Over-nutrition contributes to insulin resistance, which is a clinically important manifestation of human type 2 diabetes mellitus (T2DM). Furthermore, it has clearly demonstrated that chronic low-grade inflammation is a key factor in the initiation, propagation, and deterioration of metabolic diseases [1, 2]. In traditional views, accumulation of inflamed macrophages within adipose tissue is the major source of cytokines in diet-induced obesity [3-5]. However, both liver and adipose tissue possess resident macrophages to undergo local activation facing various stimuli and express distinct chemokines and cytokines. Furthermore, over-nutrition is also associated with lipid accumulation in tissues like liver and skeletal muscle [6-8], which has made it hard to pinpoint the primary sites responsible for initiating insulin resistance. Additionally, liver fat accumulation and visceral obesity are closely correlated, and both are highly related with the development and severity of insulin resistance [9].

Early in the 1990s, several investigations were made which first linked insulin resistance with macrophages [10]. Though other lymphocyte subsets like T-cells and NK cells may matter [11], reports have demonstrated the critical role of macrophages in initiating the chronic tissue inflammatory responses, which can ultimately cause insulin resistance. One of the major pro-inflammatory pathways within macrophages involves IKKβ/NF-κB system, and the other vital intracellu-
lar proinflammatory pathway is JNK/AP1 system [1]. Compared with the other organs, liver has the largest resident population of macrophages, i.e. Kupffer cells [12]. Although Kupffer cells have been studied less extensively than adipose tissue macrophages in the context of insulin resistance, they are not impossible to contribute to the production of inflammatory mediators that promote insulin resistance.

Meanwhile, growing evidence indicates a complex reciprocal relationship between autophagy pathway/proteins and inflammation [13-16]. Autophagy is a cellular degradation pathway which normally occurs at low basal levels in virtually all cells to perform homeostatic functions such as protein and organelle turnover [17]. However, excessive autophagy may lead to autophagic cell death, such as reduced β cell mass. Thus, it is still unclear as to whether autophagy plays a protective or harmful role in nutrition excess and its interaction with low-grade inflammation status. Therefore it might be beneficial to investigate the potential therapeutic targets of autophagic processes to mitigate inflammatory diseases including T2DM.

Current studies tend to answer whether Kupffer cells are primary sites of action in systemic inflammation in high-fat feeding induced insulin resistance, and try to bring to light the possible correlation between inflammation and liver autophagy. Gadolinium chloride (GdCl3) is often utilized as a tool for studying the role of Kupffer cells, since it can significantly and drastically decrease macrophage number and activity in the liver tissue [18]. Therefore, we have studied the effect of specific inhibition of Kupffer cells by GdCl3 on insulin sensitivity, systemic inflammation and liver autophagy.

Materials and Methods

Animal experiments

Six-week-old male C57BL/6J mice were purchased from Experimental Animal Center of Hubei Province and individually housed in a specific pathogen-free environment with a 12:12-h light-dark cycle (lights on at 07:00) at the Center for Laboratory Animal, Tongji Medical College, Huazhong University of Science and Technology. After a week for acclimatization, the mice were randomly divided into three groups: normal control group (NC), high-fat diet control group (HFC), and high-fat diet with intervention group (HFI). Mice were fed a normal chow diet (10% fat, 70% carbohydrate, and 20% protein) or high-fat diet (44.8% fat, 36.2% carbohydrate, and 19% protein, D12451) for 12 weeks. During the dietary treatment, mice received an i.p. injection of either NaCl 0.9% (5mL/Kg) or GdCl3 (20 mg/kg) twice a week. Animals had ad libitum access to water, and food was only withdrawn when required for experimental procedures. Orbital blood was collected after grouping and in the fourth, eighth and twelfth week. At the end of feeding/treatment regimen, IPGTT and ITT were performed. Finally, mice were fasted for 6 hours before sacrifice for collection of blood and tissue samples for further analysis.

Ethics statement

All experiments were performed according to the National Institutes of Health Guidelines for the care and use of animals and were approved by the Animal Care and Use Center of Tongji Medical College, Huazhong University of Science and Technology (Permit Number: SCXK2008-0004).

Metabolic parameters

Body weights were measured weekly and food intakes daily. Blood and tissue samples were subjected to lipid profiling using metabolic assay kits (Sigma, St. Louis, MO). The levels of serum insulin were quantified using specific ELISA kits (Alpco Diagnostics, Windham, NH). All testing processes were carried out according to the manufacturers’ recommendations. Additionally, fasting glucose and insulin were used to calculate HOMA-IR, an indicator of systemic insulin resistance, using the following equation. HOMA-IR = fasting glucose (mmol/L) × fasting insulin (mU/L)/22.5.

Glucose and insulin tolerance tests

Mice were fasted for overnight or 6 hours and received an intraperitoneal injection of glucose (2.0g/kg body weight) or insulin (1.0 U/kg body weight) (Novolin R; Novo Nordisk, Bagsværden, Denmark). For glucose tolerance tests, blood was collected from the tail to detect blood glucose before and at 30, 60 and 120 min after the injection. Similarly, for insulin tolerance tests, glucose levels were measured before and at 15, 30, 60 and 90 min after the injection. Blood glucose was measured using an automatic glucometer (One Touch, Byer). The area under the curve (AUC) was calculated as described previously [19].
2-deoxyglucose uptake in muscle tissue

Glucose uptake experiments in isolated gastrocnemius muscles were completed as described [20]. In brief, 2-DG (2 mmol/kg body weight) and insulin (1.5 units/kg body weight) were simultaneously injected into mice abdominal cavity 30 min before anesthesia and cervical dislocation. Gastrocnemius muscles were harvested and snap-frozen in liquid nitrogen. Frozen muscle (50–100 mg) was homogenized, followed by acid, alkali and heat treatment. Ultimately, the supernatant was collected to react with each assay solution. Radioactivity was measured by multifunctional microplate reader (ELX800; Bio-Tek, USA). Reagents needed were purchased from Sigma–Aldrich Co. (St. Louis, MO, USA).

Measurement of inflammation

Serum and tissue TNF-α were measured using the mouse TNF-α ELISA kit (eBioscience, San Diego, CA) following manufacturer’s instructions, so was NF-κB activity using the mouse P65 ELISA kit (R&D Systems, Minneapolis, MN, USA). All samples were run in duplicate and analyzed on the same day to minimize day-to-day variation.

Histological evaluation

Sections of liver tissue specimens, fixed in 10% formalin and embedded in paraffin wax, were stained with H&E for histological evaluation. All the images were visualized and captured with a Motic microscope BA310 (Ted Pella, Inc., Los Angeles, CA) equipped with a digital camera.

Western blotting

Tissues were homogenized in RIPA lysis buffer containing protease inhibitors and phosphatase inhibitors (Pl-78420, Thermo Scientific), followed by solubilization and centrifugation at 12,000 r.p.m. for 15 min at 4°C. Supernatants were stored at 80°C until assays were performed. Tissue protein concentrations were determined by bicinchoninic acid (BCA) Kit and fractionated on a SDS-PAGE gel. Proteins were transferred to a polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, MA, USA) by electroblotting, and membranes were blocked in TBS containing 0.05% Tween 20 (TBST) and 5% nonfat milk, then incubated with the GAPDH monoclonal mouse mAb (1:3000, #A01020), AMPKα rabbit mAb (1:1000, #2603), aporphospho-AMPKα (Thr172) rabbit mAb (1:1000, #2535), anti-light chain (LC) 3B rabbit mAb (1: 1,000, #3868) in 5% BSA overnight at 4 °C, followed by incubating with the appropriate HRP-conjugated secondary antibodies (#7074 and #A21010) in blocking solution for 1 h at room temperature, and then the bands were treated with enhanced chemiluminescence and images were captured by BioSpectrum Imaging System (BioSpectrum, USA). Antibodies were obtained from Cell Signaling Tech (Beverly, MA, USA) except GAPDH monoclonal mouse mAb (Abbkine, USA), and the reagents without source explanation above were obtained from Beyotime Institute of Biotechnology, Shanghai, China.

RNA isolation, reverse transcription, and quantitative RT-PCR

The mRNA was extracted from the liver tissues using RNA simple Total RNA Kit (Tiangen biotech (beijing) Co. LTD). cDNA synthesis by reverse transcription, and quantitative real-time PCR were performed as described previously [21]. The expression of CD68 and F4/80 genes were determined by real-time PCR using SYBR Premix Ex Taq (TaKaRa Bio, Inc., Japan) and a LightCycler®-PCR machine (Roche Diagnostics, Mannheim, Germany). Target gene expression levels were calculated after normalization to the standard housekeeping gene GAPDH, using the 2^−ΔΔCT method as described previously [22]. The PCR primer sets are listed below.

CD68, forward: 5’CTTCGGGCCATGTTTCTCTT3’;
reverse: 5’ATTGTCGTCTGCGGGTGAT3’.

F4/80, forward: 5’GAGATTGTGGAAGCATCCGAGAC’;
reverse: 5’GATGACTGTACCCACATGGCTGA3’.

GAPDH, forward: 5’GGTGAAGGTCGGTGTGAACG3’;
reverse: 5’CTCGCTCCTGGAAGATGGTG3’.

Statistical methods

Numeric data were presented as mean ± SE (Standard Error). Statistical analysis were performed using One-way ANOVA followed by least significant difference _t post hoc test for multiple comparisons with SPSS 20.0 statistical software program (SPSS Inc., IL, USA). Differences were considered significant at the P value <0.05.

Results

Kupffer cells are depleted by repeated GdCl3 injections

CD68 and F4/80 are critical immunological markers
of mouse macrophages. In liver tissues, quantitative RT-PCR showed there was a significantly increase in the mRNA expressions of CD68 and F4/80, representing hepatic Kupffer cells activation, after high-fat feeding compared to controls (1.8 folds, \(p<0.01\), and 2.7 folds, \(P<0.01\) respectively, Fig. 1). Meanwhile, mRNA levels of CD68 and F4/80 were pronounced down-regulated in HFI versus HFC, which fell approximately 70% and 85% respectively (both \(P<0.01\)). Importantly, though the alteration of CD68 mRNA did not reach statistical significance, there were also macrophages activated in adipose tissues after HFD feeding (Fig. 1), which were not much affected by GdCl3 treatment. These data clearly suggest it was Kupffer cells other than ATM that had been predominantly reduced via GdCl3 injections.

### Kupffer cells depletion modifies adiposity and hepatic steatosis induced by chronic HFD feeding

High-fat diet feeding significantly increased body weight gain (11.3±0.5 and 0.9±0.2 g for HFC and NC mice, respectively; \(p<0.01\), Table 1). Furthermore, blood lipid levels like cholesterol, triglyceride, and free fatty acids were pronounced increased; liver triglyceride as well as liver, skeletal muscle, epididymal and subcutaneous fatty acids were significantly higher in HFC as compared to NC group (Table 1). Meantime, H&E staining pictures revealed more and larger volume of lipid droplets in liver tissues of HFC mice (Fig. 2). Interestingly, upon HF dietary treatment, chronic Kupffer cells inhibition decreased body weight gain by about 30% as compared to HFC; this effect was accompanied by alleviated dyslipidemia and hepatic steatosis in different degrees. In addition, such alterations had little to do with daily food intakes which were similar between the HFC and HFI group mice (data were not shown).

### Kupffer cells depletion ameliorates insulin resistance

Hyperinsulinemic clamp or minimal model are Gold-standard methods to assess insulin resistance, but the homeostatic model assessment of insulin resistance (HOMA-IR), a surrogate indices first developed by Matthews et al. [23] in 1985 has attractive simplicity thus widely used [24]. The method assesses IR (HOMA-IR index) from basal glucose and insulin concentrations. In order to learn the dynamic change in insulin sensitivity, we measured fasting plasma glucose and insulin in orbital blood before the experiment began and thereafter every other four weeks. HFC showed advancing serum insulin concentration and increasing HOMA-IR index as compared to NC group, both up to statistical significance at 8 week (97.22 versus 56.27 pmol/L, and 1.9 versus 0.9, both \(P<0.01\), Fig. 3A and 3B) and the difference were much more pronounced at 12 week (109.10 versus 52.81 pmol/L, and 2.3 versus 0.7, \(P<0.01\) both). Importantly, chronic administration of GdCl3 significantly decreased hyperinsulinemia and HOMA-IR index in mice fed a HFD since 8th week and displayed an even more pronounced improvement at 12th week (\(P<0.05\) and 0.01, respectively).

To further verify the effect of Kupffer cells on glu-
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reversed the above decline induced by HFD with a rebound of 40.2% \((P<0.05)\), suggesting that Kupffer cells exerted a critical impact on skeletal muscle insulin resistance, a classic but silent feature of T2DM.

Kupffer cells depletion leads to alteration of inflammation induced by HFD feeding

TNF-\(\alpha\) is a pluripotent cytokine not exclusively produced from macrophages. TNF-\(\alpha\) has been proposed as a mediator in obesity induced insulin resistance because it can directly impair insulin signaling in both cultured cells and experimental animals [25]. Our data showed a varying degrees increased TNF-\(\alpha\) content in serum \((p<0.01, \text{Fig. 4A})\) and liver, gastrocnemius muscle and adipose tissue (Fig. 4B) in HFC versus NC group, from a minimum addition of 100% in liver to a maximum of 11 times in serum, all up to statistical difference. The absolute concentration of fat tissue was much higher than that in liver and skeletal muscle, which was consistent with the accepted view that fat tissue is a significant source of endogenous TNF-\(\alpha\) production. These abnormal expressions of TNF-\(\alpha\) in circulation and tissues due to high-fat feeding were significantly ameliorated except subcutaneous fat by Kupffer cells depletion.
Fig. 3  Kupffer cells depletion ameliorates diet-induced insulin resistance.
Dynamic changes of serum insulin level (A) and HOMA-IR index (B) throughout the study. IPGTT(C) and ITT (E) curves as well as the corresponding AUC (D and F) of the three group mice at 12th week. 2-DG uptake levels (G) in skeletal muscle glucose uptake experiment. N=5-7 mice per group. Error bars reflect mean ± S.E. *P <0.05, **P<0.01 versus NC.  #P<0.05, ##P<0.01 versus HFC.
Kupffer cells and insulin resistance

Kupffer cells depletion improves liver autophagy in HFD mice

Studies indicate that autophagy can exert a critical impact on the regulation of inflammation [13, 27]. We analyzed the autophagic proteins LC3-II, an ubiquitin-like protein that plays an important role in the formation of autophagosomes by western blot analysis. In confirmation with published data, our study showed a significant decrease of about 70% in LC3-II in HFC mice compared with controls (\(P<0.01\), Fig. 5). Interestingly, Kupffer cells depletion partially offset the alteration caused by HFD. These results indicate that autophagy was significantly weakened in high-fat feeding while markedly enhanced via Kupffer cells depletion, indicating that Kupffer cells mediated inflammation were critical in regulation of autophagy.

The energy/fuel sensor 5'AMP-activated protein kinase (AMPK) is regarded as a regulator of cellular energy balance owning to its multiple roles in glucose, lipid, and protein metabolism [28]. Then we

Orlikova et al. [26] held a prospective that TNF-\(\alpha\) can induce NF-\(\kappa\)B activation. And NF-\(\kappa\)B is known to exert transcriptional regulation of TNF-\(\alpha\) and other pro-inflammatory cytokines genes. On account of this knowledge, we went further to investigate the alteration of NF-\(\kappa\)B activation. The classic form of NF-\(\kappa\)B is the heterodimer of p50 and p65 subunits, which contains the transcriptional activation domain and sequestered in the cytoplasm as an inactive complex by the inhibitory proteins I\(\kappa\)Bs. P65 level was detected in insulin sensitive tissues. In line with the results of inflammatory cytokines, our study demonstrated that NF-\(\kappa\)B activity elevated in liver, epididymal fat, subcutaneous fat and skeletal muscle tissue, up to 1.7, 4.7, 7.3 and 6.0 -fold respectively (Fig. 4C). GdCl3 treatment attenuated NF-\(\kappa\)B activation by 36%-81% compared with HFC.

These data supports our hypothesis that the role of Kupffer cells is pivotal for the regulating of inflammation in relation to insulin resistance.

Fig. 4 Kupffer cells depletion leads to attenuated inflammation induced by HFD feeding. Serum and tissues TNF-\(\alpha\)concentration (A and B) as well as tissues NF-\(\kappa\)B activity (C) measured by ELISA. N=5-7 mice per group. Error bars reflect mean \(\pm\) S.E. *\(P<0.05\), **\(P<0.01\) versus NC. ¤\(P<0.05\), ¤¤\(P<0.01\) versus HFC.
Growing evidence demonstrates that inflammatory signaling pathways mediate nutrient excess induced insulin resistance in both cultured cells and in experimental animals [29]. In addition, adipose tissue-associated macrophage infiltration and activation have been confirmed on conditions of obesity-induced insulin resistance. As shown in pictures (Fig. 6), phosphorylation of AMPK decreased by 70% in gastrocnemius tissues after long time feeding on high fat diet. Notably, it was more than 5 times elevated in HFI compared to HFC, in accordance with the change of autophagy. p-AMPK alteration of liver showed almost the same tendency as skeletal muscle though much tempered.

**Discussion**

Growing evidence demonstrates that inflammatory signaling pathways mediate nutrient excess induced insulin resistance in both cultured cells and in experimental animals [29]. In addition, adipose tissue-associated macrophage infiltration and activation have been confirmed on conditions of obesity-induced insulin resistance.
Importantly, our data showed that GdCl₃ predominantly from both adipose tissue and macrophages [34]. The systemic inflammatory response primarily originates in inflammatory cytokines [33]. It is noteworthy that initiation of inflammation and primary source of proinflammatory pathways being widely studied, the NF-κB pathway [37]. Based on published data [9, 38], the former seems to be main accountable pathway downstream Kupffer cells involving dietary insulin resistance. We investigated NF-κB and connected cytokine TNF-α level in high-fat feeding and their alteration by Kupffer cells depletion. As expected, both parameters significantly increased under HFD which were greatly reversed by repeated GdCl₃ intraperitoneally injections. These results prompt a hypothesis that Kupffer cells inflammatory response may arise from NF-κB–dependent pathway, leading to elevated circulating TNF-α and other cytokines that can contribute to insulin resistance in peripheral tissues (liver, adipose tissue and muscle). The inflammatory response could be further magnified by hepatic steatosis and increased macrophage infiltration in adipose tissue and result in systemic insulin resistance. Since skeletal muscle accounts for the vast majority of glucose disposal, therefore, the muscle glucose uptake was decreased for suffering insulin resistance. Nonetheless, as numerous inflammatory cytokines are involved in the chronic inflammation of obesity, the possibility can’t be ruled out that other proinflammatory pathways and cytokines contribute. Moreover, Kupffer cells are the largest fixed macrophage population in the body and, except for fulfilling a series of other immunologic functions, is the indispensable primary innate immune barrier against exposure to foreign stimulators or antigens from the daily diet and intestinal tract [39]. Obviously it is not a wise option to conquer obesity-induced insulin resistance via simply depleting Kupffer cells. Therefore, future studies are warranted to investigate the detailed mechanisms of Kupffer cells initiated proinflammatory response to fix a specific downstream mediator as a novel potential target for the treatment of obesity-associated insulin resistance and T2DM.

On the other hand, the signaling pathways that regulate inflammatory processes now apparently have a role in the regulation of autophagy and vice versa. Moreover, Lake Li et al. [40] said resveratrol prevents NAFLD-caused hepatic injury partially via regulating autophagic and IκBα-NF-κB pathways. Very recently, there are three articles almost simultaneously published on J Clin Invest, which provides the first line of evidence establishing a protective role of autophagy against h-IAPP–induced toxicity in β cells, implicating the autophagy pathway as key therapeutic targets for treatment and prevention of T2DM [41-43]. To date, the findings in these fields have led to a view...
that autophagy defects can impair metabolic functions to promote T2DM and related diseases. But the overactive autophagy was associated with lots of cytosolic lipid droplets, a subset with colocalization of perilipin and the autophagy protein LC3/atg8, which can cause excessive fatty acid release also leading to metabolic disorders [44]. Of interest, our data show suppressed liver autophagy in HFD mice and a completely opposite alteration by Kupffer cells depletion. All in all, we can safely draw a conclusion that chronic exposure to high energy and nutrient intake inhibits liver autophagy, and this can further promote Kupffer cells mediated inflammation response via somewhat mechanisms such as dampened ER stress [44, 45], where future studies are necessary to bring it to light.

In addition, published literatures prove that AMPK, as a principal energy-preserving intracellular enzyme, can induce autophagy through various mechanisms such as AMPK/ULK1 pathway [46], AMPK/mTOR pathway [47], and AMPK/SIRT1 pathway [48]. Our study demonstrates AMPK induces autophagy in over-nutrition conditions, maybe through signaling pathways like phosphorylation of its downstream target Raptor and consequent inhibition of mTOR [28].

In conclusion, our study revealed the beneficial and therapeutic effects of Kupffer cells mediated inflammation on insulin resistance pathogenesis in a HFD murine model. Kupffer cells depletion after-treatment effectively alleviated most typical features of insulin resistance. The therapeutic functions of Kupffer cells reduction were partially mediated by autophagic and NF-κB pathways. Although our findings add crucial information to deeper understanding of the network of inflammation, autophagy and insulin resistance, the specific molecular mechanisms remain to be extended and identified.

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Disclosure

The authors declare that there is no conflict of interest.

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