

Intelectin1 ameliorates macrophage activation *via* inhibiting the nuclear factor kappa B pathway

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Abstract. Intelectin1 (*Itln1*) is an adipokine that is abundantly expressed in intestine, ovary, and lung. The expression levels of ITLN1 are decreased in the presence of diabetes or obesity, but the mechanisms of its production and function are still controversial. The aim of this study is to elucidate the mechanisms of ITLN1 synthesis and ITLN1-associated macrophage activation. To analyze the effects of high fat and high-carbohydrate diet (HFHCD) on the expression of ITLN1 in the intestine, the mice were fed a HFHCD for 8 weeks. HFHCD feeding enhanced the endoplasmic reticulum (ER)-stress in the intestine and inhibited the expression of *Itln1* in the intestinal endocrine cells and lowered circulating ITLN1 levels. In contrast, treatment with a chemical chaperone and reduction of ER-stress restored the expression of *Itln1* in the intestine of HFHCD-fed mice. Furthermore, *in vitro* studies indicated that ITLN1 physically interacts with adiponectin receptor 1 and suppresses lipopolysaccharide-induced mRNA expressions of pro-inflammatory cytokines and phagocytosis activities *via* inhibition of the nuclear factor kappa B-signaling pathway in macrophages. These results suggest that diet-induced ER-stress decreases circulating ITLN1 *via* inhibition of its synthesis in the intestine, and a reduction of circulating ITLN1 might enhanced the expression of proinflammatory cytokines and macrophage activation, following exacerbate the chronic inflammation of metabolic syndrome.

Key words: ER-stress, Inflammation, Nuclear factor kappa B, Macrophage

OBESITY is a worldwide health problem that is associated with several lifestyle-related diseases, including not only severe cardiovascular disease but also orthopedic disorders. A high-fat and high-carbohydrate diet (HFHCD) is associated with an increased risk of obesity [1], diabetes, metabolic syndrome (MeS), and cancer [2]. Recent studies have indicated that obesity is a low-grade inflammatory disease. Indeed, a large number of pro-inflammatory cytokines common to inflammation [3] and obesity have been found: interleukin (*Il*)1, *Il*6, angiopoietin-like protein 2 [4], and tumor necrosis factor α . The activation of chronic low-grade inflammation in individuals with obesity promotes the clinical progression of obesity-related pathologies such as insulin resis-

tance, type 2 diabetes, and ischemic heart disease (IHD) [5].

Nuclear factor kappa B (NF κ B) transcription factors drive the expression of target genes that mediate cell proliferation and the release of proinflammatory cytokines, and are conserved coordinating regulators of immune and inflammatory responses [6]. Biomolecular experiments have indicated that pathogen-associated molecules, such as lipopolysaccharide (LPS), ligate to cell surface receptors and initiate signaling cascades that converge on the activation of the inhibitor of kappa B kinase (IKK) complex. IKK phosphorylation of inhibitor of kappa B alpha (IKBA) molecules promotes their degradation and releases NF κ B, which translocates to the nucleus to promote the transcription of target genes in macrophages [7, 8].

Omentin/intelectin1 (*Itln1*) is a secretory glycoprotein consisting of 295 amino acids that show high levels of homology between humans and mice [9]. Several reports indicated that Intl is highly expressed in visceral adipose tissue compared with subcutaneous adipose tissue [10,

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11]. Other recent studies also indicated that ITLN1 is abundantly expressed in the intestine [12] and visceral adipose tissues [9]. Circulating ITLN1 levels are decreased in patients with obesity or type 2 diabetes [11], and decreased levels of ITLN1 are associated with the incidence and severity of IHD [13]. It has also been reported that ITLN1 can inhibit pulmonary inflammation and endothelial injury after LPS-induced acute respiratory distress syndrome in mice [14]. These reports indicate that ITLN1 is an anti-inflammatory adipokine, and its reduction is associated with the exacerbation of chronic inflammation. However, the link between ITLN1 and inflammation-induced macrophage activation and its mechanisms are still controversial.

In this study, we demonstrated that HFHCD feeding enhanced the intestinal endoplasmic reticulum (ER)-stress which suppressed the expression of ITLN1, and that this is partly involved in the exacerbation of systemic inflammation. We also found the anti-inflammatory effects of ITLN1 on macrophages.

Materials and Methods

Cell culture

RAW264.7 cells were purchased from ATCC and cultured in RPMI1640 supplemented with 10% FBS. LPS was purchased from Sigma-Aldrich (L2018). Cells were incubated at 37°C in a humidified 5% CO₂ environment. RAW264.7 cells were transfected with the pGluc-NFKB plasmid which contained 5 copies of an NFKB response element (Promega, Madison, WI) and the *Renilla* luciferase plasmid using the jetPRIME® transfection reagent (Polyplus, New York, NY) according to the manufacturer's instructions. Transient co-transfections were carried out in triplicate. Dual luciferase assays were carried out according to the manufacturer's instructions (Promega).

RAW 264.7 cells were incubated with 200 ng/mL of Int11 or 10 ng/mL of LPS. The concentration of ITLN1 was determined by the previous reports that analyze the effects of ITLN1 [14, 15]. The concentration of LPS were determined referred by previous *in vitro* report [16].

Animal studies

The animal study protocol was approved by the Institutional Animal Care and Use Committee of the University of Yamanashi. C57BL6 mice were fed a control regular diet (RD) or a high-fat and high-carbohydrate diet (HFHCD) consisting of 41% fat, 43% carbohydrate, and 17% protein content on an energy basis (D12079B, Research Diets Inc., New Brunswick, NJ). Mice were fed a RD or HFHCD for 8 weeks starting at 5 weeks of age. For 4-phenylbutyric acid (PBA) studies, mice were

administered it in drinking water (20 mM) [17] for 8 weeks. Mouse serum ITLN1 concentrations were measured using a RayBio® mouse ITLN1 enzyme-linked immunosorbent assay kit (Ray Biotech, Norcross, GA). The detailed protocol for immunochemical analysis was previously described [7].

Western blot analysis and real-time PCR

The protocols for western blot analysis and real-time PCR were previously described [7, 18]. Nuclear fractions were prepared using a NE-PER kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's protocol. The primary antibodies were as follows: anti-inhibitor of KB (IKB), NFKB, tubulin, and histone antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and anti-ITLN1 antibody was purchased from Proteintech (Rosemont, IL). Total RNA was extracted by using TRIzol (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Total RNA (200 ng) was used in real-time reverse transcription-PCR (RT-PCR) as described previously [19]. Specific primers were purchased from Thermo Fisher Scientific.

Electrophoretic mobility shift assay (EMSA)

NFKB DNA-binding activities were measured using an EMSA according to a previously described protocol with modifications [20]. RAW264.7 cells that were treated with LPS and/or omentin were harvested and the nuclear fractions prepared. Oligonucleotide for the *Nfkb* consensus binding sequence, 5'-AGTTGAGGGACTT TCCCAGGC-3', was end-labeled with [γ -³²P] ATP and T4 polynucleotide kinase (Promega). Nuclear extract (5 μ g) was incubated in a 10 μ l reaction volume for 20 min at room temperature in the following buffer: 10 mM Tris HCl, pH 7.6, 50 mM KCl, 5 mM MgCl₂, 1 mM DTT, 1 mM EDTA, 12.5% glycerol, 0.1% Triton X-100, and 1 μ g poly(dI-dC). Labeled probe, 50,000 cpm, was added and incubated an additional 20 min at room temperature. DNA-protein complexes were separated on an 8% native polyacrylamide gel.

Phagocytosis activities assay

RAW264.7 cells were plated on glass coverslips (Fisher Scientific, Hampton, NJ) at a density of 1×10^5 cells/coverslip. One day after plating, the cells were cultured with or without 200 ng/mL of ITLN1. After 24 h of incubation, the cells were treated with or without 10 ng/mL of LPS. Latex beads-rabbit IgG-FITC complex (Cayman Chemical, Ann Arbor, MI) was added directly to the culture medium at 1:200 dilution and incubated at 37°C for 2 h.

ITLN1-binding protein assay

Recombinant ITLN1 are kindly gifted from Dr. Tsuji, Yokohama Cancer Center [9]. To prepare activate esterified ligand-immobilized beads, 1 mg of COOH beads (Tamagawa Seiki Co, Ltd. Nagano, Japan) was incubated with 1 M succinimide to activate esterification, following incubation with 50 µg of recombinant ITLN1 or recombinant human Adiponectin (Pepro Tech Inc, Rocky Hill, NJ). Then, the ligand-immobilized beads were incubated with 1 M aminoethanol to mask ligand-unbound carboxyl ones. Two hundred micrograms of the ligand-immobilized beads were incubated with 200 µg of the cell soluble membrane fraction for 30 min at 37°C and washed three times. The eluted protein was subjected to SDS page, and western blotting was performed using mouse monoclonal anti-Adiponectin receptor 1 (ADIPOR1) antibody (sc-518030, Santa Cruz Biotechnology).

Statistics

Comparisons between groups were performed by one-way ANOVA for normally distributed variables and by the Mann-Whitney *U* test for nonparametric distributions. Data analysis was performed using STATA 14.2 (Stata Corp LLC, College Station, TX).

Results

High-fat and high-carbohydrate diet-intake accumulates ER-stress and inhibits ITLN1 expression

To analyze the linkage of high-fat and high-carbohydrate diet (HFHCD) intake and the production of ITLN1, C57BL6 mice were fed with a HFHCD for 8 weeks and the expression of ITLN1 was analyzed in the small intestine, where it is mainly produced [12]. Immunohistochemical analyses indicated that ITLN1 expression was observed in intestinal endocrine cells that co-expressed NEUROD (a marker for endocrine cells) of RD-fed mice (Fig. 1A a–c). The expression of ITLN1 was decreased in intestinal endocrine cells of HFHCD-fed mice (Fig. 1A d–f). Fifty percent of intestinal endocrine cells expressed *Itn1*, while only 10 % of endocrine cells expressed ITLN1 in 8 weeks of HFHCD-fed mice. Body weight was significantly increased in HFHCD-fed mice compared with RD-fed mice (31.0 ± 2.6 g vs. 25.0 ± 1.1 g and 1.2 ± 0.3 g vs. 0.4 ± 0.05 g, respectively, $p < 0.05$) (Fig. 1B). Serum ITLN1 levels were significantly decreased in HFHCD-fed mice (32.1 ± 3.3 ng/mL vs. 24.6 ± 1.9 ng/mL, $p < 0.05$) (Fig. 1B). HFHCD-feeding decreased the expression of ITLN1 in small intestinal tissues (Fig. 1C, lanes 1 and 2 vs. 3 and 4), accompanied by the expression of CHOP, which induces cell death under ER-stress [21].

To analyze the association of HFHCD-induced ER-stress and expression of ITLN1 in mice, HFHCD-fed mice were administrated with PBA, which is a chemical chaperone that attenuates ER-stress [17], and analyzed the expression of ITLN1 in the small intestine by western blot analysis. In HFHCD-fed mice, the protein expression of ITLN1 was significantly increased by PBA-treatment (Fig. 1C, lanes 5 and 6 vs. 7 and 8). In HFHCD-fed mice, there was no difference in the abundance of ITLN1 protein expression in adipose tissue with and without PBA-treatment (Fig. 1D). The expression of CHOP was significantly decreased in mice with PBA-treatment compared to mice without PBA-treatment. Serum ITLN1 levels were significantly elevated in PBA-treated mice (Fig. 1B). There were no differences in the body weights of mice with and without PBA-treatment. Furthermore, *Itn1* mRNA expression was decreased in the intestine of HFHCD-fed mice, while PBA-treatment enhanced the expression of *Itn1* mRNA (Fig. 1E). These results suggest that HFHCD-induced ER-stress directly regulates the expression of CHOP and might be involved in the suppression of ITLN1 in small intestine and serum.

ITLN1-bound adiponectin receptor 1 inhibits LPS-induced NFKB signaling pathway

To explore the function of ITLN1, we focused on its anti-inflammatory effects, because recent reports have indicated that a high-fat diet can induce obesity and insulin resistance *via* intestinal inflammation [22, 23]. These reports supported the hypothesis that the reduction of ITLN1 might be partly involved in the regulation of inflammation. Indeed, adiponectin is known as an anti-inflammatory adipokine, and ligand-bound ADIPOR1 inhibits the LPS-stimulated IKBA/NFKB pathway [24]. Consistent with this, recombinant adiponectin-immobilized beads interacted with ADIPOR1 (Fig. 2A, lane 4). To analyze the molecule targets of ITLN1, ITLN1-binding proteins were purified from RAW264.7 cells extracts by using ITLN1-immobilized beads. ADIPOR1 was detected as an ITLN1-binding protein, which was expressed in the membranes of RAW246.7 cells (Fig. 2A, lane 7).

IKBA molecules sequester NFKB in the cytosol of resting cells and prevent its nuclear localization and transcriptional activation. Extracellular stimuli-induced degradation of IKBA protein is a prerequisite for nuclear translocation of NFKB [25]. To analyze whether ITLN1-bound ADIPOR1 regulates the anti-inflammatory process, we analyzed the effects of ITLN1 on the LPS-activated IKBA/NFKB pathway. LPS treatment increased the phosphorylation of IKBA and then induced nuclear translocation of p65, while ITLN1-treatment

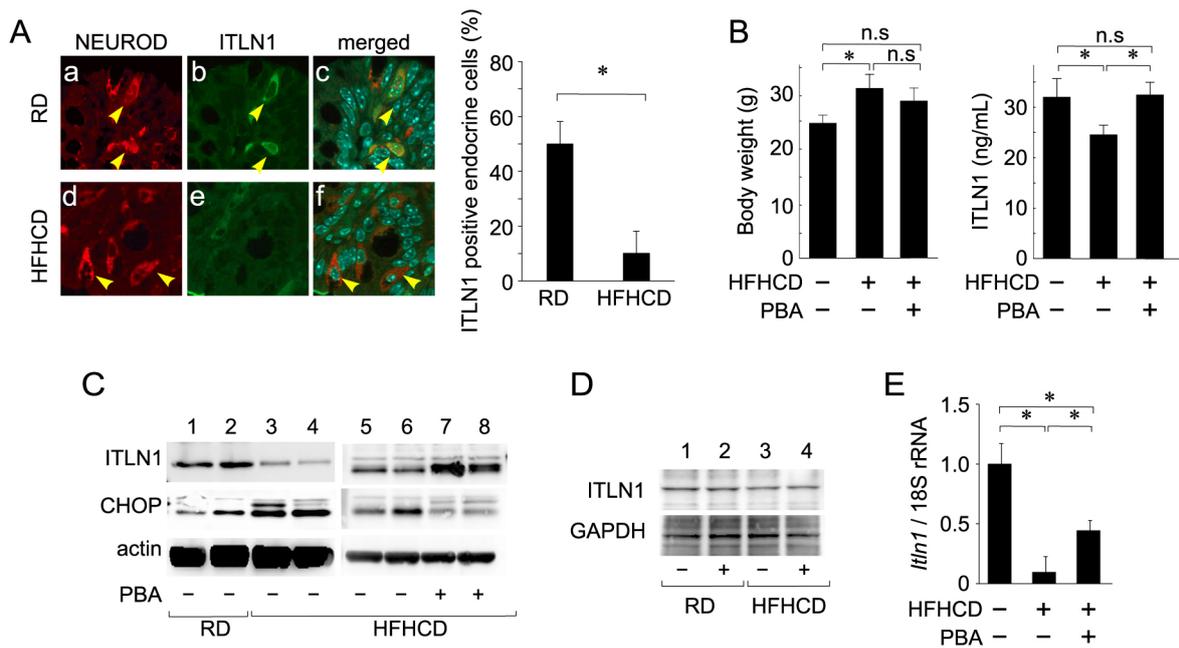


Fig. 1 A HFHCD inhibits the expression of ITLN1 in the intestine

A. The intestines of regular-diet or western-type diet fed-mice stained for NEUROD (a marker for endocrine cells) and ITLN1 were analyzed by fluorescence microscopy. NEUROD-expressing cells were visualized using an Alexa Fluor 555-conjugated secondary antibody (red) (a and d). ITLN1-positive cells were visualized with an Alexa Fluor 488-conjugated secondary antibody (green) (b and e). Nuclei were stained with DAPI, and merged images are represented in c and f. The arrows identify the endocrine cells, which are NEUROD- and ITLN1-positive cells. The percentage of ITLN1-expressed cells in NEUROD-positive endocrine cells. All data are mean \pm SD (error bars). *, $p < 0.05$. B. Body weights and serum ITLN1 concentrations. All data are mean \pm SD (error bars). *, $p < 0.05$, compared with regular diet-fed mice. Experiments were performed in triplicate. C. Expressions of ITLN1, CHOP, and actin in whole tissue lysates of small intestines of RD or HFHCD with duplicates. D. Expressions of ITLN1 and GAPDH in whole tissue lysates of adipose tissue of RD or HFHCD. E. The expression of ITLN1 in the intestine as analyzed by real time PCR. The data represent the means \pm SD (error bars). *, $p < 0.05$. RD, regular diet fed mice; HFHCD, high-fat and high-carbonate diet fed mice; PBA, 4-phenylbutyric acid. n.s., statistically no significant differences.

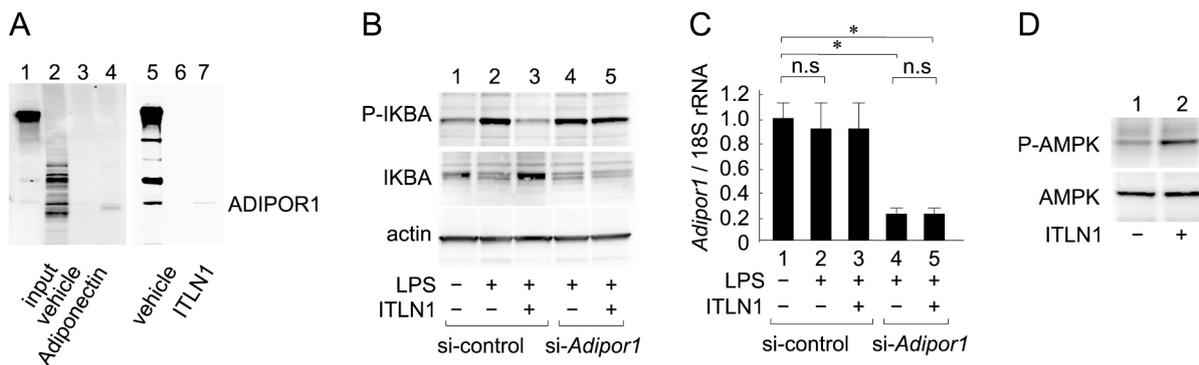


Fig. 2 ITLN1 bound to Adiponectin receptor 1

A. Two hundred micrograms of membranous fraction proteins were incubated with Adiponectin 1-immobilized beads, ITLN1-immobilized beads, or control beads (vehicle), and the bead-bound proteins were analyzed by western blot using anti-Adiponectin receptor 1 (ADIPOR1) antibody. Lane 2 shows the corresponding input of 20 μ g of membranous protein. Lanes 1 and 5 show molecular weight markers. B. The effects of siRNA knockdown of *Adipor1* (or control siRNA) on the expressions of P-IKBA, total IKBA, or actin were analyzed by western blot. C. The effects of siRNA knockdown of *Adipor1* (or control siRNA) on the expression of ADIPOR1 as analyzed by real time PCR. The data represent the means \pm SD (error bars). *, $p < 0.05$. Experiments were performed in triplicate. D. Expressions of phosphorylated AMPK (Adenosine monophosphate-activated protein kinase) and AMPK were analyzed by western blot.

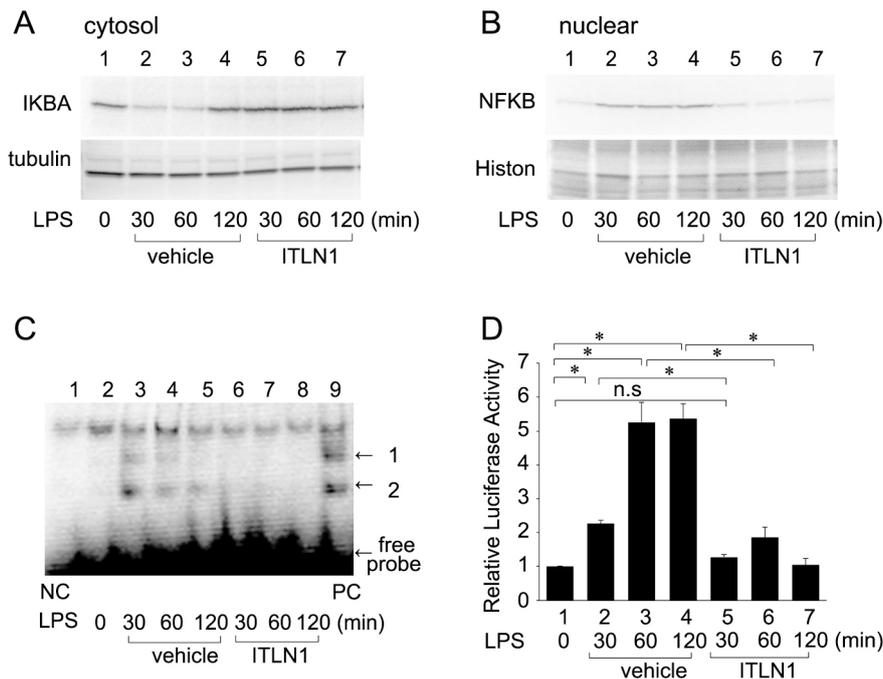


Fig. 3 ITLN1 inhibits LPS-induced activation of the NFKB pathway

The expressions of IKB in whole cell lysates (A) and p65 in nuclear fractions (B) were analyzed by western blot. The expression of tubulin or histone was used as a loading control, respectively. C. LPS-induced accumulation of DNA-binding NFKB in nuclei of RAW264.3 cells was detected by EMSA. Specific bindings are shown by arrows. NC, negative control incubation with cold probe. Nuclear extracts of HeLa cells were used as positive controls (PC). D. Effects of ITLN1 on luciferase activity in LPS-treated RAW264.3 cells that were transfected with an NFKB-driven Luc reporter and incubated with or without ITLN1. Luciferase activity was normalized to *Renilla* activity and is expressed as induction over the control. Relative signals were determined by arbitrarily setting the value for control nontreated cells to 1. The data represent the means \pm SD (error bars). *, $p < 0.05$.

inhibited the LPS-induced phosphorylation of IKBA (Fig. 2B, lanes 1–3). In *si-Adipor1*-transfected cells, in which the expression of *Adipor1* mRNA was inhibited (Fig. 2C, lanes 4 and 5), the ITLN1-induced suppression of IKBA-phosphorylation, which was induced by LPS-treatment, was not observed (Fig. 2B, lanes 4 and 5). Adenosine monophosphate-activated protein kinase (AMPK) is phosphorylated and activated by the ligand-bound ADIPOR1 [26]. ITLN1 induced the phosphorylation of AMPK in RAW246.7 cells (Fig. 2D). These results suggest that ITLN1 bound to ADIPOR1 following suppression of the IKBA/NFKB pathway and acted as an anti-inflammatory adipokine in macrophages.

The abundance of IKBA was decreased by 30 and 60 min of LPS treatment; in contrast, the LPS-induced decline in IKBA was inhibited by the presence of ITLN1 (Fig. 3A, lanes 2 and 3 vs. 5 and 6). The nuclear distribution of p65 protein was significantly enhanced in 30 and 60 min LPS-treated RAW264.7 cells; in contrast, ITLN1 inhibited the nuclear distribution of p65 (Fig. 3B, lanes 2 and 3 vs. 5 and 6). These results suggest that ITLN1-induced modification of IKBA degradation was involved in the suppression of translocation and transcriptional activities of NFKB in macrophages.

EMSA demonstrated that NFKB-DNA binding complexes, p50/p50 homodimers (Fig. 3C, arrow 1) and p50/p65 heterodimers (Fig. 3C arrow 2), were in RAW264.7 cells following 30 min of LPS stimulation, while treatment with ITLN1 significantly reduced the binding complexes (Fig. 3C, lanes 3–5 vs. 6–8). These results suggest that ITLN1 diminished the physical affinity of p50 or p65 subunits to the NFKB consensus sequence in macrophages.

Next, we analyzed the molecular linkages of ITLN1 and the transcriptional activity of NFKB and a coordinating regulator of immune and inflammatory response [6]. RAW264.7 cells were transfected with a luciferase reporter plasmid that was expressed under the control of a promoter containing an NFKB-binding sequence and treated with LPS in the presence or absence of ITLN1. LPS increased the luciferase activity by 5.3-fold, and ITLN1 substantially inhibited the LPS-induced increase in luciferase activity (Fig. 3D, lanes 2–4 vs. 5–7).

ITLN1 ameliorates LPS-stimulated macrophage activation.

To assess the function of ITLN1 in the anti-inflammatory process, LPS-stimulated RAW264.7 cells

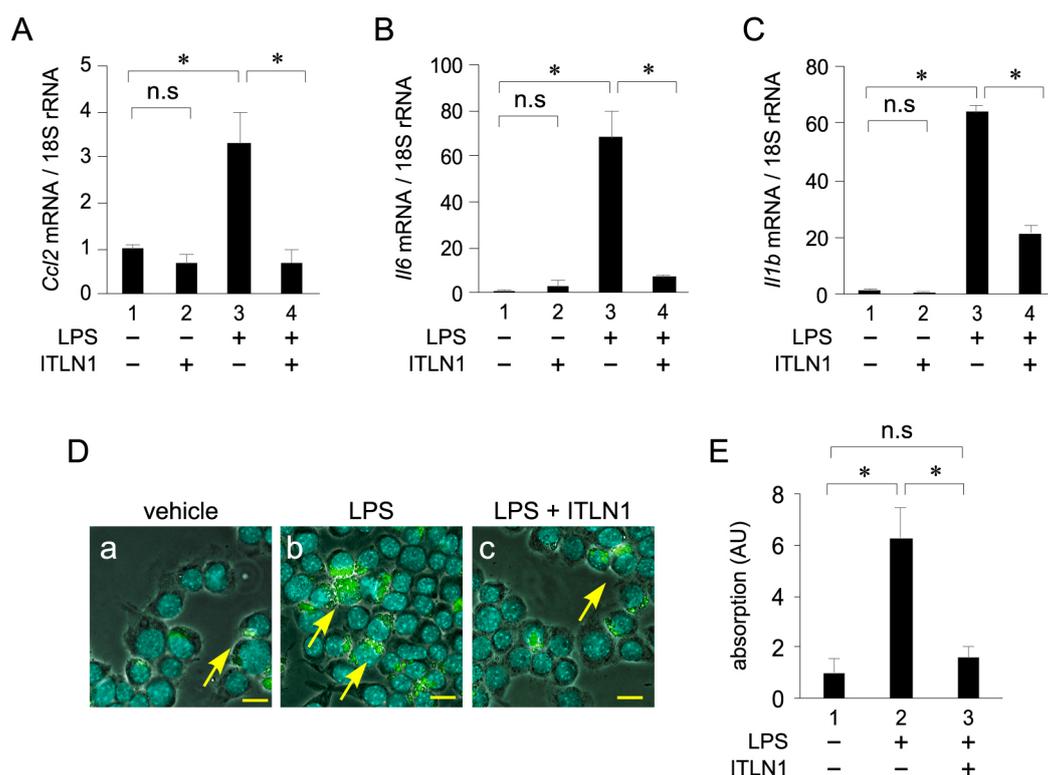


Fig. 4 Expression of pro-inflammatory cytokines and phagocytosis activities

The mRNA expressions of *Ccl2*, *Il6*, *Il1b*, and 18S were determined using real-time RT-PCR with 100 ng of cDNA. Relative mRNA expression levels were determined by arbitrarily setting the value for control cells to 1 (A–C, respectively). Data are expressed as means \pm SD (error bars). *, $p < 0.05$. D. The photo-images show representative RAW264.3 cells that phagocytosed fluorescence-conjugated beads. Phase contrast images were merged and are represented in a–c. Arrows indicate phagocytosed beads. Scale bar, 5 μ m. E. The quantifications of fluorescence signals of cells represent the means \pm SD (error bars). *, $p < 0.05$. These experiments were performed in triplicate.

were treated with ITLN1, and the expression of pro-inflammatory cytokines was analyzed. Recombinant ITLN1 treatment had no effect on chemokine (C-C motif) ligand 2 (*Ccl2*), *Il6*, or *Il1b* mRNA (Fig. 4A–C, lane 1 vs. 2). LPS treatment significantly enhanced the expression of *Ccl2*, *Il6*, and *Il1b* mRNA in RAW264.7 cells (Fig. 4A–C, lanes 1 vs. 3). These LPS-induced expressions of *Ccl2*, *Il6*, and *Il1b* were significantly inhibited by co-treatment with ITLN1 (Fig. 4A–C, lane 3 vs. 4). LPS-treated cells demonstrated enhanced phagocytosis of FITC-conjugated beads compared with vehicle treatment (Fig. 4D, a vs. b; and 4E, lane 1 vs. 2). These LPS-induced phagocytic activities were completely inhibited by co-treatment with ITLN1 (Fig. 4D, b vs. c; and 4E, lane 2 vs. 3). These results suggest the possibilities that ITLN1 directly inhibits IKBA/NF κ B signaling pathway and ameliorates activation of macrophages.

Discussion

The current study indicated that HFHCD feeding induced the accumulation of ER-stress and suppressed

the expression of ITLN1 in the intestinal endocrine cells. HFHCD-fed mice also characterized an obesity and reduced serum ITLN1. Treatment with a chemical chaperone that attenuates ER-stress restored the expression of ITLN1 in the intestine of HFHCD-fed mice. Recombinant ITLN1 ameliorated the macrophage activation *via* inhibiting the NF κ B pathway. These our findings suggested that HFHCD-induced ER-stress disturbed the endocrine system in the intestine and exacerbated obesity-related chronic inflammation.

ER-stress in intestinal epithelial cells has been shown to be a pathological component of many chronic diseases, including inflammatory bowel disease [27] and MeS [3, 28]. A dense mucus layer prevents inflammation in the intestine by shielding the underlying epithelium from luminal microbes. The major macromolecular component of this barrier is mucin glycoprotein and is produced by intestinal goblet cells [29]. In this study, HFHCD enhanced the expression of CHOP, which is induced by ER-stress and involved in cytokine production, accompanied by the suppression of ITLN1 expression in intestinal epithelial cells. In contrast,

downregulation of ER-stress by treatment with PBA enhanced the expression of ITLN1 in intestinal epithelial cells. Furthermore, intestinal expression of ITLN1 were reflected in the serum ITLN1 levels. The expression of ITLN1 were not differenced in the adipose tissues of 8 weeks HFHCD-fed mice, compared with RD-fed mice. The differences of intestinal epithelial cells and adipocytes might be caused by direct effects of HFHCD and feeding interval (8 week). These results suggested that HFHCD-intake induced the overproduction of glycoproteins, and post-translational modification at the ER might induce ER-stress following enhanced expression of CHOP in intestinal endothelial cells. The HFHCD-induced accumulation of ER-stress in the intestine causes the dysfunction of intestinal epithelial cells as an endocrine organ and is involved in the resulting systemic inflammatory environment.

NFKB proteins are regulators of immune and inflammatory responses [6] and are under the control of signaling from extracellular stimuli. Pathogen-associated molecules can ligate to cell surface receptors and initiate NFKB signaling cascades that converge on the activation of the IKK complex. IKBA is phosphorylated by IKK and promotes their degradation and releases p65, which translocates to the nucleus to promote the transcription of cytokine genes. *Adipor1*, which are plasma membrane proteins with 7 transmembrane domains, are expressed in skeletal muscle, macrophages, endothelial cells, and pancreatic β -cells [30]. ADIPOR1 could bind several molecules; adiporon, schisandrin A, arctiin, or gramine and activate down-stream pathway [31, 32]. Agonist-bound *Adipor1* activates AMPK, which promotes insulin-sensitizing effects and mediates anti-inflammatory effects that regulate the NFKB signaling pathway [33-35]. Activation and phosphorylation of AMPK inhibits the phosphorylation of IKK and IKB and the nuclear translocation of p65 in macrophages and induces anti-inflammatory effects [35]. While basic local alignment search tool finds no regions of similarity between the sequences of ITLN1 and Adiponectin, our results suggested that ITLN1 could bind with ADIPOR1 and

activate down-stream signaling pathway.

In our studies, the concentration of ITLN1 was determined referred by previous studies which analyzed the effects of recombinant ITLN1 on the PI3K/AKT signaling pathway in macrophages or pulmonary endothelial cells [14, 15]. This concentration was differed in the mice serum level of ITLN1. These differences might cause by lower bioactivities of recombinant ITLN1, compared with secreted ITLN1.

Our results are the first finding that ITLN1 influences the nuclear translocation and transcriptional activation of NFKB *via* activation of *Adipor1*. Under the presence of ITLN1, the LPS-induced reduction of IKBA was inhibited, suggesting that IKBA and NFKB complex formation was sustained and that the expressions of pro-inflammatory cytokines were completely inhibited in LPS-treated macrophages. Furthermore, ITLN1 inhibited the phagocytosis activities of macrophages. These results suggest that ITLN1 directly acts as an anti-inflammatory cytokine *via* inhibition of the NFKB signaling pathway.

A HFHCD is an aggravating factor of MeS-enhanced ER-stress in intestinal epithelial cells. Furthermore, our study indicated that downregulated ITLN1 exacerbates the inflammatory response in macrophages in MeS patients. MeS is usually classified as a non-inflammatory disease; however, genome-wide transcriptome analysis studies consistently indicate the presence of inflammatory signaling pathways in the context of MeS. It has been established that chronic low-grade inflammation plays a key role in the initiation and progression of MeS. Consistent with its central role in coordinating inflammatory responses, our study implicates the transcription factor NFKB in the development of such diseases, thereby further establishing inflammation as a critical factor in their etiology and offering hope for the development of new therapeutic approaches for their treatment using ITLN1.

Acknowledgments

N/A.

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