

## ORIGINAL ARTICLE

Large adipocytes function as antigen-presenting cells to activate CD4<sup>+</sup> T cells via upregulating MHCII in obesityL Xiao<sup>1,3</sup>, X Yang<sup>2,3</sup>, Y Lin<sup>2</sup>, S Li<sup>1</sup>, J Jiang<sup>1</sup>, S Qian<sup>1</sup>, Q Tang<sup>1</sup>, R He<sup>2</sup> and X Li<sup>1</sup>

**BACKGROUND/OBJECTIVES:** Although obesity is associated with low-grade inflammation and metabolic disorders, clinical studies suggested some obese people were metabolically healthy with smaller adipocyte size compared with metabolically abnormal obese (MAO). This indicated adipocyte size may be an important predictor underlay the distinction between MAO and metabolically healthy obese. As recent study has shown that adipocytes expressed class II major histocompatibility complex (MHCII), which functioned as APCs during obesity. However, the relationship between adipocyte hypertrophy and MHCII expression was not involved. Here we hypothesize that hypertrophic adipocytes could be associated with upregulating MHCII to influence adipose tissue metabolism.

**METHODS:** Adipocytes were sorted by fluorescence-activated cell sorting (FACS) according to the cell size from MAO mice. The activation of MHCII, T cells and related signaling molecules were examined by FACS, ELISA and western blotting. 3T3-L1 cell line and primary adipocytes were used to examine the effect of free fatty acids (FFA) on adipocytes enlargement and MHCII expression.

**RESULTS:** MAO mice had a significant increase in adipocytes size and FFA concentration. The large adipocytes from both obese and non-obese mice expressed higher levels of MHCII than small adipocytes. Importantly, large adipocytes from obese mice stimulated CD4<sup>+</sup> T cells to secrete more interferon (IFN)- $\gamma$ . Furthermore, the activation of the JNK-STAT1 pathway was involved in upregulation of MHCII in large adipocytes. *In vitro* FFA treatment promoted adipocyte hypertrophy and expression of MHCII-associated genes.

**CONCLUSIONS:** This study demonstrates that large adipocytes highly express MHCII and function as APC to stimulate IFN- $\gamma$ -expressing CD4<sup>+</sup> T cells, in which FFA may have important roles before IFN- $\gamma$  elevated. These findings suggest that adipocyte hypertrophy, rather than overall obesity, is the major contributor to adipose tissue inflammation and insulin resistance.

*International Journal of Obesity* (2016) 40, 112–120; doi:10.1038/ijo.2015.145

## INTRODUCTION

The prevalence of obesity has become a worldwide public health problem as it increases the risk of developing metabolic disorders such as type 2 diabetes (T2D) and cardiovascular disease.<sup>1,2</sup> Insulin resistance and elevated intrahepatic triglyceride content are the core features of these disorders.<sup>3,4</sup> However, more studies have demonstrated that obesity did not always translate into increased risk for these comorbidities.<sup>4–6</sup> Approximately 30% of obese individuals were insulin sensitive similar to healthy lean individuals despite having a higher level of body fat.<sup>5</sup> These individuals are often referred as metabolically healthy obese, whereas obese individuals with metabolic disorders are referred as metabolically abnormal obese.

Increased adipose tissue mass is a characteristic feature of obesity and is caused by an increase in the number (hyperplasia) and/or size (hypertrophy) of its constituent adipocytes.<sup>7,8</sup> According to cross-sectional studies, metabolically healthy obese individuals had smaller-sized adipocytes than metabolically abnormal obese patients, suggesting that adipocytes hypertrophy was associated with the development of metabolic disorders.<sup>5,6</sup> Besides, it has been proposed that enlarged subcutaneous abdominal adipocytes size, but not obesity itself, was a significant predictor for the future development of T2D.<sup>9</sup> Furthermore, cumulative studies indicated that enlarged adipocytes expressed high levels of pro-inflammatory factors and low levels of anti-inflammatory

factors.<sup>10–12</sup> The increased secretion of pro-inflammatory factors is a main contributor to the initiation of chronic low-grade inflammation in adipose tissue with obesity. However, other studies have demonstrated the infiltration of immune cells, which secreted pro-inflammatory factors, also played an important role in the inflammation process. With the discovery of infiltrated macrophages, which increased from 10% to more than 50% of total cells within adipose tissue during the progression of obesity, more researchers paid great attention to immune cells (such as dendritic cells (DCs), macrophages, T cells, eosinophils and B cells) in adipose tissue.<sup>13</sup> Recent studies have demonstrated that T cells also increased in adipose tissue during a high-fat diet (HFD) challenge and interacted with both adipocytes and macrophages to modulate adipose metabolism.<sup>14–16</sup> Among various T-cell subsets, polarization of pro-inflammatory type 1 helper T-cell (Th1) cells promoted obesity-induced inflammation by presenting antigens via class II major histocompatibility complex (MHCII) on antigen-presenting cell (APC) and secreting cytokines such as interferon (IFN)- $\gamma$ .<sup>16–19</sup> With the discovery of increased expression of MHCII in obese compared with that in lean adipose tissues,<sup>20</sup> a recent study showed that the MHCII-restricted signals from macrophages played a central role in regulating maturation of CD4<sup>+</sup> T cells and obesity-induced inflammation in visceral fat.<sup>21</sup> However, another study has demonstrated that adipocytes, rather

<sup>1</sup>Key Laboratory of Metabolic Molecular Medicine, Ministry of Education, Department of Biochemistry and Molecular Biology, Fudan University Basic Medical School, Shanghai, PR China and <sup>2</sup>Key Laboratory of Medical Molecular Virology of Ministries of Education and Health, Department of Immunology, Fudan University Basic Medical School, Shanghai, PR China. Correspondence: R HE, Key Laboratory of Medical Molecular Virology of Ministries of Education and Health, Department of Immunology, Fudan University Basic Medical School, 138 Yi Xue Yuan Road, PO Box 238, Shanghai 200032, PR China or Dr X Li, Key Laboratory of Metabolic Molecular Medicine, Ministry of Education, Department of Biochemistry and Molecular Biology, Fudan University Basic Medical School, 138 Yi Xue Yuan Road, PO Box 238, Shanghai 200032, PR China.  
E-mail: ruihe@fudan.edu.cn or lixi@shmu.edu.cn

<sup>3</sup>These authors contributed equally to this work.

Received 10 March 2015; revised 28 June 2015; accepted 26 July 2015; accepted article preview online 7 August 2015; advance online publication, 22 September 2015

than APC, expressed MHCII and directly activated T cells during obesity, which aggravated the process of adipose inflammation.<sup>22</sup>

MHCII molecules, which are mainly expressed on the surface of APC, such as DCs, macrophages and B cells, present exogenous antigens to CD4<sup>+</sup> T lymphocytes to activate adaptive immunity in conjunction with co-stimulatory signals.<sup>23</sup> However, certain non-APC cells, such as fibroblasts, astrocytes, endothelial cells and epithelial cells, also express MHCII during infection, inflammation or trauma. Although study has demonstrated that adipocytes expressed MHCII in obese individuals,<sup>22</sup> it is very necessary to identify whether all adipocytes or just hypertrophic adipocytes in obesity highly express MHCII and the underlying mechanism of MHCII expression to activate T cells during the early stage of obesity. As studies have reported that adipocyte hypertrophy was related to adipose tissue dysfunction and adipocytes size was positively correlated to insulin resistance during obesity,<sup>24,25</sup> the presence of MHCII on adipocytes in obese individuals may be due to adipocytes hypertrophy rather than overall obesity.

In this study, we aim to demonstrate that large, rather than all, adipocytes highly express MHCII and function as APC to stimulate IFN- $\gamma$ -expressing CD4<sup>+</sup> T cells and explore the signaling pathway involved. Furthermore, studies *in vitro* confirm free fatty acids (FFA) promotes adipocytes hypertrophy and expression of MHCII-associated genes.

## MATERIALS AND METHODS

### Mouse studies

Four-week-old C57BL/6J male mice were purchased from Chinese Academy of Science (Shanghai) and kept in a specific pathogen-free environment. Mice were fed with either normal diet (ND; 12% kcal in fat) or HFD (51% kcal in fat, beginning at age 8 weeks). All studies were approved by the Animal Care and Use Committee of the Fudan University Shanghai Medical College. Mice were randomly assigned to experimental groups and blinding was not possible.

### Cell culture

3T3-L1 preadipocytes were induced to differentiate as previously described.<sup>26</sup> After 7 days of induction, mature adipocytes were trypsinized, plated at 50% density and cultured in Dulbecco's modified Eagle's medium containing 10% (vol/vol) fetal bovine serum (Gibco, Grand Island, NY, USA), then treated with palmitic acids or bovine serum albumin (both from Sigma-Aldrich, St Louis, MO, USA) until day 20. Co-culture of adipocytes and T cells was performed as previous described.<sup>22</sup> C57BL/6 J mice were intraperitoneally injected with 1 mg Imject Alum (Thermo Fisher Scientific, Rockford, IL, USA) and 50  $\mu$ g ovalbumin (OVA) (Sigma-Aldrich) once a week for 14 days to induce OVA-specific T cells. Splenic T cells were isolated by using CD4<sup>+</sup> T-Cell isolation Kits (Miltenyi Biotec, Bergisch, Germany). CD45-depleted adipocytes ( $0.2 \times 10^6$  cells per well) and T cells ( $2 \times 10^6$  cells per well) isolated from OVA-immunized C57BL/6 J mice were incubated with 500  $\mu$ g ml<sup>-1</sup> OVA for 72 h in 12-well plates, as single cultures or co-cultures in direct contacted with T cells. Cultured cells were continuously shaken gently to ensure mixing of T cells and floating adipocytes.

### ELISA

IFN- $\gamma$  concentrations in supernatants of co-cultured adipocytes and T cells or in adipose tissue were analyzed by Mouse Ready-SET-Go! ELISA kits from eBioscience (San Diego, CA, USA). The supernatants of cultured adipocytes from ND and HFD C57BL/6J mice were collected after 24 h and the concentrations of interleukin (IL)-6 and tumor necrosis factor (TNF)- $\alpha$  were measured by using Mouse Ready-SET-Go! ELISA kits from eBioscience. For the detection of free fatty acid, adipocytes from ND and HFD C57BL/6J mice were harvested and homogenized in chloroform solution and the concentrations, which were relative to the DNA content, were measured by using free fatty acid quantitation kit (Sigma-Aldrich).

### Glucose tolerance test and insulin tolerance test

For the glucose tolerance test, mice were injected intraperitoneal (i.p.) with D-glucose (2 mg g<sup>-1</sup> body weight) after an overnight fast, and tail blood glucose levels were monitored at 0, 30, 60, 90 and 120 min after injection. For the insulin tolerance test, mice fed *ad libitum* were injected i.p. with

human insulin (Eli Lilly, Indianapolis, IN, USA; 0.75 mU g<sup>-1</sup> body weight) around 1400 hours, and tail blood glucose levels were monitored at 0, 15, 30, 45 and 60 min after injection.

### Hematoxylin and eosin (H&E) staining and cell size quantitation

Standard H&E staining was performed on 5- $\mu$ m paraffin sections of gonadal adipose tissue. Cell diameter of H&E-stained sections was measured in the 3–5 fields of 6–8 individual samples in each group using Image J (NIH, Bethesda, MD, USA). For the cell size quantitation by flow cytometry, polystyrene particles (Spherotech, Chicago, IL, USA) were used to define the scale of flow cytometry.

### Isolation of adipocytes

Gonadal adipose tissue was cut into small pieces and treated with enzymatic digestion (collagenase VIII; Sigma-Aldrich). The digested tissue was filtered through a 100- $\mu$ m mesh filter to remove debris and was centrifuged. The adipocytes floated above the supernatant after centrifugation, and then incubated with fluorochrome-labeled mouse anti-CD45 (from eBioscience 30-F11) and CellTracker 5-chloromethylfluorescein diacetate (from Life Technologies, Carlsbad, CA, USA) to confirm its purity and vitality.

### Flow cytometry analysis

After isolation of adipocytes, single-cell suspensions were treated with anti-CD16/32 for 15 min at room temperature to block non-specific binding, followed by surface staining with the following antibodies for 30 min at room temperature: fluorochrome-labeled mouse anti-CD45 (30-F11), MHCII (M5/114.15.2), CD86 (GL1) or isotype control antibody (all from eBioscience). Intracellular staining was performed with the following antibodies: fluorochrome-labeled mouse anti-c-Jun N-terminal kinase (JNK; pT-183/pY185) and signal transducer and activator of transcription 1 (STAT1; pY-701; from BD Biosciences, Franklin Lakes, NJ, USA). The process of staining was performed according to the manual protocol from eBioscience or BD Biosciences. The cells were analyzed on a CyAn (from Beckman Coulter, Fullerton, CA, USA), and the data analysis was performed by using FlowJo 7.6 version. Adipocytes were sorted by fluorescence-activated cell sorting according to the cell size and then collected for co-culture or RNA extract.

### RNA isolation and quantitative real time-PCR (qRT-PCR)

Total RNAs were extracted with RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany), and the mRNA was reverse transcribed by using a Revert Aid first-strand cDNA synthesis kit (Fermentas, Waltham, MA, USA). Then, the cDNAs were analyzed by using the Power SYBR green PCR kit with the ABI Prism 7500 qPCR machine (Applied Biosystems, Foster City, CA, USA). All qRT-PCR data were normalized to the 18 s results. Primer sequences for qRT-PCR were from PrimerBank (<http://pga.mgh.harvard.edu/primerbank/>).

### Western blotting

Western blot analysis in adipocytes was performed as previously described.<sup>26</sup> The membranes were incubated with primary antibodies (MHCII and ClITA from Abcam (Cambridge, UK); Phospho-SAPK/JNK (Thr183/Tyr185), SAPK/JNK and Phospho-Stat1 (Tyr701) from Cell Signaling Technology (Beverly, MA, USA);  $\beta$ -actin from Sigma-Aldrich, Stat1 and HSP90 from Santa Cruz Biotechnology (Santa Cruz, CA, USA), 422/aP2 from the Department of Biological Chemistry of the Johns Hopkins University School of Medicine) overnight.

### Statistical analysis

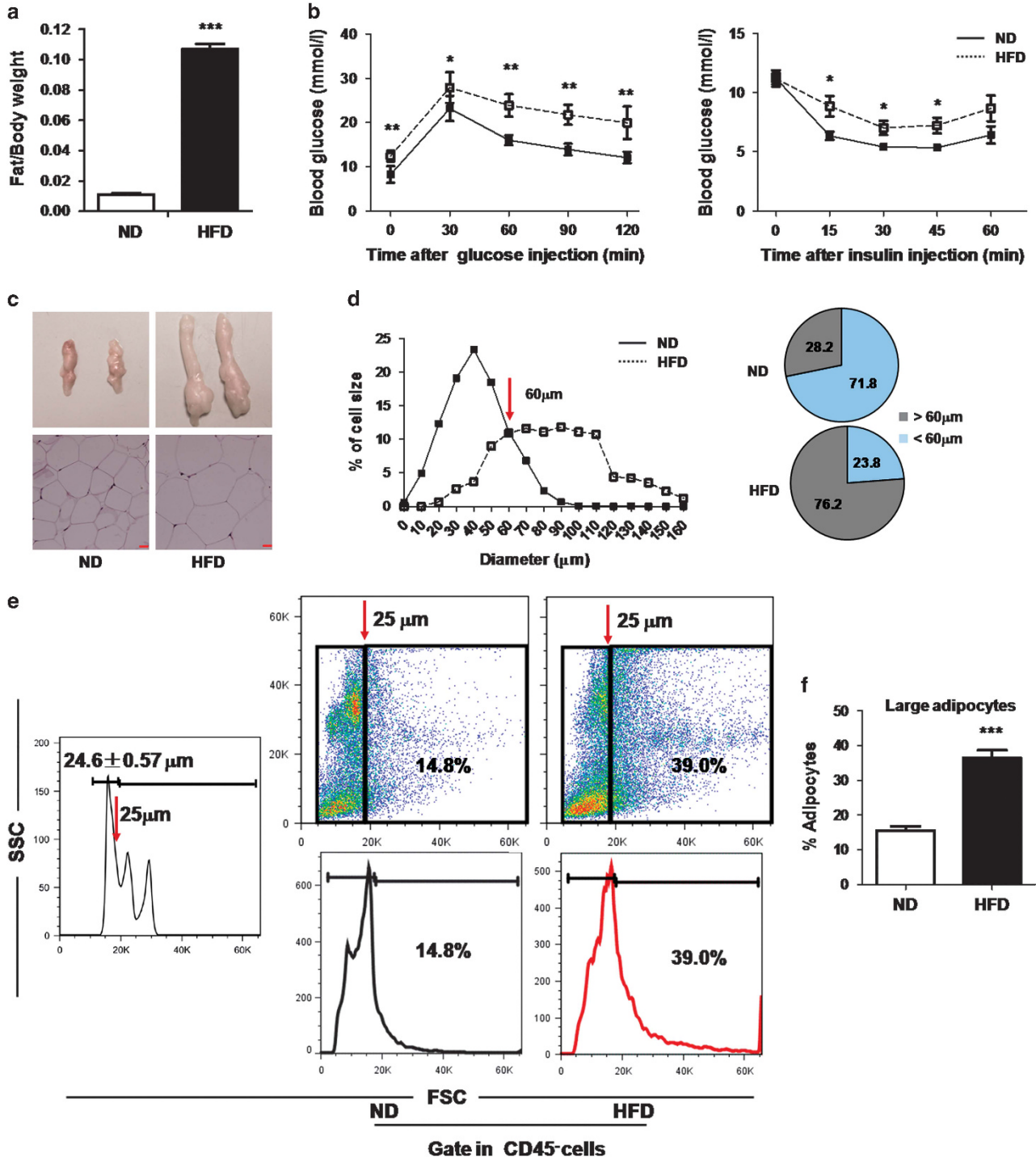
The statistical analysis was performed with GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA). All results were presented as means  $\pm$  s.e.m. Comparisons between groups were made by using an unpaired two-tailed Student's *t*-test. A *P* value < 0.05 was considered statistically significant. All experiments were repeated at least three times, and representative data are shown.

## RESULTS

Adipocytes size increases in insulin-resistant mice fed with HFD

To clarify the role of adipocytes hypertrophy and obesity in the development of insulin resistance, obese mice were generated by HFD. Because visceral adiposity was more closely related to insulin resistance than to subcutaneous adiposity,<sup>3,27</sup> we focused on

gonadal adipose tissue (visceral adipose tissue). As shown in Figure 1a, the fat/body weight of HFD-fed mice increased significantly more than their ND-fed littermates. Because obese are classified into metabolically healthy obese and metabolically abnormal obese, we then tested the intraperitoneal glucose and insulin tolerance of HFD-fed mice. The results showed deteriorated



**Figure 1.** Adipocytes size increases in insulin-resistant mice fed with HFD. (a) Fat index (percentage of fat pad weight relative to the whole body weight); (b) glucose concentrations during glucose tolerance test or insulin tolerance test; (c) morphologic comparison and H&E staining (scale bar = 20 μm); (d) H&E staining sections quantification of adipocytes diameter; (e) polystyrene particles and representative adipocytes flow cytometric analysis, three polystyrene particles with mean size at 24.6, 33.5 and 43.6 were used. (f) Percentage of large adipocytes by flow cytometric analysis. All the results detected in mice fed with ND or HFD ( $n = 6-7$ ). All the mice fed with ND or HFD for 20 weeks if not indicated otherwise. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ . SSC, side scatter.

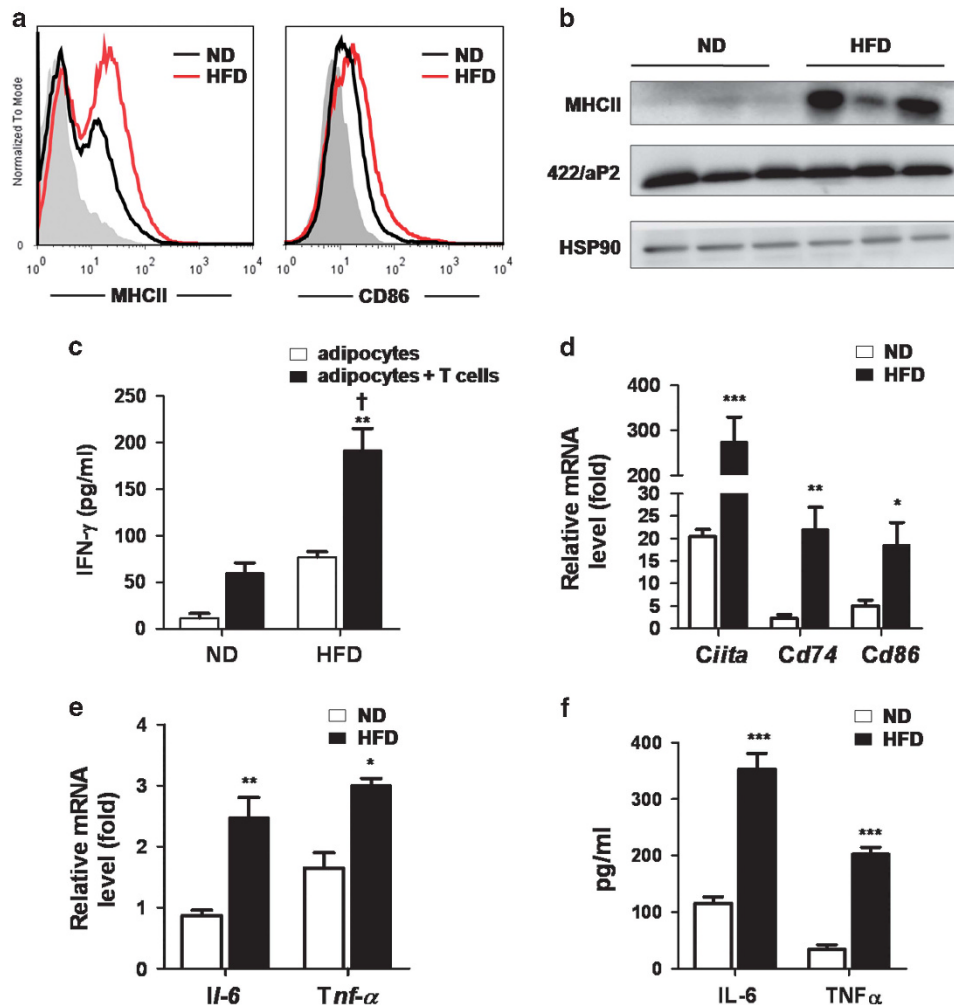
insulin sensitivity in HFD-fed mice than in ND-fed mice (Figure 1b). Consistent with this, H&E-stained sections revealed an apparent morphologic increase in adipocytes size of obese versus lean mice (Figure 1c). Adipocyte diameters measured on the H&E-stained sections displayed a near Gaussian distribution, and the line charts of the two groups of mice intersected at the 60- $\mu\text{m}$  point (Figure 1d). Thus, small adipocytes were defined as those with diameter < 60  $\mu\text{m}$ , whereas large adipocytes as those with diameter > 60  $\mu\text{m}$  in H&E-stained sections. As presented in the pie chart, the proportion of large adipocytes was approximately threefold higher in the HFD-fed mice than in the ND-fed mice.

To further identify the actual size of adipocytes *in vivo*, we measured them using flow cytometry (Figures 1e and f). To eliminate the effects of other cells and oil droplets, isolated adipocytes were stained with 5-chloromethylfluorescein diacetate dye, CD45 and Nile red dye (data not show), which confirmed they were pure adipocytes. In addition, we used polystyrene particles with diameter among 18.0–52.0  $\mu\text{m}$  to define the scale of flow cytometry and identify the size of adipocytes. As shown in Figure 1e, the peaks of adipocytes from ND and HFD were both around 25  $\mu\text{m}$ , so we defined small adipocytes as those with diameter < 25  $\mu\text{m}$ , whereas large adipocytes as those with diameter > 25  $\mu\text{m}$ . We found the proportion of large adipocytes

was approximately 2.5-fold greater in HFD-fed mice than in the ND-fed mice, which was consistent with H&E staining result. The differences of cell diameter between H&E-stained sections and flow cytometry may be due to the squeeze and deformation of H&E staining process. As flow cytometry was the main technique to divide adipocytes into two groups (small adipocytes and large adipocytes) in our study, we used 25  $\mu\text{m}$  as a boundary point of adipocytes in the following study.

MHCII expression is elevated in adipocytes of HFD-fed mice

Agreed with previous studies, we confirmed the expression of MHCII in adipocytes of gonadal adipose tissue in HFD-fed mice was higher than in ND by using flow cytometry and western blotting (Figures 2a and b). To investigate the ability of adipocytes to activate CD4<sup>+</sup> T cells, adipocytes isolated from lean and obese mice were co-cultured, respectively, with splenic T cells. Result showed IFN- $\gamma$  was significantly higher in the supernatant of co-culture from obese mice compared with those from the lean or culture alone (Figure 2c). Consistent with these results, the mRNA expression of *CIITA* (the master regulator of MHCII), *CD74* (the MHCII invariant chain peptide) and *CD86* (co-stimulatory factors) increased significantly with obesity (Figure 2d). In addition, classic



**Figure 2.** The expression of MHCII elevates in adipocytes of HFD-fed mice. (a) Representative flow cytometric analysis of MHCII with CD86 and (b) western blot analysis of MHCII in adipocytes of mice fed with ND or HFD. Similar results were obtained from 6 to 7 individual mice. 422/aP2 and HSP90 were used to identify mature adipocytes and loading control, respectively. (c) IFN- $\gamma$  concentrations in supernatants of adipocytes were cultured alone or co-cultured with T cells ( $n=4-6$ ). (d and e) *CIITA*, *CD74*, *CD86*, *IL-6* and *TNF $\alpha$*  mRNA level in adipocytes; (f) concentrations of *IL-6* and *TNF- $\alpha$*  in supernatants of cultured adipocytes. The results of c–f detected in mice fed with ND or HFD ( $n=6$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  for ND vs HFD-fed. † $P < 0.05$  for adipocytes were cultured alone vs co-cultured with T cells in HFD fed.

pro-inflammatory cytokines such as IL-6 and TNF- $\alpha$  were also substantially higher in the adipocytes of obese mice and the cell culture supernatant of these adipocytes (Figures 2e and f). Collectively, these results suggested that the expression of MHCII increased with obesity and was accompanied by increased pro-inflammatory factors.

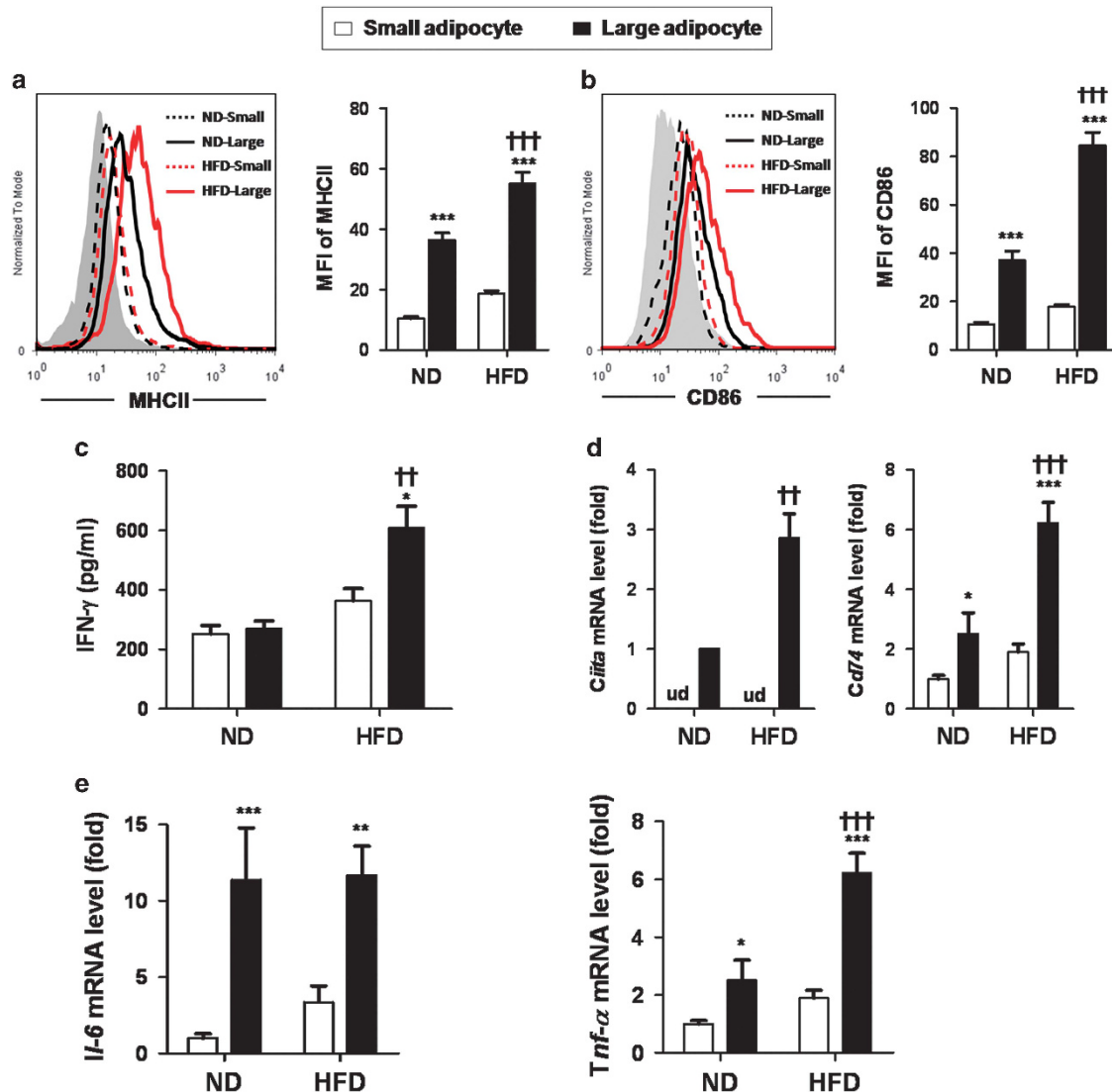
Large adipocytes express higher levels of MHCII than small adipocytes and stimulate CD4<sup>+</sup> T cells to secrete more IFN- $\gamma$  in HFD-fed mice

Because adipocytes hypertrophy is closely related to insulin resistance, we hypothesized that MHCII was highly expressed in hypertrophic adipocytes but not in small adipocytes in obese mice. To investigate whether only large adipocytes had a high expression of MHCII, adipocytes were divided into two groups based on their size measured by flow cytometry analysis (Figures 1e and f). The results revealed large adipocytes (> 25  $\mu$ m) had a higher expression of MHCII and CD86 than small adipocytes in both HFD- and ND-fed mice (Figures 3a and b).

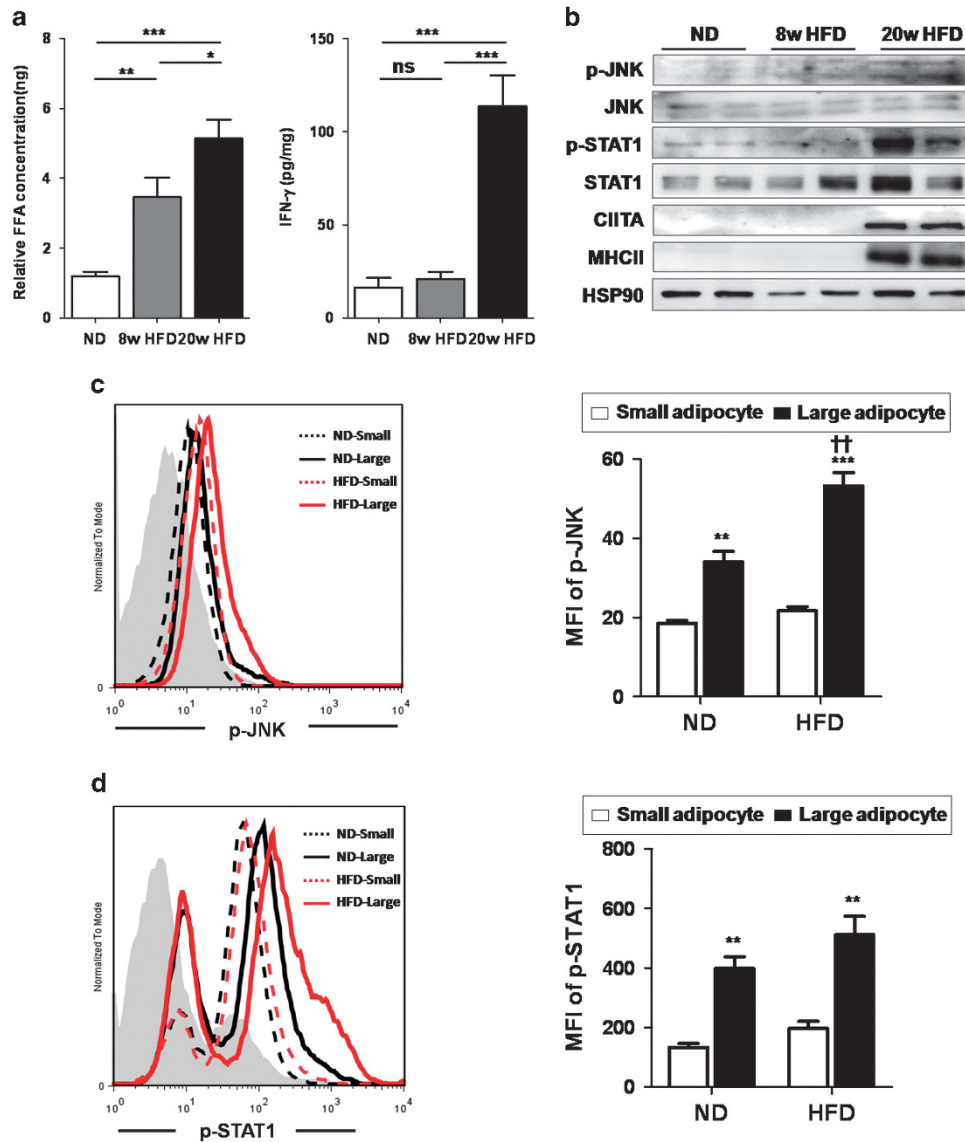
To further test the ability of adipocytes with different size to activate CD4<sup>+</sup> T cells, the sorted adipocytes were co-cultured with CD4<sup>+</sup> T cells, and then the cytokines in the supernatant were analyzed. Large adipocytes of HFD-fed mice co-cultured with T cells secreted more IFN- $\gamma$  (Figure 3c), although this increase in IFN- $\gamma$  secretion was not observed in ND-fed mice. We speculated large adipocytes in ND-fed mice did not secrete additional IFN- $\gamma$  because the expression of MHCII was too low to reach the threshold for CD4<sup>+</sup> T cell activation. Consistent with these results, qRT-PCR analysis revealed greater expression of CIITA and CD74 in large adipocytes than in small adipocytes (Figure 3d), and expression of pro-inflammatory cytokines IL-6 and TNF- $\alpha$  were higher in large adipocytes than in small adipocytes (Figure 3e).

Activation of the JNK-STAT1 pathway is involved in upregulated expression of MHCII and CIITA in large adipocytes of HFD-fed mice with elevated FFA

It is well known that IFN- $\gamma$  could induce MHCII expression. To identify whether IFN- $\gamma$  could also induce MHCII expression in



**Figure 3.** Large adipocytes express higher levels of MHCII than small adipocytes and stimulate CD4<sup>+</sup> T cells to secrete more IFN- $\gamma$  in HFD-fed mice. (a and b) Flow cytometric analysis of MHCII and CD86 expression level ( $n=6-7$ ); (c) IFN- $\gamma$  concentrations in supernatants of adipocytes co-cultured with T cells ( $n=4-6$ ); (d and e) CIITA, CD74, CD86, IL-6 and TNF- $\alpha$  mRNA level. All the results detected in small or large adipocytes in mice fed with ND or HFD. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  for small vs large adipocytes. † $P < 0.01$ , †† $P < 0.001$  for large adipocytes from ND- vs HFD-fed mice.



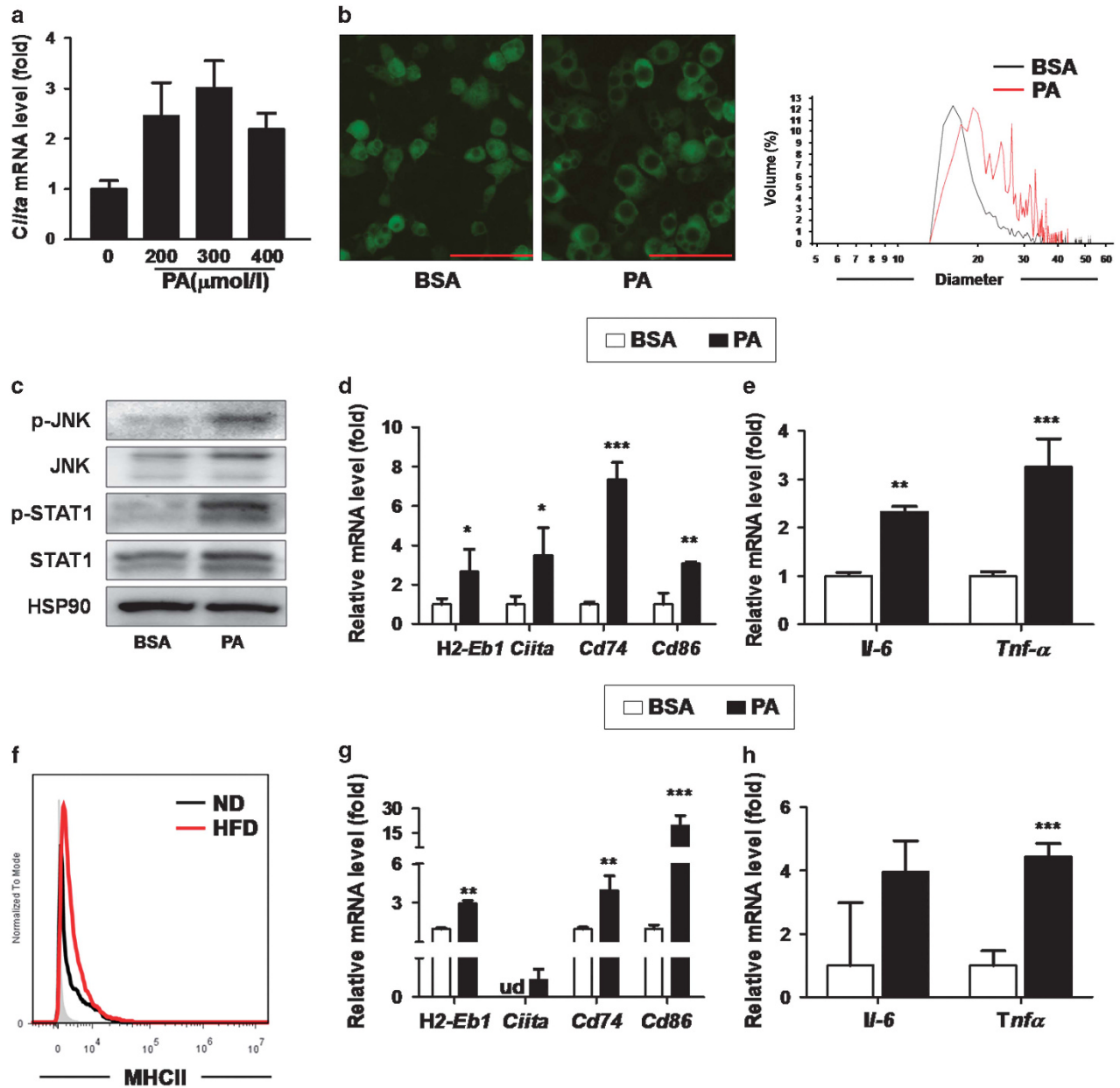
**Figure 4.** Activation of JNK-STAT1 pathway is involved in large adipocytes of HFD-fed mice with elevated FFA. **(a)** The relative contents of IFN- $\gamma$  (left) and FFA (right) in adipose tissue ( $n = 10-13$ ) and **(b)** western blot analysis of p-JNK, JNK, p-STAT1, STAT1, CIITA and MHCII expression levels in adipocytes of mice fed with ND-, HFD for 8 weeks, HFD for 20 weeks ( $n = 4-6$ ). Mice fed a HFD for 8 weeks were presented as a gradient control. **(c and d)** Flow cytometric analysis of p-JNK and p-STAT1 expression levels in small or large adipocytes of mice fed with ND or HFD ( $n = 6$ ). \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  for small vs large adipocytes. †† $P < 0.01$  for large adipocytes from ND- vs HFD-fed mice.

adipose tissue, we detected the content of IFN- $\gamma$  in mice and found it was low in the early stage of obesity (8 weeks of HFD) showed in Figure 4a. Many studies have shown that large adipocytes have increased lipolytic activity, which contributed to increased flux of FFA.<sup>13</sup> Elevated plasma FFA was a typical feature of obesity, which was closely related to insulin resistance.<sup>28,29</sup> Thus, we hypothesized elevated FFA in large adipocytes triggered inflammatory signaling pathways to increase MHCII expression. As expected, the FFA content in intracellular adipose tissue increased with the development of obesity (Figure 4a).

As previous studies demonstrated increased FFA activated JNK,<sup>30-32</sup> whereas JNK was reported to activate STAT1,<sup>33-35</sup> we detected phosphorylation of JNK and STAT1 in adipocytes. Similar to previous studies, both JNK and STAT1 were activated with obesity and were consistent with the high expression of CIITA and MHCII in adipocytes (Figure 4b). To further clarify the activation occurred primarily in large adipocytes, the adipocytes were divided into small and large groups by using flow cytometry.

Paralleled with the expression of MHCII, p-JNK and p-STAT1 were primarily activated in large adipocytes of both HFD- and ND-fed mice (Figures 4c and d).

Excess saturated fatty acids increase adipocytes size and expression of CIITA and MHCII in 3T3-L1 and primary adipocytes. To clarify the underlying mechanism of MHCII expression in adipocytes during obesity, we checked whether palmitic acid, the most abundant saturated fatty acid in the blood,<sup>36</sup> could induce the enlargement of adipocytes and expression of MHCII *in vitro*. According to previous studies and our data (Figure 5a), we incubated 3T3-L1 mature adipocytes with 300  $\mu\text{M}$  palmitic acid for 13 days. Before harvest, we measured cell size and found palmitic acids could enlarge the adipocytes (Figure 5b). Consistent with the results *in vivo*, phosphorylation of JNK and STAT1 increased in 3T3-L1 adipocytes treated with palmitic acids (Figure 5c). Although MHCII cannot be detected by western blotting, palmitic acids increased mRNA expression of MHCII (H2-Eb1), CIITA, CD74 and



**Figure 5.** Saturated fatty acids increase adipocytes size and expression of CIITA and MHCII in 3T3-L1 and primary adipocytes. Results of **a–e** detected in 3T3-L1 adipocytes treated with bovine serum albumin (BSA) or palmitic acid (PA). (**a**) palmitic acid gradient response of CIITA mRNA expression; (**b**) 5-chloromethylfluorescein diacetate (CMFDA) staining (left) and cell size measurement (right, scale bar = 100 μm); (**c**) western blot analysis of p-JNK, JNK, p-STAT1 and STAT1; (**d** and **e**) indicated genes mRNA level in mature adipocytes. Results of **f–h** detected in primary adipocytes of 8-week-old mice. (**f**) Flow cytometric analysis of MHCII; (**g** and **h**) indicated genes mRNA level in primary adipocytes. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  for BSA vs palmitic acid treated.

CD86, which were accompanied by an increase in the pro-inflammatory factors IL-6 and TNF- $\alpha$  (Figures 5d and e). We speculated the reasons of these results might be that 3T3-L1 is classic and simple adipocytes, which lacked the essential condition to synthesize the protein of MHCII or the expression was too low to be detected by conventional approaches. However, protein levels of MHCII were increased in primary adipocytes of 8-week-old mice treated with palmitic acids (Figure 5f). The mRNA expressions of associated genes were increased simultaneously (Figures 5g and h). Collectively, these results suggested that saturated fatty acids induced adipocytes hypertrophy and expression of CIITA and MHCII.

## DISCUSSION

Recent study has shown that adipocytes can express MHCII to directly activate T cells in promoting adipose inflammation during the progression of obesity,<sup>22</sup> which uncovered a new insight of adipocytes acting as antigen-presenting cells. However, there are still some questions to be answered, such as which type of adipocytes highly express MHCII and the underlying mechanism. In this study, we demonstrate that MHCII is highly expressed in large adipocytes but not in small adipocytes and the activation of the JNK-STAT1 pathway is involved in it. The expression of MHCII in large adipocytes from obese mice stimulate CD4<sup>+</sup> T cells to secrete more IFN- $\gamma$  and polarize to Th1 in conjunction with CD86,

which in return escalate this process and inflammation. These findings would help us to understand why metabolically healthy obese containing small adipocytes is in low inflammation and less insulin resistance.

Studies have shown that obese people with enlarged subcutaneous abdominal adipocytes size were more susceptible for glucose intolerance and hyperinsulinemic than those with relatively smaller adipocytes size but with a similar degree of adiposity.<sup>37,38</sup> In addition, other groups have demonstrated large adipocytes had a high expression of immune-related genes such as leptin and serum amyloid A, which may link hypertrophic adipocytes to insulin resistance.<sup>12</sup> Here we report that large adipocytes rather than small adipocytes highly express MHCII in both HFD- and ND-fed mice and can function as APC to activate T cells. However, the number of large adipocytes in ND-fed mice is much lower compared with HFD-fed mice, so the activation of T cell is absent in ND-fed mice. These results may be of value as we demonstrate the phenotype change of adipocytes other than immune cells is also associated with obesity-related inflammation.

Previous study reported that during the progression of obesity, increased leptin promoted activation and polarization of T cells in adipose tissue, which secreted IFN- $\gamma$  to induce expression of CIITA and MHCII in adipocytes.<sup>22</sup> However, other studies showed that T-cell enrichment and IFN- $\gamma$  gene induction occurred in later periods of obesity development and that early T-cell infiltration might be a protective process to temper adipose tissue inflammation.<sup>18,39</sup> Although we cannot exclude the possibility that IFN- $\gamma$  could increase MHCII expression in adipocytes, it is less likely that increase of MHCII in adipocytes is due to IFN- $\gamma$  during the early periods of obesity, as our study showed that FFA, but not IFN- $\gamma$ , elevated during the early periods of obesity. As high expression of MHCII is particularly in large adipocytes, elevated FFA is closely associated with adipocyte hypertrophy and insulin resistance,<sup>40</sup> we hypothesize FFA, rather than IFN- $\gamma$ , may be an initial stimulus of MHCII expression. But it is still possible that, with the propagation of obesity, IFN- $\gamma$  secreted by activated T cell may work together with FFA to induce more MHCII expression in adipocytes to worsen adipose inflammation.

According to previous studies, elevated intracellular FFA led to endoplasmic reticulum stress, which activated JNK signaling cascades that contributed to the development of insulin resistance.<sup>30–32</sup> In our study, the increased intracellular FFA in adipocytes is associated with the activation of JNK and STAT1. As reports have demonstrated that phosphorylated JNK can activate STAT1,<sup>33–35</sup> while STAT1 can further activate CIITA (the key regulator for MHCII expression and location),<sup>23,41</sup> we propose that the expression of MHCII and CIITA are regulated by p-STAT1 through p-JNK signaling in large adipocytes and our results confirmed it. Besides, adipocyte hypertrophy might lead to local adipose tissue hypoxia, which induced expression of hypoxia inducible factor 1 and related genes. Hypoxic adipose tissue displayed increased activity of the JNK1 pathway and expression of genes involved in inflammation and endoplasmic reticulum stress,<sup>28,42,43</sup> this may be another mechanism to explain the high expression of MHCII in large adipocytes and need to further study. Moreover, we cannot exclude the role of classical JAK-STAT pathway in the activation of MHCII in adipocytes, as some studies demonstrated JAK1 was involved in JNK-STAT1 activation<sup>35</sup> and our data were consistent with it (Supplementary Figure). It will be interesting to address the exact mechanism by using adipocyte-specific JNK- or JAK1-deficient mice in the future study.

Although our study reveals a novel function of large adipocytes in T-cell activation in the adipose tissue of obese mice, we agree that macrophages/DCs are able to activate T cells to secrete IFN- $\gamma$ , which in turn polarize resident macrophages into inflammatory M1, contributing to ectopic fat deposition and systemic insulin resistance.<sup>21,44</sup> However, the expression of multiple MHCII genes

did not change in adipose tissue macrophages of lean versus obese subjects according to previous study.<sup>22</sup> As for DCs, they constituted a small proportion in adipose tissue (around 0.4–0.6%, data not shown) and existed reports indicated that their function focused on the induction of naïve T-cell activation, Th17-cell responses and macrophage infiltration.<sup>45,46</sup> In contrast to macrophages and DCs, the adipocytes are the major component in adipose tissue. Thus, our study suggested that, although DC or macrophage could be more capable of activating T cells on a single-cell level, enlarged adipocytes may have an important role in activating T cells during early stage of obesity.

Activation of T cells by large adipocytes will promote an immune reaction against the antigen, such as autoimmune reaction. Compared with type 1 diabetes, a typical autoimmune disease, obesity-related T2D is more complex. Although T2D has not been defined as an autoimmune disease, accumulating evidence supported autoimmune reactions in the adipose tissue of obese mice.<sup>47–49</sup> For example, several studies showed that, in visceral adipose tissue of HFD-fed mice, T cells displayed biased T cell receptor-V $\alpha$  repertoires, suggesting the antigen-specific expansion.<sup>47</sup> In addition, it was reported that accumulating B cells in visceral adipose tissues of HFD-fed mice were capable of secreting IgG2c auto-antibodies to worsen insulin resistance.<sup>48</sup> Furthermore, many human studies suggested the existence of autoimmune reactions by showing the presence of diabetes antibody markers in T2D.<sup>50,51</sup> However, it remains unclear about the nature of the relevant self-antigens that are derived from the adipose microenvironment. Necrotic adipocytes have been suggested to act as the antigenic stimulus and antigens absorbed from the gut in visceral adipose also have been demonstrated to play a role in T-cell activation in mouse model.<sup>13,52–54</sup> Together with previous studies, our findings may further support the possibility that there are autoimmune reactions involving T-cell activation in the adipose tissue of obese mice.

In conclusion, our investigation highlights the role of adipocyte hypertrophy in the development of obesity-associated inflammation. This phenotype change of adipocytes may be due to excess FFA accumulated in adipocytes, which stimulate the expression of MHCII to activate T cells involving the JNK-STAT1 signaling pathway. In a word, our results provide a new therapeutic potential to attenuate obesity-associated inflammation by preventing adipocytes hypertrophy and/or removing large adipocytes.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

This work was supported by National Key Basic Research Project Grants (2013CB530601 to X Li, 2011CB910201 to Q Tang), National Natural Science Foundation of China (81270954 to X Li, 813220437 to R He, 81390350 to Q Tang), the Shanghai Rising Star Program (13QH1400800 to X Li), the Department of Biochemistry and Molecular Biology at Fudan University Shanghai Medical College is supported by the Shanghai Leading Academic Discipline Projects B110 and by 985 Project 985III-YFX0302.

## REFERENCES

- 1 Stevens GA, Singh GM, Lu Y, Danaei G, Lin JK, Finucane MM *et al*. National, regional, and global trends in adult overweight and obesity prevalences. *Popul Health Metr* 2012; **10**: 22.
- 2 Haslam DW, James WP. Obesity. *Lancet* 2005; **366**: 1197–1209.
- 3 Chergaoui R, Kassim TA, Kwagyan J, Freeman C, Nunlee-Bland G, Ketete M *et al*. The metabolically healthy but obese phenotype in African Americans. *J Clin Hypertens* 2012; **14**: 92–96.
- 4 Fabbrini E, Yoshino J, Yoshino M, Magkos F, Tiemann Luecking C, Samovski D *et al*. Metabolically normal obese people are protected from adverse effects following weight gain. *J Clin Invest* 2015; **125**: 787–795.
- 5 Blüher M. Are there still healthy obese patients? *Curr Opin Endocrinol Diabetes Obesity* 2012; **19**: 341–346.



- 6 Samocha-Bonet D, Chisholm DJ, Tonks K, Campbell LV, Greenfield JR. Insulin-sensitive obesity in humans - a 'favorable fat' phenotype? *Trends Endocrinol Metab* 2012; **23**: 116–124.
- 7 Spalding KL, Arner E, Westermark PO, Bernard S, Buchholz BA, Bergmann O et al. Dynamics of fat cell turnover in humans. *Nature* 2008; **453**: 783–787.
- 8 Arner E, Westermark PO, Spalding KL, Britton T, Ryden M, Frisen J et al. Adipocyte turnover: relevance to human adipose tissue morphology. *Diabetes* 2010; **59**: 105–109.
- 9 Weyer C, Foley JE, Bogardus C, Tataranni PA, Pratley RE. Enlarged subcutaneous abdominal adipocyte size, but not obesity itself, predicts type II diabetes independent of insulin resistance. *Diabetologia* 2000; **43**: 1498–1506.
- 10 Bahceci M, Gokalp D, Bahceci S, Tuzcu A, Atmaca S, Arikian S. The correlation between adiposity and adiponectin, tumor necrosis factor alpha, interleukin-6 and high sensitivity C-reactive protein levels. Is adipocyte size associated with inflammation in adults?. *J Endocrinol Invest* 2007; **30**: 210–214.
- 11 Skurk T, Alberti-Heber C, Herder C, Hauner H. Relationship between adipocyte size and adipokine expression and secretion. *J Clin Endocrinol Metab* 2007; **92**: 1023–1033.
- 12 Jernas M, Palming J, Sjöholm K, Jennische E, Svensson PA, Gabrielson BG et al. Separation of human adipocytes by size: hypertrophic fat cells display distinct gene expression. *FASEB J* 2006; **20**: 1540–1542.
- 13 Odegaard JI, Chawla A. Alternative macrophage activation and metabolism. *Annu Rev Pathol* 2011; **6**: 275–297.
- 14 Finlay D, Cantrell DA. Metabolism, migration and memory in cytotoxic T cells. *Nat Rev Immunol* 2011; **11**: 109–117.
- 15 Wu H, Ghosh S, Perrard XD, Feng L, Garcia GE, Perrard JL et al. T-cell accumulation and regulated on activation, normal T cell expressed and secreted upregulation in adipose tissue in obesity. *Circulation* 2007; **115**: 1029–1038.
- 16 Kintscher U, Hartge M, Hess K, Foryst-Ludwig A, Clemenz M, Wabitsch M et al. T-lymphocyte infiltration in visceral adipose tissue: a primary event in adipose tissue inflammation and the development of obesity-mediated insulin resistance. *Arterioscler Thromb Vasc Biol* 2008; **28**: 1304–1310.
- 17 O'Rourke RW, White AE, Metcalf MD, Winters BR, Diggs BS, Zhu X et al. Systemic inflammation and insulin sensitivity in obese IFN-gamma knockout mice. *Metabolism* 2012; **61**: 1152–1161.
- 18 Strissel KJ, DeFuria J, Shaul ME, Bennett G, Greenberg AS, Obin MS. T-cell recruitment and Th1 polarization in adipose tissue during diet-induced obesity in C57BL/6 mice. *Obesity* 2010; **18**: 1918–1925.
- 19 Yang H, Youm YH, Vandanmagsar B, Ravussin A, Gimble JM, Greenway F et al. Obesity increases the production of proinflammatory mediators from adipose tissue T cells and compromises TCR repertoire diversity: implications for systemic inflammation and insulin resistance. *J Immunol* 2010; **185**: 1836–1845.
- 20 Klimcakova E, Roussel B, Marquez-Quinones A, Kovacova Z, Kovacikova M, Combes M et al. Worsening of obesity and metabolic status yields similar molecular adaptations in human subcutaneous and visceral adipose tissue: decreased metabolism and increased immune response. *J Clin Endocrinol Metab* 2011; **96**: E73–E82.
- 21 Cho KW, Morris DL, DelProposto JL, Geletka L, Zamarron B, Martinez-Santibanez G et al. An MHC II-dependent activation loop between adipose tissue macrophages and CD4+ T cells controls obesity-induced inflammation. *Cell Rep* 2014; **9**: 605–617.
- 22 Deng T, Lyon CJ, Minze LJ, Lin J, Zou J, Liu JZ et al. Class II major histocompatibility complex plays an essential role in obesity-induced adipose inflammation. *Cell Metab* 2013; **17**: 411–422.
- 23 Ting JP, Trowsdale J. Genetic control of MHC class II expression. *Cell* 2002; **109**: S21–S33.
- 24 Larson-Meyer DE, Heilbronn LK, Redman LM, Newcomer BR, Frisard MI, Anton S et al. Effect of calorie restriction with or without exercise on insulin sensitivity, beta-cell function, fat cell size, and ectopic lipid in overweight subjects. *Diabetes Care* 2006; **29**: 1337–1344.
- 25 McLaughlin T, Sherman A, Tsao P, Gonzalez O, Yee G, Lamendola C et al. Enhanced proportion of small adipose cells in insulin-resistant vs insulin-sensitive obese individuals implicates impaired adipogenesis. *Diabetologia* 2007; **50**: 1707–1715.
- 26 Qian SW, Tang Y, Li X, Liu Y, Zhang YY, Huang HY et al. BMP4-mediated brown fat-like changes in white adipose tissue alter glucose and energy homeostasis. *Proc Natl Acad Sci USA* 2013; **110**: E798–E807.
- 27 Wronska A, Kmiec Z. Structural and biochemical characteristics of various white adipose tissue depots. *Acta Physiol* 2012; **205**: 194–208.
- 28 Schenk S, Saberi M, Olefsky JM. Insulin sensitivity: modulation by nutrients and inflammation. *J Clin Invest* 2008; **118**: 2992–3002.
- 29 Boden G. Obesity and free fatty acids. *Endocrinol Metab Clin North Am* 2008; **37**: 635–646.
- 30 Hirosumi J, Tuncman G, Chang L, Gorgun CZ, Uysal KT, Maeda K et al. A central role for JNK in obesity and insulin resistance. *Nature*, 2002; **420**: 333–336.
- 31 Ozcan U, Cao Q, Yilmaz E, Lee AH, Iwakoshi NN, Ozdelen E et al. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science* 2004; **306**: 457–461.
- 32 Sabio G, Das M, Mora A, Zhang ZY, Jun JY, Ko HJ et al. A Stress Signaling Pathway in Adipose Tissue Regulates Hepatic Insulin Resistance. *Science* 2008; **322**: 1539–1543.
- 33 Cui G, Qin X, Zhang Y, Gong Z, Ge B, Zang YQ. Berberine differentially modulates the activities of ERK, p38 MAPK, and JNK to suppress Th17 and Th1 T cell differentiation in type 1 diabetic mice. *J Biol Chem* 2009; **284**: 28420–28429.
- 34 Mandrekar P, Szabo G. Signalling pathways in alcohol-induced liver inflammation. *J Hepatol* 2009; **50**: 1258–1266.
- 35 Gorina R, Font-Nieves M, Marquez-Kisinousky L, Santalucia T, Planas AM. Astrocyte TLR4 activation induces a proinflammatory environment through the interplay between MyD88-dependent NFkappaB signaling, MAPK, and Jak1/Stat1 pathways. *GLIA* 2011; **59**: 242–255.
- 36 Frohner BI, Jacobs Jr DR, Steinberger J, Moran A, Steffen LM, Sinaiko AR. Relation between serum free fatty acids and adiposity, insulin resistance, and cardiovascular risk factors from adolescence to adulthood. *Diabetes* 2013; **62**: 3163–3169.
- 37 Salans LB, Knittle JL, Hirsch J. The role of adipose cell size and adipose tissue insulin sensitivity in the carbohydrate intolerance of human obesity. *J Clin Invest* 1968; **47**: 153–165.
- 38 Stern JS, Batchelor BR, Hollander N, Cohn CK, Hirsch J. Adipose-cell size and immunoreactive insulin levels in obese and normal-weight adults. *Lancet* 1972; **2**: 948–951.
- 39 Duffaut C, Galitzky J, Lafontan M, Bouloumie A. Unexpected trafficking of immune cells within the adipose tissue during the onset of obesity. *Biochem Biophys Res Commun* 2009; **384**: 482–485.
- 40 Aboufajali G, Shtaynberg N, Wetz R, Costantino T, Abela GS. Current concepts in triglyceride metabolism, pathophysiology, and treatment. *Metabolism* 2010; **59**: 1210–1220.
- 41 Reith W, LeibundGut-Landmann S, Waldburger JM. Regulation of MHC class II gene expression by the class II transactivator. *Nat Rev Immunol* 2005; **5**: 793–806.
- 42 Ye J, Gao Z, Yin J, He Q. Hypoxia is a potential risk factor for chronic inflammation and adiponectin reduction in adipose tissue of ob/ob and dietary obese mice. *Am J Physiol Endocrinol Metab* 2007; **293**: E1118–E1128.
- 43 Hosogai N, Fukuhara A, Oshima K, Miyata Y, Tanaka S, Segawa K et al. Adipose tissue hypoxia in obesity and its impact on adipocytokine dysregulation. *Diabetes* 2007; **56**: 901–911.
- 44 Chawla A, Nguyen KD, Goh YP. Macrophage-mediated inflammation in metabolic disease. *Nat Rev Immunol* 2011; **11**: 738–749.
- 45 Bertola A, Ciucci T, Rousseau D, Bourlier V, Duffaut C, Bonnafous S et al. Identification of adipose tissue dendritic cells correlated with obesity-associated insulin-resistance and inducing Th17 responses in mice and patients. *Diabetes* 2012; **61**: 2238–2247.
- 46 Stefanovic-Racic M, Yang X, Turner MS, Mantell BS, Stolz DB, Sumpter TL et al. Dendritic cells promote macrophage infiltration and comprise a substantial proportion of obesity-associated increases in CD11c+ cells in adipose tissue and liver. *Diabetes* 2012; **61**: 2330–2339.
- 47 Winer S, Chan Y, Paltser G, Truong D, Tsui H, Bahrami J et al. Normalization of obesity-associated insulin resistance through immunotherapy. *Nat Med* 2009; **15**: 921–929.
- 48 Winer DA, Winer S, Shen L, Wadia PP, Yantha J, Paltser G et al. B cells promote insulin resistance through modulation of T cells and production of pathogenic IgG antibodies. *Nat Med* 2011; **17**: 610–617.
- 49 Sell H, Habich C, Eckel J. Adaptive immunity in obesity and insulin resistance. *Nat Rev Endocrinol* 2012; **8**: 709–716.
- 50 Niskanen LK, Tuomi T, Karjalainen J, Groop LC, Uusitupa MI. GAD antibodies in NIDDM. Ten-year follow-up from the diagnosis. *Diabetes Care* 1995; **18**: 1557–1565.
- 51 Hathout EH, Thomas W, El-Shahawy M, Nahab F, Mace JW. Diabetic autoimmune markers in children and adolescents with type 2 diabetes. *Pediatrics* 2001; **107**: E102.
- 52 Ghoshal S, Witta J, Zhong J, de Villiers W, Eckhardt E. Chylomicrons promote intestinal absorption of lipopolysaccharides. *J Lipid Res* 2009; **50**: 90–97.
- 53 Wang Y, Ghoshal S, Ward M, de Villiers W, Woodward J, Eckhardt E. Chylomicrons promote intestinal absorption and systemic dissemination of dietary antigen (ovalbumin) in mice. *PLoS One* 2009; **4**: e8442.
- 54 Wang Y, Li J, Tang L, Wang Y, Charnigo R, de Villiers W et al. T-lymphocyte responses to intestinally absorbed antigens can contribute to adipose tissue inflammation and glucose intolerance during high fat feeding. *PLoS One* 2010; **5**: e13951.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivs 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>

Supplementary Information accompanies this paper on International Journal of Obesity website (<http://www.nature.com/ijo>)