Cajanonic acid A regulates the ratio of Th17/Treg via inhibition of expression of IL-6 and TGF-β in insulin-resistant HepG2 cells

Yanfeng Gong, Huanbing Liu, Liming Tao

Department of Geriatrics, The First Affiliated Hospital of Nanchang University, No. 17, Yongwai Zheng Street, Nanchang, Jiangxi 330006, China

Correspondence to: Liming Tao, Department of Geriatrics, the First Affiliated Hospital of Nanchang University, No. 17, Yongwai Zheng Street, Nanchang, Jiangxi 330006, China. Email: tao961129@hotmail.com

Running title: Cajanonic acid A protects cells from insulin resistance
Abstract

**Background:** The objectives of this study are to investigate whether cajanonic acid A (CAA) can reduce insulin resistance in HepG2 cells and to gain a preliminary understanding of the mechanisms underlying this effect.

**Methods:** Following induction of insulin resistance in HepG2 cells, we tested the regulatory effect of CAA on glucose consumption and evaluated hepatocyte production of IL-6, TGF-β, and key molecules in the insulin transduction pathway. A transwell co-culturing system was used to assess the effect of CAA on insulin resistance in HepG2 cells during the differentiation of CD4+ T cells by calculating the ratio of Th17/Treg. We evaluated the effect of CAA on the expression of IL-17RC cells and HepG2 cell apoptosis by immunofluorescence and flow cytometry assay.

**Results:** CAA improved dexamethasone-induced reduction of glucose consumption in HepG2 cells, inhibited hepatocyte production of IL-6 and TGF-β, increased the expression of IL-17RC cell, and increased cellular apoptosis in insulin-resistant HepG2 cells. When co-cultured with CD4+ T cells, insulin-resistant HepG2 cells induced a decreased in the ratio of Th17/Treg, but CAA dampened the effect. Application of IL-6 and TGF-β, together with CAA, reversed the effect of CAA on insulin-resistant HepG2 cells. Overexpression of IL17R, however, counteracted the effect of IL-6 neutralizing antibody within the culture system.

**Conclusion:** CAA can regulate the ratio of Th17/Treg by mediating the expression of IL-6 and TGF-β in insulin-resistant HepG2 cells.

**Key words:** Cajanonic acid A, Th17/Treg, IL-6, TGF-β, insulin resistance
**Introduction**

Insulin resistance is critical in the development of type 2 diabetes mellitus (T2DM) [1], which is characterized by reduced responsiveness of cells to normal circulating concentrations of insulin. The liver plays an important role in carbohydrate metabolism, which adjusts glucose production according to the energy balance via the regulation of insulin. Insulin binds to its receptors that are expressed on hepatocytes and suppresses hepatic glucose production via activation of PI3K signal transduction [2, 3]. Insulin resistance results in the suppression of glucose uptake and glycogenesis, which in turn lead to hyperinsulinemia and glucose in tolerance [2, 3]. In vitro studies in HepG2 cells revealed that insulin resistance in HepG2 cells is mainly associated with deficient glycogenesis, impaired hepatic glucose production and dysfunction of the insulin signal transduction pathway [3, 4]. Insulin-resistant HepG2 cells have fewer total glucose transporters (GLUT) and changes in other critical molecules involved in glucose metabolism, which leads impaired glucose uptake in cells [5-8].

The immune system was reported to be involved in the development of insulin resistance in T2DM. Increased infiltration of activated T lymphocytes has been found in the liver of the patients with T2DM. Along with accumulation of T cells, there is an imbalance between the pro-inflammatory T cell subset known as T helper 17 (Th17) and the T helper 1 and anti-inflammatory T cell subset known as regulatory T cells (Treg). Th17 cells secrete a series of cytokines including IL-17A (IL-17), IL-17F, IL-22, IL-6, and TNF-α [9, 10]. The shift in the ratio of Th17/Treg from T cells is mediated by IL-6 and TGF-β [11, 12]. In HepG2 with insulin resistance, a series of inflammatory responses also occur. Studies have identified the presence of secreted cytokines such as IL-6, TNF-β in insulin-resistant HepG2 cells [3, 4]. Meanwhile, an up-regulation of expression of cytokine receptors such as IL-17 receptors occurs, along with IL-17 and other pro-inflammatory cytokine exacerbate hepatic steatosis.
and apoptosis of liver cells [13-15]. These studies suggest that increased inflammatory sensitivity in insulin-resistant hepatocytes may trigger an alteration in the immune system, which in turn promotes the inflammatory response and deteriorates insulin resistance.

Cajanonic acid A (CAA) is a novel stilbenes isolated from the leaves of Pigeonpea [Cajanus cajan (L.) Millsp] by Sheng-Xiang Qiu et al. in 2008 [16]. CAA was reported to have hypoglycemic activity in db/db mice and inhibit PTP-1B and PPARg with high potency, which may prevent the development of side-effects found with classical thiazolidinediones PPARg activators that are used in practice [16]. In this study, we further investigated whether CAA can reduce insulin resistance in HepG2 cells and used our findings to propose hypothetical mechanisms underlying this effect. We hypothesize that CAA might alleviate insulin resistance by regulating the ratio of Th17/Treg via inhibition of the expression of IL-6 and TGF-β in HepG2 cells.

Materials and methods

Main Reagents and antibodies

CAA was dissolved in dimethyl sulfoxide (DMSO, Sigma, St. Louis, MO, U.S.A.) to a concentration of 200 μM and stored at −20 °C as a stock solution. The stock solution was diluted with Dulbecco’s-modified Eagle’s medium (DMEM, Hyclone, U.S.A) to a final concentration of 50 μM before use. Dexamethasone (Cell Signaling Technology, #9668) was diluted with DMEM medium to a final concentration of 1 μM. DMSO was added in the vehicle control group. TGF-β (240-B; R&D Systems, MN, USA) was diluted to a concentration of 6.4 ng/mL, and IL-6 (206-IL/CF, R&D Systems,
MN, USA) was diluted to a concentration of 8 ng/mL before use. Insulin (Cell Signaling Technology, #9668), human IL-6 High Sensitivity enzyme-linked immunosorbent assay (ELISA) Kit (Abcam, ab46042, U.S.A), human IL-17 High Sensitivity ELISA Kit (Abcam, ab46042, U.S.A), propidium iodide (Cell Signaling Technology, #9668), 4′,6-diamidino-2-phenylindole (DAPI; Cell Signaling Technology, #9668), primary antibodies including anti-IL-17RC, anti-IL-6, anti-p-IR (Cell Signaling Technology, #9542), anti-GLUT1 (Cell Signaling Technology, #9542), and monoclonal mouse anti-GAPDH (Santa Cruz, CA, USA), and horseradish peroxidase (HRP)-conjugated polyclonal goat anti-mouse and anti-rabbit (both from Santa Cruz, CA, USA) were used in this study. Human IL-17RC adenovirus was purchased from Vector Biolabs (Malvern, PA, USA).

**Experiment design**

In experiment 1, glucose consumption by cells in the Dex group (IR) or control group was determined by the glucose oxidase and peroxidase (GOD-POD) method. In experiment 2, cells with Dex disposal were divided into a CAA+Dex group or Dex group in which CAA (50 μM) or isometric DMSO was added into the medium. Glucose consumption, protein levels of p-IR, GLUT1, level of IL-6 and TGF-β were determined by GOD-POD assay, Western blotting, and ELISA, respectively. In experiment 3, dexamethasone-induced IR HepG2 (abbreviated as IR) cells were allocated into 3 groups (IR group, IR+CAA group, and IR+CAA+IL-6 AB group). The vehicle, CAA (50 μM) and CAA (50 μM), IL-6 neutralizing monoclonal antibody (Siltuximab, CNTO328, 10 ng/mL) were added, respectively. The activated CD4+T cells were cultured with the IR group, IR+CAA group and IR+CAA+IL-6 AB group at ratio of 5/1 in transwell system. HepG2 cells were seeded into in upper chambers and CD4+T cells
were seeded into the lower chambers. The expression of IL-17RC and IL6, protein levels of p-IR, GLUT1, apoptotic cell ratio, and glucose consumption in HepG2 cells groups were determined by immunofluorescence assay, Western blotting assay, flow cytometry, and GOD-POD assay, respectively. The ratio of Th17/Treg in CD4+ T cells was determined by flow cytometry. ELISA was used to evaluate the level of IL-17 in the culture medium. Cells treated with equivalent volume solvent (DMSO) was regarded as control group.

**Cell culture**

HepG2 cells were cultured in DMEM containing 4.5 g/l d-glucose and 10% heat-inactivated fetal bovine serum in an incubator at 37 °C and 5% CO₂ (Thermo Forma, U.S.A). Two days after reaching confluence, 1 μM Dex or vehicle was added to the growth medium, allowing another 48 hours for induction of insulin resistance (IR). After Dex induction, the cells were processed.

CD4+ T cells was isolated from human peripheral blood using MojoSort™ CD4 T Cell Isolation Kit (BioLegend, Cat#48000) and then CD4+ T cells were resuspended in RPMI-1640 media (Flowlab, Australia) with 100 IU/mL of penicillin, 100 μg/mL of streptomycin (Flowlab, Australia), and 10%, v/v fetal bovine serum at density of 1×10⁴ cell/mL. CD4+ T cell number was determined by cell counting with equal volume of trypan blue. CD4+ T cells were exposed to anti-CD3 and anti-CD28 antibodies for 2 days to induce differentiation into CD4+T cells.

HepG2 cell seeding in the upper chamber, and activated CD4+ T cells were co-cultured in the Transwell system at ratio of T/E at 5/1 and incubated at 37 °C, 90% humidity, and 5% CO₂ for 24 h. After the co-culture incubation period, cells were collected and analyzed.
Transduction

Human IL-17RC adenovirus was transduced into the CAA-treated insulin-resistant HepG2 cells after the DMEM medium was removed. Fresh complete DMEM medium was then added to the cells, which were incubated at 37°C overnight. After 24 h, the medium containing virus was removed and fresh, complete DMEM culture medium was replaced. Cells were harvested after 48 h post transduction.

Western blot assay

HepG2 cells were washed with PBS twice before collection. Cells were lysed in RIPA buffer and centrifuged for 30 min at 13,000×g at 4 °C. The supernatant was heated with 4× loading buffer at 95 °C for 5 min. Proteins were electrophoresed in a 12% sodium dodecyl sulfate–polyacrylamide gel. Proteins were then transferred onto 0.45μm PMSF membranes for 1 h after electrophoresis and blocked in 5% skim milk for 1 h. PMSF membranes were exposed to primary antibodies against p-IR, GLUT1 or GADPH in blocking buffer at 1:500 or 1:1000 dilutions overnight at 4 °C. The membranes were then incubated with secondary antibody conjugated with HRP at 1:5000 dilutions for 1 h. The proteins were visualized autoradiographically with enhanced chemiluminescence (ECL), and scanned using a bio-imaging analyzer (Bio-Rad, USA).

Glucose consumption assay
The glucose consumption assay was performed using a GOPOD kit (Rongsheng Biotech, Shanghai, China) according to the manufacturer’s instructions. Medium from HepG2 cells in different treatment groups was spun down in a centrifuge column, and glucose concentrations before and after the 24-hour treatment were determined using the kits.

**Immunofluorescence assay**

Cells were fixed and processed into paraffin-embedded slides. After antigen retrieval, 3% H2O2 were used to inactivate endogenous peroxidase. The slides were blocked in 1% bovine serum albumin phosphate buffered saline (BSA PBS) solution. For the immunofluorescence assay, slides were incubated with primary antibodies overnight at 4 °C. The slides were incubated with biotinylated–modified secondary antibody at 25 °C for 1 hour, and then incubated with conjugated HRP-labeled streptavidin (Dako, Glostrup, Denmark) at 25 °C for 30 minutes. Substrate diaminobenzidine (DAB; Sigma, St. Louis, MO, USA) was used as the chromogen. Photographs were using the BioRad Lasersharp MRC500 scanning confocal microscopy system.

**Flow cytometric**

An apoptosis detection kit was used to measure apoptosis in cells pre-labeled with propidium iodide and annexin V–fluorescein isothiocyanate (FITC; Invitrogen, Burlington, Canada) according to the manufacturer’s instructions. For the detection of Treg and Th17, cells were labeled with PE-labelled
anti-human CD25 Abs and FITC-labelled CD4 Abs. To analysis the Treg and Th17, after cells were fixation and permeabilization, cell was then stained with Foxp3 (APC labelled) or IL-17 (APC labelled) to detect Treg and Th17, respectively. Samples were analyzed via flow cytometry and the results were recorded using matching Cell Quest software (Becton Dickinson, San Jose, CA, USA).

**Statistical analyses**

The data were expressed as the mean ± standard deviation (SD) of at least three independent experiments. All of the statistical analyses were performed using the SPSS 19.0 software. Student’s t-test was used to analyze differences between two groups, and one-way ANOVA was used to determine the significant differences among multiple groups. P values less than 0.05 were considered statistically significant.

**Results**

CAA improved dexamethasone-induced decrease in glucose consumption by HepG2 cells with insulin resistance.

In this study, we induced insulin resistance in HepG2 cells via dexamethasone to lipoblast, and oil red O staining was used to confirmed this induction (Figure 1A). As shown in Figure1B, glucose consumption in dexamethasone treated HepG2 cells group (Dex group) was significantly decreased compared with the control group (Figure1B). Besides, IC50 of CAA to HepG2 was measured (Figure...
and results showed that IC50 value was 100 nM. Therefore, 0.5-fold of IC50 (50 nM) was used for further experiments. Using an established cell model of insulin resistance, we evaluated the effect of CAA on regulation of insulin resistance. The glucose consumption assay revealed that cells in the control group and CAA-treated group (IR+CAA group) significantly reversed the decrease in 2-DG level in the IR group (Figure 1D).

It is believed that IL-6 and the TGF-β signal pathway are involved in the development of insulin resistance. We evaluated the levels of IL-6 and TGF-β by ELISA in the control group, the IR group, and the CAA group. The level of IL-6 was significantly decreased in the IR group compared with the control group and CAA group (P<0.05, Figure 1E). IL-6 and TGF-β were further measured in IR+CAA group. We found that IL-6 was then increased compared with the IR group, but TGF-β showed the opposite trend (Figure 1E and F). These findings indicated that CAA may mediate hepatocyte production of IL-6 and TGF-β in insulin-resistant HepG2 cell.

To further confirm these findings, we used Western blotting to determine the protein levels of GLUT1 and p-IR which are critical for insulin signal transduction pathway. We found that CAA treatment improved the changes in GLUT1 and p-IR induced by dexamethasone exposure in HepG2 cells (Figure 1G).

CAA mediates hepatocyte production of IL-6 and TGF-β in HepG2 cells with insulin resistance.

To further confirm that CAA regulates insulin resistance by altering the Th17-to- Treg ratio and to determine whether this involved inhibition of the TGF-β-IL-6 pathway, we performed a transwell co-culture assay of HepG2 and CD4+ T cells. The glucose consumption assay revealed a significantly
higher level of 2-DOG in the CAA group compared with the IR group, while application of IL-6 AB blunt the effect of CAA (Figure2A). Western blotting assay showed that the protein levels of GLUT1 and p-IR were decreased in the IR+CAA+IL-6 AB group compared with the IR+CAA group, which indicates a defect in the insulin signal transduction pathway (Figure2B). Furthermore, measurement of IL-6 and TGF-β levels by ELISA showed that CAA elevated IL-6 level and that addition of IL-6 AB reversed this trend; a significantly lower level was detected in cells treated with IL-6 AB (Figure2C).

As shown in Figure 2D, TGF-β level was attenuated by CAA treatment but was further increased by IL-6 AB.

**Treg/Th17 ratio and cell apoptosis was reversed by IL-6 Ab in insulin-resistant HepG2 cell.**

IL-6 AB is reportedly involved in the regulation of CD4+ T cell differentiation. Therefore, we evaluated the ratio of Th17/Treg in the IR group, IR+CAA group and IR+CAA+IL-6 AB group. As shown in Figure3A and B, the flow cytometry assay revealed a significant increase in the Th17/Treg ratio in CD4+ T cells interacting with HepG2 cells in IR+CAA group compared with the IR group and the IR+CAA+IL-6 AB group (Figure3D). We also detected the expression of IL-17RC in HepG2 cells and found that compared with IR+CAA group, cells in the IR group and IR+CAA+IL-6 AB group showed decreased immunofluorescence of IL-17RC (Figure3C). The concentration of IL-17 was significantly higher in the IR+CAA compared with the IR group. However, it was then significantly suppressed in the IR+CAA+IL-6 Ab group compared with the IR+CAA group (Figure 3E).

In the flow cytometry assay, a significantly higher percentage of apoptotic cells was found in the IR group (early apoptosis, 6.5% of cells; late apoptosis, 23.6% of cells) and the IR+CAA+IL-6 AB
group (early apoptosis, 8.7% of cells; late apoptosis, 33.4% of cells) compared with the IR+CAA group (early apoptosis, 3.7% of cells; late apoptosis, 10.1% of cells; Figure 3F).

Overexpression of IL17R counteracted the effect of IL-6 AB on CAA-treated HepG2 cells.

We then validated the effect of CAA by overexpressing IL17R in CAA-treated insulin-resistant HepG2 cells. Glucose consumption was elevated by IL-17R overexpression compared with IR+CAA+IL-Ab group (Figure 4A). Likewise, Western blotting indicated that the expression of IL-17R in IR+CAA+IL-6 Ab+IL17R group was to some extent weakened compared to expression in IR+CAA group, with downregulation of levels of p-IR, GLUT1 and IL-17RC, but expression was remarkably higher than in IR+CAA+IL-6 Ab group (Figure 4B). Of note, the flow cytometry assay revealed a significant increase of the Th17/Treg ratio in the IR+CAA+IL17 group compared to the IR group, which had results that were similar to the IR+CAA group. The ratio of Th17/Treg after overexpression of IL17R was significantly increased compared to that in the IR+CAA+IL-6 AB group (Figure 4C and 4D), suggesting that the overexpression of IL17R generally counteracted the effect of IL-6 AB in CAA-treated insulin-resistant HepG2 cells. Our results from ELISA showed that the level of IL-17 in CAA-treated cells was significantly increased compared with that in IR group, but was statistically greater than in the IR+CAA+IL-6 AB group (Figure 4E).

Discussion

In this study, we investigated whether CAA could reduce insulin resistance in HepG2 cells and
used those findings to propose preliminary hypotheses regarding the underlying mechanism. We found that CAA could improve dexamethasone-induced insulin resistance in HepG2 cells, promoted hepatocyte production of IL-6 and suppressed hepatocyte production of TGF-β, increased expression of IL-17RC, and increased cell apoptosis in insulin-resistant HepG2 cell. When co-cultured with CD4+ T cells, insulin-resistant HepG2 induced a decrease in the ratio of Th17/Treg, and CAA dampened that effect. The application of IL-6 and TGF-β accompanied with CAA could reverse the effect of CAA on HepG2 cells with insulin resistance.

In many liver diseases and other hepato pathological conditions such as non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, and insulin resistance, hepatic immune responses play critical roles [17-20]. In this study, secretion of IL-6 and TGF-β in insulin-resistant HepG2 and CAA could potentially affect these processes. Studies have shown that serum levels of IL-6 correlate with the development of T2DM [1, 21]. IL-6 as well as TGF-β were found to be involved in the inflammatory process in hepatocytes; in the acute phase of inflammation, hepatocytes secrete IL-6 [4]. A study by Tang et al. reported an increase in secretion of IL-6 in HepG2 cells following stimulation off reefatty acid [22]. Our results are in accordance with the previous study. The effect of CAA is diminished when applied with IL-6. These results suggest that CAA may target IL-6 and TGF-β in the regulation of insulin resistance.

There is study indicated that insulin resistance is associated with through Treg/Th17 [23]. In this study, we hypothesize that CAA regulates the ratio of Th17/Treg by mediating the expression of IL-6 and TGF-β in insulin-resistant HepG2 cells. In support of our hypothesis, insulin-resistant HepG2 cells co-cultured with CD4+ T cells induced a decreased in the ratio of Th17/Treg. Th1, Th2 and Treg are subsets of T cells derived from CD4+T cells and are essential regulators in immune responses and
inflammatory diseases [24], TGF-β and IL-6 are also critical in the differentiation of naive T cells into Th17 in both humans and mice via activation of the transcription factors retinoid-related or phan receptor (ROR) γt and RORα [9, 25, 26], and inhibition of Treg differentiation [27-29]. Compared with Treg, which acts as an anti-inflammatory regulator to maintain immune homeostasis and prevent uncontrolled inflammation response, Th17 cells secrete a series of cytokines, (eg, IL-17A (IL-17), IL-17F, IL-22, IL-6, and TNF-α) and act as a proinflammatory regulator [9, 13, 14, 30, 31]. In our study, we found that CAA could diminish the shift in the ratio of Th17/Treg in CD4+ T cells interacting with insulin-resistant HepG2 cells. This effect was blunted by the application of IL-6 as well as the overexpression of IL17R. Besides, we found that expression of GLUT1 and p-IR were regulated by ratio of Th17/Treg. As a matter of fact, GLUT1 has been identified as a factor acting key role in insulin resistance [32]. These results supported the hypothesis that CAA regulates ratio of Th17/Treg by inhibiting the expression of IL-6 in insulin-resistant HepG2 cells, characterized by expression changing of GLUT1 and p-IR. However, relationship among GLUT1, p-IR, Th17/Treg IL-6 and TGF-β still need further explorations.

Previous studies have reported that increased IL-17, an important proinflammatory cytokine, is involved in cell apoptosis via the IL-17/IL-17R-JAK2/STAT3 pathway and other signaling pathways [10, 33]. In this study, we observed an increase in IL-17RC and apoptosis of the IR HepG2 cell which is reversed by the CAA treatment. Our study also suggested that the increase in IL-17RC and apoptosis are closely related to the increase in IL-6 and TGF-β, and that CAA blunt this effect, while the overexpression of IL17R further validated the role of CAA. These results indicated that insulin resistance in HepG2 cells leads to increases in hepato cellular IL-6 and TGF-β production that induce a shift in the ratio of Th17/Treg in CD4+ T cells. Meanwhile, the Th17 cells, which are increased in
number, secreted IL-17 that act with the increased IL-17RC and promoted cell apoptosis and inflammatory response in insulin-resistant HepG2 cells. CAA reduced insulin resistance and inhibited this positive feedback between Th17/Treg and secretion of IL-6 and TGF-β induced by insulin resistance.

**Conclusion**

The present work investigated the antidiabetic activity of CAA in an insulin-resistant HepG2 cell line. The glucose uptake assay showed significant glucose uptake in CAA-treated cells at a concentration of 50μM and demonstrated blunted secretion of IL-6. Transwell co-culture assay with HepG2 cell and CD4+ T cells revealed that CAA regulates the ratio of Th17/Treg via inhibition of IL-6 and TGF-β expression in insulin-resistant HepG2 cells. Hence, our results imply that CAA can enhance glucose uptake and insulin sensitivity via the IL-6 and TGF-β pathway and suggest a promising treatment for T2DM.

**Conflicts of interest**

The authors declare that there was no conflict of interest regarding the publication of this paper.

**Author contribution statement**

Yanfeng Gong: designing and performance of experiments; manuscript writing work. Huanbing Liu:
performance of experiments, data collecting, and manuscript writing work. Liming Tao: administrative support, manuscript review. All authors read and approved the final manuscript version before submitted.

Acknowledgement

This research was supported by the foundation of Science and Technology Department of Jiangxi Province (No. 2011ZBAB204033) and The foundation of cultivating young teachers of Nanchang university (NO. PY201817).
References

[1] Pradhan AD, Manson JE, Rifai N, Buring JE and Ridker PM. C-reactive protein, interleukin 6, and risk of developing type 2 diabetes mellitus. Jama 2001; 286: 327-334.

[2] Inoue H. Central insulin-mediated regulation of hepatic glucose production [Review]. Endocr J 2016; 63: 1-7.

[3] Ramnanan CJ, Kraft G, Smith MS, Farmer B, Neal D, Williams PE, Lautz M, Farmer T, Donahue EP, Cherrington AD and Edgerton DS. Interaction between the central and peripheral effects of insulin in controlling hepatic glucose metabolism in the conscious dog. Diabetes 2013; 62: 74-84.

[4] Streetz KL, Luedde T, Manns MP and Trautwein C. Interleukin 6 and liver regeneration. Gut 2000; 47: 309-312.

[5] Aravinthan A, Challis B, Shannon N, Hoare M, Heaney J and Alexander GJ. Selective insulin resistance in hepatocyte senescence. Exp Cell Res 2015; 331: 38-45.

[6] Le Marchand-Brustel Y, Tanti JF, Cormont M, Ricort JM, Gremeaux T and Grillo S. From insulin receptor signalling to Glut 4 translocation abnormalities in obesity and insulin resistance. J Recept Signal Transduct Res 1999; 19: 217-228.

[7] Petersen KF and Shulman GI. Etiology of insulin resistance. Am J Med 2006; 119: S10-16.

[8] Ren ZQ, Zhang PB, Zhang XZ, Chen SK, Zhang H, Lv DT, Zhuang BQ, Wen YQ, Hu HH, Ding WC and Zhang C. Duodenal-jejunal exclusion improves insulin resistance in type 2 diabetic rats by upregulating the hepatic insulin signaling pathway. Nutrition 2015; 31: 733-739.

[9] Harrington LE, Hatton RD, Mangan PR, Turner H, Murphy TL, Murphy KM and Weaver CT.
Interleukin 17-producing CD4+ effector T cells develop via a lineage distinct from the T helper type 1 and 2 lineages. Nat Immunol 2005; 6: 1123-1132.

[10] Wu J, Guo J, Cao Q, Wang Y, Chen J, Wang Z and Yuan Z. Autophagy impacts on oxaliplatin-induced hepatocarcinoma apoptosis via the IL-17/IL-17R-JAK2/STAT3 signaling pathway. Oncol Lett 2017; 13: 770-776.

[11] Marshall KM, He S, Zhong Z, Atkinson C and Tomlinson S. Dissecting the complement pathway in hepatic injury and regeneration with a novel protective strategy. J Exp Med 2014; 211: 1793-1805.

[12] Schmidt-Arras D and Rose-John S. IL-6 pathway in the liver: From physiopathology to therapy. J Hepatol 2016; 64: 1403-1415.

[13] Rolla S, Alchera E, Imarisio C, Bardina V, Valente G, Cappello P, Mombello C, Follenzi A, Novelli F and Carini R. The balance between IL-17 and IL-22 produced by liver-infiltrating T-helper cells critically controls NASH development in mice. Clin Sci (Lond) 2016; 130: 193-203.

[14] Shi W, Zhu Q, Gu J, Liu X, Lu L, Qian X, Shen J, Zhang F and Li G. Anti-IL-17 antibody improves hepatic steatosis by suppressing interleukin-17-related fatty acid synthesis and metabolism. Clin Dev Immunol 2013; 2013: 253046.

[15] Tang Y, Bian Z, Zhao L, Liu Y, Liang S, Wang Q, Han X, Peng Y, Chen X, Shen L, Qiu D, Li Z and Ma X. Interleukin-17 exacerbates hepatic steatosis and inflammation in non-alcoholic fatty liver disease. Clin Exp Immunol 2011; 166: 281-290.

[16] S.X. Qiu XLS. Cajanus cajan L. natural medicine with blood sugar reduction and weight reduction function. CN 101422450A 2008;
[17] Machado MV and Cortez-Pinto H. Diet, Microbiota, Obesity, and NAFLD: A Dangerous Quartet. Int J Mol Sci 2016; 17: 481.

[18] Radwan MM, Radwan BM, Nandipati KC, Hunter WJ, 3rd and Agrawal DK. Immunological and molecular basis of nonalcoholic steatohepatitis and nonalcoholic fatty liver disease. Expert Rev Clin Immunol 2013; 9: 727-738.

[19] Tarantino G, Costantini S, Finelli C, Capone F, Guerriero E, La Sala N, Gioia S and Castello G. Carotid intima-media thickness is predicted by combined eotaxin levels and severity of hepatic steatosis at ultrasonography in obese patients with Nonalcoholic Fatty Liver Disease. PLoS One 2014; 9: e105610.

[20] Wang XA, Zhang R, Zhang S, Deng S, Jiang D, Zhong J, Yang L, Wang T, Hong S, Guo S, She ZG, Zhang XD and Li H. Interferon regulatory factor 7 deficiency prevents diet-induced obesity and insulin resistance. Am J Physiol Endocrinol Metab 2013; 305: E485-495.

[21] Matsubara T, Mita A, Minami K, Hosooka T, Kitazawa S, Takahashi K, Tamori Y, Yokoi N, Watanabe M, Matsuo E, Nishimura O and Seino S. PGRN is a key adipokine mediating high fat diet-induced insulin resistance and obesity through IL-6 in adipose tissue. Cell Metab 2012; 15: 38-50.

[22] Nakamura A, Osonoi T and Terauchi Y. Relationship between urinary sodium excretion and pioglitazone-induced edema. J Diabetes Investig 2010; 1: 208-211.

[23] Gilleron J, Bouget G, Ivanov S, Meziat C, Ceppo F, Vergoni B, Djedaini M, Soprani A, Dumas K, Jacquel A, Yvan-Charvet L, Venteclef N, Tanti JF and Cormont M. Rab4b Deficiency in T Cells Promotes Adipose Treg/Th17 Imbalance, Adipose Tissue Dysfunction, and Insulin Resistance. Cell Rep 2018; 25: 3329-3341.e3325.
Erratum: Borderud SP, Li Y, Burkhalter JE, Sheffer CE and Ostroff JS. Electronic cigarette use among patients with cancer: Characteristics of electronic cigarette users and their smoking cessation outcomes. Cancer. doi: 10.1002/cncr.28811. Cancer 2015; 121: 800.

Langrish CL, Chen Y, Blumenschein WM, Mattson J, Basham B, Sedgwick JD, McClanahan T, Kastelein RA and Cua DJ. IL-23 drives a pathogenic T cell population that induces autoimmune inflammation. J Exp Med 2005; 201: 233-240.

Park H, Li Z, Yang XO, Chang SH, Nurieva R, Wang YH, Wang Y, Hood L, Zhu Z, Tian Q and Dong C. A distinct lineage of CD4 T cells regulates tissue inflammation by producing interleukin 17. Nat Immunol 2005; 6: 1133-1141.

Yang L, Anderson DE, Baecher-Allan C, Hastings WD, Bettelli E, Oukka M, Kuchroo VK and Hafler DA. IL-21 and TGF-beta are required for differentiation of human T(H)17 cells. Nature 2008; 454: 350-352.

Manel N, Unutmaz D and Littman DR. The differentiation of human T(H)-17 cells requires transforming growth factor-beta and induction of the nuclear receptor RORgammat. Nat Immunol 2008; 9: 641-649.

Miossec P, Korn T and Kuchroo VK. Interleukin-17 and type 17 helper T cells. N Engl J Med 2009; 361: 888-898.

Gagliani N, Amezcuia Vesely MC, Iseppon A, Brockmann L, Xu H, Palm NW, de Zoete MR, Licona-Limon P, Paiva RS, Ching T, Weaver C, Zi X, Pan X, Fan R, Garmire LX, Cotton MJ, Drier Y, Bernstein B, Geginat J, Stockinger B, Esplugues E, Huber S and Flavell RA. Th17 cells transdifferentiate into regulatory T cells during resolution of inflammation. Nature 2015; 523: 221-225.
[31] Yang J, Sundrud MS, Skepner J and Yamagata T. Targeting Th17 cells in autoimmune diseases. Trends Pharmacol Sci 2014; 35: 493-500.

[32] Fang P, Yu M, Zhang L, Wan D, Shi M, Zhu Y, Bo P and Zhang Z. Baicalin against obesity and insulin resistance through activation of AKT/AS160/GLUT4 pathway. Mol Cell Endocrinol 2017; 448: 77-86.

[33] Su SA, Yang D, Zhu W, Cai Z, Zhang N, Zhao L, Wang JA and Xiang M. Interleukin-17A mediates cardiomyocyte apoptosis through Stat3-iNOS pathway. Biochim Biophys Acta 2016; 1863: 2784-2794.
Figure legends

Figure 1. CAA improved dexamethasone induced decrease of glucose consumption in HepG2 cells with insulin resistance. A, oil red O staining diagram. B, dexamethasone treatment induced decreased glucose consumption in HepG2 cell. C, IC50 detection of HepG2 on CAA. D, glucose consumption in Control group, IR group and IR+CAA group. E, IL-6 concentration in Control group, IR group, IR+CAA group detected by ELISA assay. F, TGF-β concentration in Control group, IR group, IR+CAA group detected by ELISA assay. G, representative band of p-IR, and GLUT1 in Control group, IR group and IR+CAA group. *P < 0.05 versus Control group, †P < 0.05 versus IR group. Data represent as mean ± SD. IR, insulin resistance; IR, insulin resistance; CAA, cajanonic acid A; SD, standard deviation.

Figure 2. CAA mediated hepatocyte production of IL-6 and TGF-β in HepG2 cells with insulin resistance. A, glucose consumption in HepG2. B, representative band of p-IR, and GLUT1 IR group, IR+CAA group and IR+CAA+IL-6 AB group. C, IL-6 concentration in IR group, IR+CAA group and IR+CAA+IL-6 Ab group were detected by ELISA assay. D, TGF-β concentration in in IR group, IR+CAA group and IR+CAA+IL-6 Ab group detected by ELISA assay. *P < 0.05 versus IR group, †P < 0.05 versus IR+CAA group. Data represent as mean ± SD. IR, insulin resistance; CAA, cajanonic acid A; SD, standard deviation.

Figure 3. Treg/Th17 ratio and cells apoptosis was reversed by IL-6 Ab in insulin-resistant HepG2 cell. A, B and D, Ratio of Th17 and Treg cells was investigated. C, immunofluorescent
staining of IL-17RC in IR group, IR+CAA group and IR+CAA+IL-6 AB group. **E**, IL-17 concentration in IR group, IR+CAA group and IR+CAA+IL-6 AB group detected by ELISA assay. **F**, percentage of apoptotic HepG2 cells in IR group, IR+CAA group and IR+CAA+IL-6 AB group by flow cytometry assay. *P < 0·05 versus IR group, *P < 0·05 versus IR+CAA group. IR, insulin resistance; CAA, cajanonic acid A.

**Figure 4. Overexpression of IL17R counteracted the effect of IL-6 AB on CAA-treated HepG2 cells.** **A**, glucose consumption in HepG2. **B**, representative band of IL-17R, p-IR, and GLUT1 in IR group, IR+CAA group, IR+CAA+IL-6 AB group, and IR+CAA+IL-6 AB+IL17R group detected by Western blotting detection. **C and D**, Th17/Treg ratio cells was investigated by flow cytometry assay. **E**, IL-17 concentration in the IR group, IR+CAA group, IR+CAA+IL-6 AB group, and IR+CAA+IL-6 AB+IL17R group detected by ELISA assay. *P < 0·05 versus IR+CAA+IL-6 AB group. Data are represented as the mean ± SD. IR, insulin resistance; CAA, cajanonic acid A; SD, standard deviation.
A

Radioactivity (nmol 2-DOG/mg protein/min)

IR
IR + CAA
IR + CAA + IL-6 Ab

B

IR
IR + CAA
IR + CAA + IL-6 Ab

P-IR
GLUT1
GAPDH

C

IL-6 pg/mL

IR
IR + CAA
IR + CAA + IL-6 Ab

D

TGF-β pg/mL

IR
IR + CAA
IR + CAA + IL-6 Ab
