific) as recommended by the manufacturer.

Cell cycle analysis (flow cytometry)

After being treated for 18 hours without or with LCB (1:150 cells:LCB) in the D/A medium, 3T3-L1 differentiating cells were detached using the Accutase solution (Innovative Cell Technologies) and fixed with 70% (v/v) cold ethanol at -20 °C for 24 hours. Then, the fixed cells were collected by centrifugation at 1,000 rpm for 3 minutes. The cell pellet was resuspended with the guava® cell cycle reagent (Merck) and incubated for 30 minutes at room temperature. Subsequently, the cell suspension samples were transferred to a new tube for cell cycle analysis using BD FACSCanto II Flow Cytometer (BD Bioscience) and the data were analyzed using BD FACSDiva software (BD Bioscience).

Statistical analysis

All data were performed at least three times independently and expressed as the mean ± S.D. The statistical significance was determined by the statistical software R (version 3.6.2) using one-way ANOVA with Duncan's multiple range tests ($p < 0.05$).

Results

LCB inhibits the D/A medium-induced 3T3-L1 adipogenesis in a dose-dependent manner

To test the effect of LCB on adipogenesis, we introduced an increasing amount of LCB to the D/A medium-induced differentiating 3T3-L1 adipocytes. The preadipocytes (Fig. 1A), which are voided of lipid accumulation, was included as a negative control, while the fully differentiated adipocytes or the untreated (UT) set (Fig. 1B) served as a positive control for the staining assay. The results show that the percentage of lipid accumulation in the LCB-treated differentiating adipocytes decreases as the amount of LCB added to the cells increases (Fig. 1E-H), suggesting that LCB dose-dependently inhibits adipogenesis. The uncoated beads (UC)-treated differentiating adipocytes reveal a comparable level of lipid accumulation with the untreated set (Fig. 1C, H), suggesting that the effect of LCB was conferred by laminarin (b-glucan) on the beads but not the beads themselves. In addition, lipopolysaccharide (LPS) which was previously shown to inhibit the differentiation of 3T3-L1 adipocytes [19], shows a significant

reduction in the lipid accumulation (Fig. 1D and H). Thus, LCB confers a similar adipogenic inhibitory effect to LPS.

To further confirm this effect of the LCB, the total amount of triglyceride (TG) accumulation in adipocytes was determined using a triglyceride (TG) assay. Consistent with the Oil Red O staining results, LCB inhibits the accumulation of triglycerides in 3T3-L1 adipocytes in a dose-dependent manner (Fig. 1I). Collectively, these data suggest that LCB inhibits adipogenesis in a dose-dependent fashion.

LCB attenuates the D/A medium-induced expression and production of the key adipogenic marker genes in differentiating 3T3-L1 adipocytes in dose- and time-dependent manners

To investigate how LCB inhibits adipogenesis, we analyzed the expression of the key adipogenic transcription factors by mean of qPCR assay. The results show that the expression of *Pparg*, *C/ebpa,* and *Srebp-1* decreases as the mount of LCB added to the differentiating cells increases (Fig. S1B-D), suggesting a dose-dependent effect of the LCB. In addition, we find that LCB dose-dependently inhibits the expression of genes involved in adipogenesis, including *Fas*, *Lpl,* and *Fabp4* (Fig. S1E-G). LPS, which was included as an inhibitor of adipogenesis, could also significantly suppress the gene expression of those transcription factors and adipogenic enzymes (Fig. S1B-G). Unlike the expression of those genes, the expression of *C/ebpb* is not affected by LPS or an increasing amount of LCB (Fig. S1A). Consistently, a coffee extract, which was shown to inhibit adipogenesis, downregulated the protein expression but not the gene expression of *C/ebpb*[20]. Because C/EBPb regulates the expression of *Pparg* and *C/ebpa*[21], we think the decrease in the expression of those genes is not directly caused by LCB, but rather the effect on C/EBPb. Because the transcript of *C/ebpb* remains unchanged, this suggests that LCB may not affect the expression of *C/ebpb* in the transcription and post-transcriptional levels but it may inhibit the protein expression of C/EBPb in a post-translational level.

To test this hypothesis, we performed Western blotting to analyze the protein level of those adipogenic transcription factors and enzymes in LCB-treated differentiating 3T3-L1 adipocytes. First, we analyzed the kinetic expression of C/EBPb, C/EBPa, PPARg, FAS, FABP4, and acetyl-CoA carboxylase (ACC) proteins on day 0, 1, 3, 5, and 8 by mean of Western blotting. We find that C/EBPb protein is expressed as early as day 1 after the start of differentiation and the protein level gradually decreases throughout the course of differentiation, whereas C/EBPa, PPARg, and FABP4 proteins appear on the 3rd day differentiation (Fig. 2A, C and F). Although FAS and ACC proteins can be detected even before the differentiation starts, their protein expression is upregulated in the late stage of differentiation (Fig. 2A, E and G). However, the treatment with LCB from the starting period of adipogenesis severely decreased the amount of C/EBPb, as well as C/EBPa, PPARg, FAS, FABP4, and ACC proteins (Fig. 2). To further illustrate the effect of LCB on gene expression, we treated the differentiating cells with an increasing dose of LCB for 2 days and analyzed the protein levels of C/EBPb, C/EBPa, PPARg and FAS through Western blotting. We show that an increase amount of LCB results in a reduction of those proteins, suggesting a dose-dependent effect of the LCB (Fig. 3). LPS, which was included as a positive control, also significantly reduces those

proteins (Fig. 3). Therefore, these data demonstrate that LCB promotes C/EBPb protein degradation, causing the downregulation of the other transcription factors and enzymes.

To show that this effect is conferred by b-glucan on the beads, we also treated the cells with uncoated beads and free laminarin. Treatment with uncoated beads does not affect the production of those proteins. In addition, only laminarin at 1 mg/mL concentration reduced protein expression (Fig. 3). As we previously showed that the concentration of laminarin in 1:150 cells:beads in a 48-well was approximately at 4.03 mg/mL [18], the 1 mg/mL concentration, which causes a noticeable effect to the protein expression, is far more concentrated than the amount of b-glucan on the beads. Therefore, b-glucan must be presence on the beads to inhibit adipogenesis through the reduction of C/EBPb protein, which may subsequently affect gene expression and protein production of other adipogenic genes such as *C/ebpa*, *Pparg*, *Fabp4*, *Fas*, *Lpl*, and *Srebp-1*.

LCB suppresses the differentiation of 3T3-L1 adipocytes in the early phase of adipogenesis

The effect of LCB on adipogenesis led us to investigate whether the introduction of LCB at different stages of adipogenesis would equally suppress adipogenesis. Therefore, we treated differentiating adipocytes with LCB (1:150 cells:LCB) at different time periods: day 0, day 2, day 4, or day 6 after the start of differentiation (Figure 4A). Then, the lipid accumulation and the expression of adipogenic transcription factors (*Pparg* and *C/ebpa*) of LCB-treated cells at each time period were analyzed by mean of the Oil Red O staining and qPCR assays, respectively. We find that LCB could effectively suppress adipogenesis and the expression of the transcription factors when introduced on day 0 but had very little or no effect when the LCB treatment was initiated on day 2 or later (Fig 4B-D). These results suggest that LCB affects the early stage of adipogenesis, most likely through the reduction of C/EBPb.

Because LCB can only inhibit adipogenesis in the early stage of differentiation, we wondered if an inflammatory response induced by LCB must also happen in the early stage. To answer this question, we analyzed the expression of *Il-6* and *Mcp-1* genes in differentiating adipocytes treated with LCB by following a similar experimental setup for adipogenesis as described above. After 3 hours of treatment with LCB, the results reveal that the expression of *Il-6* and *Mcp-1* genes is relatively high in all conditions and reached the highest levels when LCB was introduced on day 0 and day 6 of differentiation, respectively (Fig. 4E and F). This observation is not unexpected since we showed that LCB induced an inflammatory response in the differentiated 3T3-L1 adipocytes [18]. Therefore, while LCB stimulates an inflammatory response at any stage of adipogenesis, it could only effectively induce adipogenesis inhibition only at the early stage of differentiation.

LCB induces cell cycle arrest, suppresses the mitotic clonal expansion (MCE), and downregulates cell cycle-related gene expression during the early phase of 3T3-L1 differentiation

During the early stage of adipogenesis, differentiating adipocytes undergo mitotic clonal expansion (MCE), in which the cells enter approximately 2 rounds of cell division in the first 2 days of differentiation [22]. The cell cycle division is tightly controlled by cyclin and cyclin-dependent kinases (CDK). For example,

the G1 to S cycle progression requires the collaboration of cyclin D-CDK4/6 complex [5]. The activation of CDK4/6 leads to phosphorylation of Rb, a negative regulator of E2F proteins, resulting in the release of E2F factors. Free E2F factors promote transcription of cyclin E, an S-phase cyclin. Cyclin E binds CDK2 to promote hyperphosphorylation of Rb to enable the G1/S transition [23].

Given that LCB inhibits adipogenesis, LCB may have an effect on cell cycle. Previous studies analyzed the effects of adipogenic inhibitors on cell cycle progression and showed that coffee extract, dimethylfumarate, and sulforaphane promoted cell cycle arrest at the G1 phase [20,24,25]. These studies led us to hypothesize that LCB may promote a similar effect to the cell cycle. To test our hypothesis, we investigated the effect of LCB on cell cycle progression in differentiating 3T3-L1 adipocytes by using a flow cytometry technique. Preadipocytes were induced into differentiation with the D/A medium in the absence or the presence of uncoated beads or an increasing dose of LCB. While most of the cell population in preadipocytes remains in the G0/G1 phase, the differentiating cells exit the G0/G1 phase and progress into the S and G2/M phases (Fig. 5A-B and G). However, the presence of LCB in the D/A medium dose-dependently increases the number of cells in G0/G1 but decreases the cell population in the G2/M phases (Fig. 5D-G). In addition, we show that LCB inhibits the protein expression of cyclin D1 (Fig. 5H), a factor necessary for G1/S transition [5]. Together, these results suggest that LCB promotes the G0/G1 cell cycle arrest and decreases cyclin D1 protein expression.

The effect on cell cycle progression likely causes a decrease in the total number of differentiated cells. To test this idea, we counted the number of preadipocytes and the D/A medium-stimulated differentiating adipocytes treated without or with the presence of either uncoated beads or an increased dose of LCB for 2 days. We show that the number of preadipocytes does not greatly increase during the 48 hours of culturing, confirming that they did not undergo MCE. In contrast, the number of differentiating adipocytes and the differentiating adipocytes treated with the uncoated beads (UC) increase approximately 2-fold during the 48 hours of culturing with the D/A medium, suggesting that treatment with the D/A medium alone stimulates MCE (Fig. 5I). However, the presence of LCB induces a reduction in the total cell number proportionally to the amount of LCB added, suggesting a dose-dependent effect of LCB (Fig. 5I). The loss in the number of the cells is not due to the beads themselves because the uncoated beads produced no effect. To further illustrate the effect of LCB on MCE, we performed an assay to quantify the amount of double-stranded DNA in preadipocytes, differentiating adipocytes, and differentiating adipocytes treated with the uncoated beads or an increased dose of LCB. Consistent with the data in Fig. 5I, an increased dose of LCB causes an increasing loss in the total amount of dsDNA (Fig. 5J).

During the early adipogenesis, the key transcriptional activator C/EBPb stimulates the expression of many genes involved in DNA replication and cell division, including cell division cycle 45 homolog (*Cdc45l*), mini-chromosome maintenance complex component 3 (*Mcm3*), GINS complex subunit 1 (*Gins1*), and cell division cycle 25 homolog c (*Cdc25c*) [26]. Consistent with this notion, we show that the D/A medium-induced differentiating 3T3-L1 adipocytes (untreated/UT) upregulates the expression of all 4 genes (Fig. 5K-N). Because LCB suppresses the protein expression of *C/ebpb* (Fig. 2A-B), we think the

expression of those cell-cycle regulatory genes must be affected by the treatment with LCB. To test this idea, we analyzed whether LCB would suppress the expression of *Cdc45l*, *Mcm3*, *Gins1*, and *Cdc25c* genes in differentiating adipocytes by mean of qPCR. Differentiating 3T3-L1 adipocytes were induced with D/A medium in the presence of the increasing dose of LCB for 48 hours prior to being harvested for qPCR analysis. We find that the greater the amount of LCB that is added, the greater the inhibition of the expression of those 4 genes results (Fig. 5K-N), suggesting the dose-dependent inhibitory effect of LCB. Together, these results demonstrate the ability of LCB to inhibit MCE through the promotion of G0/G1 cell cycle arrest and the downregulation of cyclin D1, causing a reduction of the cell number and inhibition of adipogenesis.

IRAK and SYK signaling pathways mediate an inflammatory-stimulating and adipogenic suppressing effects of LCB on differentiating 3T3-L1 adipocytes

To obtain a clue for which of the b-glucan receptors involve in b-glucan recognition in the differentiating cells, we performed the qPCR assay to check the expression of *Tlr2*, *Clec7a/Dectin-1*, *Cd36*, and *Tlr4* in differentiating adipocytes treated with LCB. If any of these receptors is important for the cellular response, then their expression should change upon the treatment with LCB. The results show that the expression of *Tlr2* and *Clec7a/ Dectin-1* genes in the differentiating adipocytes transiently upregulates as the incubation time with LCB progresses from 1-24 hours (Fig. S2A-B). In addition, we show that the expression of both genes in the cells is increased proportionally to the amount of LCB added (Fig. S2E-F). In contrast, the expression of *Cd36* and *Tlr4* genes remain unchanged upon the treatment with LCB (Fig. S2C-D & G-H). These results suggest that TLR2 and CLEC7A/Dectin-1 but not CD36 and TLR4 receptors are important or may involve in the response of adipocytes to LCB activation.

Previous studies showed that TLR2 and Dectin-1 signaling pathways require interleukin-1 receptor-associated kinase 1 and 4 (IRAK1 and IRAK4) and spleen tyrosine kinase (SYK) for their signal transduction, respectively [27,28]. Therefore, we investigated the involvement of IRAK and/or SYK proteins in mediating the effect of LCB in differentiating adipocytes by using IRAK 1/4 and SYK inhibitors that inhibit IRAK and SYK proteins, respectively. If IRAK or SYK pathways are required for LCB recognition, then the IRAK1/4 and/or SYK inhibitors should reverse any effect of LCB on the differentiating cells, including an inflammatory response and inhibition of adipogenesis.

We began the analysis by investigating whether the inhibitors could block LCB-induced inflammatory response in the differentiating adipocytes. We find that the expression of nuclear factor kappa B subunit 1 (*Nfkb1*), monocyte chemoattractant protein-1 (*Mcp-1*), prostaglandin-endoperoxide synthase 2 (*Cox-2*), interleukin 6 (*Il-6*), and inducible nitric oxide synthase 2 (*Nos2*) genes in the differentiating adipocytes, which are induced by the presence of LCB, is significantly suppressed in the presence of either IRAK1/4 or SYK inhibitor (Fig. S3A-E). The IKK-2 inhibitor, which was recently shown to inhibit the ability of the inhibitor of IκB kinase β (IKKβ) to degrade IkBa protein for an inflammatory response in adipocytes triggered by LCB [18], serves as a positive control and could efficiently prevent the effect of LCB (Fig. S3A-E). To show that IRAK and SYK proteins regulate IkBa protein stability, we performed a Western blotting

assay to test whether each of the inhibitors can prevent IkBa degradation. The cells were collected for Western blotting after treating with LCB and the inhibitors for 30 minutes. The results show that IkBa protein stability is increased when either IRAK1/4 or SYK inhibitor was included in the LCB-treated differentiating adipocytes, and it is further enhanced when both IRAK1/4 and SYK inhibitors were combined (Fig. S3F). Thus, these data demonstrate that the IRAK and SYK proteins mediate the LCB-induced inflammatory response through the NF-kB complex in the differentiating adipocytes.

To analyze the role of IRAK and SYK signaling pathways in the LCB-triggering inhibition of adipogenesis, we employed the same strategy of using those inhibitors to cripple the pathways. Throughout differentiation (8 days), the presence of either IRAK1/4 or SYK inhibitor significantly increases the lipid accumulation in the LCB-treated differentiating adipocytes, and the amount of lipid accumulation is further increased in the presence of the IKK-2 inhibitor (Fig. 6A). These results suggest that the inhibitors relief the transcriptional inhibitory effect of LCB on the key markers for adipogenesis. To test this idea, we performed a qPCR assay in the LCB-treated differentiating adipocytes for 2 days, without or with the presence of IRAK1/4, SYK, and IKK-2 inhibitors. We find that the expression of *C/ebpa,Fapb4*, *Fas*, *Pparg*, *Lpl*, and *Srebp-1*, the key markers for adipogenesis whose expression that are normally repressed by the presence of LCB are derepressed when either IRAK1/4 or SYK inhibitor is included in the LCB-treated differentiating adipocytes and the derepression is further elevated when the IKK-2 inhibitor was used (Fig. 6B-G). The effects on gene expression mostly likely lead to the effect on protein expression. Therefore, we analyzed the levels of PPARg and C/EBPa proteins, which are the key proteins for adipogenesis. As expected, the stability of those proteins in the LCB-treated adipocytes is enhanced by either IRAK1/4 or SYK inhibitors. Furthermore, the co-treatment of the IRAK1/4 and SYK inhibitors further enhances the stabilization of PPARg and C/EBPa (Fig. 6H). Together, these results suggest the role of IRAK and SYK proteins in mediating the inhibitory effect of LCB, including the LCB-mediated inhibition of adipogenesis and LCB-induced inflammatory response through the NF-kB complex.

LCB activates the AMP-activated protein kinase (AMPK) and acetyl-CoA carboxylase (ACC) phosphorylation at the early stage of adipogenesis

AMPK, a serine/threonine kinase complex, functions in response to a variety of conditions that increase the ratio of AMP/ATP such as exercise, fasting, starvation, and hypoxia [29,30]. This complex consists of a catalytic α-subunit (α1 and α2), a scaffolding β-subunit (β1 and β2) and a regulatory γ-subunit (γ1, γ2, and γ3). Upon the activation by cellular or metabolic stress that increases the AMP/ATP ratio, Thr172 residue on the a-subunit of AMPK is phosphorylated, which stimulates the kinase activity of the complex [31]. Thus, the phosphorylation of AMPK a-subunit at the Thr172 residue is considered the hallmark of AMPK activation. One of the protein targets of AMPK is ACC protein, which catalyzes the formation of fatty acid for adipogenesis [32]. AMPK downregulates the function of ACC enzyme by promoting Ser79 phosphorylation, resulting in the inhibition of adipogenesis [32].

The findings above suggest the role of AMPK as a negative regulator for adipogenesis. To explore whether LCB exerts its adipogenic inhibition through the promotion of AMPK activity, we performed a

Western blotting assay to analyze the phosphorylation of AMPK and ACC proteins in the differentiating 3T3-L1 adipocytes with or without the presence of LCB. Consistent with the notion that AMPK inhibits adipogenesis, we find that LCB enhances the Thr172 phosphorylation on the a-subunit of AMPK from the first hour of incubation with LCB and the level of Thr172 phosphorylation is maintained until at least 6 hours after the incubation (Fig. 7A). Furthermore, LCB also stimulates Ser79 phosphorylation of the ACC enzymes (Fig 7A). Thus, these results suggest that LCB may inhibit adipogenesis through the activation of the AMPK complex and phosphorylation of ACC protein at the early stage of adipogenesis.

To analyze whether the activation of AMPK is promoted through the IRAK and SYK kinases, we used the IRAK1/4 and SYK inhibitors with the LCB-treated differentiating adipocytes. We observed a small but significant inhibition of the AMPK and ACC protein phosphorylation; however, the inhibition of the protein phosphorylation is evidence when the cells were treated with both IRAK1/4 and SYK inhibitors (Fig. 7B). Thus, these results suggest that both IRAK and SYK signaling pathways mediate the activation of AMPK in the LCB-treated adipocytes.

Discussion

Recently, we reported that LCB is an inflammatory elicitor for 3T3-L1 adipocytes, which stimulated the expression of many inflammatory-related genes (e.g., *Il-6*, *Mcp-1*, and *Cox-2*) in dose- and time-dependent manners through the activation of classical NF-kB pathway [18]. In the present study, we further analyzed the mechanistic effect of LCB on differentiating 3T3-L1 adipocytes. We find that upon the treatment during the early stage of adipogenesis, LCB promotes cell cycle arrest and inhibits MCE, which result in a significant decrease in cell number. Furthermore, LCB suppresses gene and protein expression of the key adipogenic transcription factors and enzymes and stimulated phosphorylation of AMPK and ACC proteins, leading to the repression of adipogenesis in 3T3-L1 adipocytes. The mechanistic effect of LCB on the differentiating cells is summarized in Fig. 8.

Possible b-glucan receptors on 3T3-L1 adipocytes

One of the most important questions that we attempted to answer is what the specific b-glucan receptor on the cells is. Our investigation reveals that the pathways that rely on IRAK and SYK proteins involve the recognition of b-glucan on LCB. We find that any effect that LCB causes to the cells, including an LCB-induced inflammatory response and LCB-mediated inhibition of adipogenesis could be inhibited by the IRAK1/4 or SYK inhibitors. IRAK proteins (IRAK1, IRAK2, IRAK3, and IRAK4) are serine-threonine kinases involved in toll-like receptors (TLRs) and interleukin-1 (IL-1R) signaling pathways [33]. Upon the activation of TLRs or IL-1R, IRAK4 recruits IRAK1 to the receptors and the kinase activities of these two IRAK proteins are required for the activation of mitogen-activated protein kinases (MAPKs) and the production of TNF-a in macrophages [34,35]. In addition to the IRAK proteins, SYK is a kinase protein required for the receptors that contain the immunoreceptor tyrosine activation (ITAM) domains, such as the classical immune receptors (e.g., Fc-gamma receptor/FcgR) and the c-type lectin receptors (e.g., CLEC7A/Dectin-1 and CLEC2) [36]. SYK protein was previously shown to mediate CLEC7A/Dectin-1 signaling for activation

of NF-kB complex and a release of TNF-a in human peripheral blood mononuclear cells [37,38]. Thus, both IRAK and SYK proteins regulate the production of inflammatory cytokines.

Although IRAK and SYK proteins function in multiple receptors, only TLR2 and CLEC7A/Dectin-1, respectively, are the IRAK- and SYK-dependent receptors that bind to b-glucan [39]. In the context of immune cells, it was shown that CLEC7A/Dectin-1 synergized with TLR2 for the degradation of IkBa, and the production of TNF-a [37], and this synergy requires both CLEC7A/Dectin-1 and TLR2 to be within a close proximity [40]. Consistently, either IRAK1/4 or SYK inhibitor suppresses the effect of LCB and the enhanced inhibitory effects were observed when both inhibitors were added together (Fig. 6-7), illustrating that the IRAK- and SYK-dependent pathways synergize with each other. Thus, the synergy between IRAK and SYK pathways resembles the cooperation between Dectin-1 and TLR2 signaling pathways in the immune cells, suggesting Dectin-1 and TLR2 may be the receptors for b-glucan on differentiating adipocytes.

Interestingly, a recent report suggested cluster of differentiation 36 (CD36), a scavenger receptor, as a candidate for a b-glucan receptor on the surface of macrophages and differentiated adipocytes [17]. CD36 plays a major role as a receptor that mediates the uptake of fatty acids in skeletal muscles and adipose tissues [41]. In addition, CD36 was reported as a b-glucan receptor for zymosan recognition in J744 and human monocyte U937 cells [42,43]. However, we find that the expression of CD36 and TLR4 receptors in the cells was not affected by the incubation with LCB (Fig. S3), suggesting that both CD36 and TLR4 may not involve in the recognition of b-glucans in the cells. Despite the discrepancy, our results did not rule out the possibility that the CD36 might be a b-glucan receptor for the differentiated cells because our study was conducted using the differentiating cells. Therefore, based on the data from our study, at least in the context of the differentiating 3T3-L1 adipocytes, Dectin-1 and TLR2 mostly likely play a role in b-glucan recognition. Nonetheless, future experimentation is required to identify the b-glucan receptor on both differentiating and differentiated adipocytes.

Mechanism of LCB inducing an inflammatory response and adipogenic inhibition in differentiating 3T3-L1 adipocytes

An inflammatory response and adipogenesis are the two related events. We show that LCB can trigger an inflammatory response in differentiating adipocytes at any stage of differentiation (Fig. 4). Other inflammatory elicitors such as TNF-a and LPS can inhibit adipogenesis [19,44]. Unlike an inflammatory response, LCB could only efficiently inhibit adipogenesis when it is introduced at the early stage of differentiation (Fig. 4). Consistently, many compounds or extracts were reported to effectively inhibit adipogenesis when they were incubated with the differentiating cells from the beginning [45–48]. These results suggest that many adipogenic inhibitors may share a common target, which is probably active during the early stage of adipogenesis. Additionally, we show that the IKK-2/SC-514, which inhibits IKKb, efficiently reverses the effects of LCB (Fig. 6 and S3), suggesting that the NF-kB complex regulates both inflammation and adipogenesis in adipocytes.

The most likely explanation for the mechanism of how LCB inhibits adipogenesis and stimulates inflammation is that it activates the NF-kB complex, which subsequently targets the C/EBPb, a key transcription factor for adipogenesis. At the early stage of adipogenesis, C/EBPβ is expressed rapidly after the hormonal inducers from day 1, whereas PPAR and C/EBPa appear from day 3 onward (Fig. 2). This observation is consistent with the previous finding that C/EBPb is required for the subsequent induction of PPARg and C/EBPa [49]. By treating the differentiating adipocytes with LCB at the early stage of adipogenesis, the C/EBPβ protein but not mRNA expression is significantly inhibited (Fig. 2-3), leading to a downregulation of the major adipogenic transcription factors and enzymes (e.g., PPARg, C/EBPa, FAS, and FABP4) (Fig. 2, 3, and Fig. S1). It was shown that C/EBPβ forms a dimer to stabilize itself through the basic leucine-rich zipper domain (b-zip) [50]. Thus, LCB may cause an instability to the C/EBPb dimer or promote the monomeric form of C/EBPb, causing C/EBPb to be degraded via the ubiquitin-proteasome pathway [50]. Nonetheless, exactly how LCB promotes the protein degradation of C/EBPb requires further investigation.

C/EBPβ also plays an important role in the mitotic clonal expansion (MCE) [51]. A previous study showed that mouse embryo fibroblasts (MEFs) from C/EBPβ-deficient mice could not undergo MCE, as well as adipogenesis [51]. Similarly, the RNA interference (RNAi)-mediated knockdown of C/EBPβ also inhibited MCE and differentiation of 3T3-L1 adipocytes [52], suggesting the requirement of C/EBPβ for MCE and adipogenesis. These data are consistent with our finding that LCB inhibits MCE in differentiating 3T3-L1 cells by blocking the cell cycle at the G1/S transition through suppressing the expression of cell cycle regulatory proteins (e.g., cyclin D1) (Fig. 5). Thus, it is highly likely that LCB exerts the inhibitory effects on MCE and adipogenesis at the early stage of differentiation, mainly by promoting the degradation of C/EBPb protein.

In addition to C/EBPb, LCB acts through the AMPK protein complex by promoting the T172 phosphorylation at the a-subunit (Fig. 7A). AMPK activation using 5-aminoimidazole-4-carboxamide ribonucleoside (AICAR) inhibited adipogenesis [53]. Furthermore, AMPK activation by metformin or A769662 in mouse embryonic fibroblast (MEF) resulted in the inhibition of adipogenesis and the suppression of C/EBPb protein [54], suggesting a mechanism of how AMPK inhibits adipogenesis. Consistent with this notion, a previous work done in hepatoma cells showed that C/EBPb was suppressed by AICAR [55], further strengthening a connection between C/EBPb and AMPK. Based on our data, AMPK is activated within the first hour upon LCB exposure (Fig. 7A), whereas C/EBPb protein level is upregulated within the first day of differentiation (Fig. 2). Thus, when LCB was added at the early stage of adipogenesis, it stimulated AMPK, which subsequently inhibits C/EBPb protein. LCB also induces the production of inflammatory cytokines through the activation of NF- kB complex. These inflammatory cytokines and the NF-kB complex may contribute to the inhibition of C/EBPb in the cells. A future experiment is required to elucidate the relationship among NF-kB, AMPK, and C/EBPb proteins in the LCB-treated differentiating adipocytes.

The notion that AMPK inhibits adipogenesis remains a controversy. A previous work by Wang and colleagues analyzed the effect of LPS on adipogenesis in 3T3-L1 adipocytes and showed that LPS inhibited AMPK Thr172 phosphorylation, suggesting a positive role of AMPK in adipogenesis [19]. Despite the differences, we noticed that Wang and colleagues investigated AMPK phosphorylation in the time period throughout the course of differentiation (day 0-8) and began to detect the lower level of AMPK Thr172 phosphorylation from day 2 onward, suggesting the role of AMPK in the intermediate and late stages of adipogenesis. In contrast, we analyzed the phosphorylation of AMPK during the first 6 hours of adipogenesis (Fig. 7), demonstrating the role of AMPK in the early stage of differentiation. Therefore, these data together suggest that AMPK has a differential effect on adipogenesis, depending on the timing throughout differentiation.

In conclusion, we have illustrated the mechanism of LCB, which was previously shown as an inflammatory elicitor for adipocytes, in the inhibition of adipogenesis and cell cycle progression in differentiating adipocytes. LCB may be recognized by both TLR2 and CLEC7A/Dectin-1 on the cell surface. However, additional experiments will be required to determine the actual b-glucan receptor on the surface of adipocytes.

Declarations

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Author contributions

C.J. designed, performed experiments, analyzed data, drafted the manuscript, and prepared raw figures. M.I. and H.O. contributed to all Western blotting, flow cytometry, and dsDNA measurement and interpreted the flow cytometry results. N.H-T. gave technical advice and mentored. R.B. contributed to Fig. 1. N.S. and P.A. contributed to Fig. S1. D.S. conceived the project, designed the concepts for experiments, acquired research grant, analyzed and interpreted data, revised all the figures, and wrote the manuscript. All authors read and approved the final manuscript.

Competing interest

The authors declare that they have no competing interest.

Data availability

All data generated or analyzed during this study are included in this published article (and its Supplementary Information files).

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