Immune profiles of T lymphocyte subsets in adipose tissue of obese mouse induced by high-fat diet

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ABSTRACT
In this study, we analyzed high-fat diet (HFD)-induced time-course changes in proportions of T lymphocyte subsets in adipose tissue. Mice were fed with normal-fat diet (NFD) or HFD for 20 weeks. An autoanalyzer was used to assay plasma concentrations of glucose, cholesterol, triglycerides, low-density lipoprotein (LDL), and high-density lipoprotein (HDL). Stromal vascular cells were isolated from epididymal adipose tissues and labeled with antibodies for cluster of differentiation (CD) 3, CD4, CD8, interferon-γ (IFN-γ), interleukin (IL)-4, and IL-17 for fluorescence-activated cell sorter. We discovered that weight of epididymal fat pads and perirenal fat in HFD group were higher than that in NFD group. Significant changes in glucose, cholesterol, triglyceride, LDL, and HDL content were detected in sera of mice fed with HFD compared with those provided with NFD. Proportions of CD3⁺, CD4⁺, and CD8⁺ T cells increased significantly in adipose tissues of HFD mice compared with those of NFD mice. Proportions of T helper (Th)1 and Th17 sublineages also increased significantly in HFD group. Long-time HFD-induced obesity can increase proportions of CD3⁺ T, CD4⁺ T, and CD8⁺ T cells in epididymal fat pads, disrupt balance of CD4⁺ T lymphocyte subsets, and induce progressive Th1 and Th17 biases.

Key words: Adipose tissue, High-fat diet, Obesity, T lymphocyte.

INTRODUCTION
Cases of obesity and its associated disorders are increasing at accelerating and alarming rate worldwide (Frederick et al., 2014). Obesity is associated with immune function impairment, affecting both innate and adaptive immune systems (Andersen et al., 2016, Exley et al., 2014). Excess fat mass leads to systemic low-grade chronic inflammatory states, particularly active local inflammation in adipose tissue (Apostolopoulos et al., 2016). Chronic low-grade inflammation is recognized as one of the key steps in pathogenesis of insulin resistance, type 2 diabetes mellitus, and atherosclerotic cardiovascular disease in obese individuals (Aarti et al., 2008, Emanuela F., 2012). However, studies do not fully demonstrate detailed events underlying inflammatory initiation and development.

Understanding pathogenesis of adipose inflammation can be achieved by studying the large numbers of immune cells residing in adipose tissues. Chronic low-grade systemic inflammation can be observed in obese patients or animal models of obesity, macrophages, T cells, B cells, and other immune cells infiltrated adipose tissues, which produce inflammatory cytokines, such as tumor necrosis factor (TNF)-α and interleukin (IL)-6, (Makki et al., 2013). However, in adipose tissues, initiation of primary inflammation is not fully understood. Two main viewpoints exist. Some researchers believe that adipose tissue macrophages act as antigen presenting cells and promote proliferation of interferon (IFN)-γ-producing T helper (Th)1 cells in adipose tissues (Morris et al., 2013). Others insisted that infiltration of CD8⁺ T cells precedes recruitment of macrophages and initiates inflammatory cascade (Nishimura et al., 2009). Therefore, balancing T cell subsets is critical for regulation of adipose inflammation.

Although research focused on functional roles of T cells in adipose tissues, until now, information is unavailable for sequence of each cell type for inflammation in adipose tissues. Little information is available on dynamic processes of Th1/Th2/Th17 cells in adipose tissues of obese mouse. In the present study, we prepared mouse fat models by high-fat diet (HFD) and compared dynamic changes in Th1/Th2/Th17 cells of adipose tissues between normal-fat diet group (NFD) and HFD group at different times.
MATERIALS AND METHODS

Mice and obesity model: Four-week- to five-week-old male C57BL/6 mice were purchased from Beijing Vital River Laboratory Animals Tech. Co. After adaptive feeding for one week, mice were randomized into control (n = 35) or obese groups (n = 42). Mice were fed with NFD (12.8% kcal from fat) or HFD (58% kcal from fat) (no. D12331, Research Diets, New Brunswick, NJ) for 20 weeks to establish the obesity model.

Determination of plasma biochemical index: All mice were fasted overnight and were then anesthetized. Blood was collected from the fundus venous plexus of each mouse at 2, 4, 6, 8, 12, 16, and 20 weeks. Blood samples were centrifuged at 2,000 rpm for 10 min at room temperature in a tabletop centrifuge. An auto-biochemical analyzer was used to assay plasma concentrations of glucose, cholesterol, triglycerides, low-density lipoprotein (LDL), and high-density lipoprotein (HDL) (Hitachi Limited 7600-020, Japan).

Determination of adipose tissue and immune organ index: Mice were sacrificed by cervical dislocation after blood collection. Then, epididymal fat pad, perirenal fat, thymus, and spleen were collected and weighed to calculate indexes of thymus and spleen. Thymus or spleen index = thymus or spleen weight / mouse weight × 100%.

Isolation of stromal vascular cells (SVCs) and fluorescence-activated cell sorter (FACS) analysis: Isolation of SVCs in epididymal fat pads was performed as previously described (Yu et al., 2014). For FACS analysis, SVCs were labeled with conjugated antibodies for CD3, CD4, CD8, and their respective isotype controls. As for Th1/Th2/Th1 FACS analysis, SVCs were stimulated with PMA (50 ng/ml) and ionomycin (1 μg/ml) for 5 h in the presence of BD GolgiStop™ Protein Transport Inhibitor, fixed with 1 mL of cold BD Cytofix™ buffer, permeabilized in 1 mL of BD Perm/Wash™ buffer, incubated at room temperature for 15 min, and finally stained with antibody cocktail of CD4 (Clone: RM4-5), IFN-γ (Clone: XMG1.2), IL-4 (Clone: 11B11), and IL-17 (Clone: TC11-18H10.1) at room temperature for 30 min. Cells were analyzed with a flow cytometer (BD FASC Aria III, America).

Statistical analysis: Results are presented as mean ± standard error of the mean (SEM). At least three independent experiments were performed, and each experiment was repeated thrice. For statistical analyses, comparison between two groups was performed using student t-tests. Calculations were performed using SPSS version 11.0 statistics software. Significance was defined as p < 0.05 or p < 0.01.

RESULTS AND DISCUSSION

Effects of HFD on body weight and weight gain: Figure 1A shows mean body weight for each group. After six weeks of feeding, body weight of HFD group (30.1±1.58 g) was significantly higher than that of NFD group (25.0±0.58 g; p < 0.05). At 20 weeks, mean body weight of HFD group reached 48.7±4.00 g, which was significantly heavier than that of NFD group (31.6±1.57 g; p < 0.01). As shown in Figure 1B, weight gain was significantly higher after five weeks HFD feeding compared with that with NFD. HFD-fed mice were significantly larger than NFD control group and were filled with abdominal cavity fat compared with the control (Figures 1C and 1D). These results indicate that we successfully established fat mice models.

Effects of HFD on fat weight and immune organs: HFD significantly affected mean weight of epididymal fat pads. In HFD group, mean weight of epididymal fat pads was 1.35 times greater than that of NFD group after two weeks of feeding (Figure 2A). At 20 weeks, for mean weight of epididymal fat pads, that of HFD group was 4.2 times greater than that of NFD group (Figure 2A). In HFD group, mean weight of perirenal fat increased 6.3 times than that in NFD group (Figure 2B). However, no significant differences were found between thymus and spleen indices of the two groups (Figures 2C and D).

Time-course changes in lipid and glucose metabolic components: Sera of HFD-fed animals presented significantly changed glucose, cholesterol, triglyceride, LDL, and HDL contents. In HFD mice, triglyceride and glucose levels in sera increased from the sixth week and were persistent throughout the observation period (Figure 3A and B). After two weeks of feeding, cholesterol, HDL-C, and LDL-C levels in sera increased, remained at high levels for 16 weeks, and decreased slightly thereafter (Figure 3C, 3D, and 3E).

Time-course changes in T cells of adipose tissues and peripheral leukocytes: Compared with the NFD group, mice fed with HFD exhibited increased proportions of CD3⁺ T, CD4⁺ T, and CD8⁺ T cells in adipose tissues (Figures 4A, 4B, and 4C). Total T cells increased after 12 weeks of HFD feeding compared with controls, whereas CD4⁺ and CD8⁺ T cells significantly increased after eight weeks of feeding. We examined ratios of Th1, Th2, and Th17 cells in CD4⁺ T cells. Data indicate that HFD generated progressive Th1 and Th17 biases (Figures 4D and 4E). Compared with the NFD group, Th1 and Th17 sublineages significantly increased in the HFD group, whereas no significant change was detected on proportions of Th2 sublineage (Figure 4F).

Adipose tissue inflammation is considered as a crucial event leading to diabetes, metabolic syndromes, and other diseases (Tateya et al., 2013). However, no current study discusses the mechanisms underlying inflammation initiation and development. Recently, experimental evidence indicated that lymphocytes are important for adipose tissue inflammation (Anderson et al., 2013, Mathis, 2013, Winer et al., 2013). Visceral adipose tissue (VAT) contains more T cells, and in VAT of human volunteers, the number of CD3⁺
Figure 1: Effects of HFD on body weight and weight gain. (A) Time-course changes at different weeks as shown by weekly body weights of mice fed with HFD or NFD. (B) Weekly weight gain of mice fed with HFD or NFD; time-course changes at different weeks. (C) Sizes of mice fed with HFD or NFD. (D) Abdominal cavity of mice fed with HFD or NFD. Data are presented means ± SEM of three experiments. *p < 0.05, **p < 0.01. Images represent at least three experiments.

T cells is correlated with body mass index (Duffaut et al., 2009). CD4⁺ T and CD8⁺ T cells are two main subsets of T cells and express pro- and anti-inflammatory cytokines. These T cells are very important factors for inflammation initiation. However, little is known about effects of time-course changes of T lymphocytes, especially CD4⁺ T cells, during adipose inflammation.

In the current study, we first observed the effects of HFD on obesity in male mice. Long-time feeding with HFD (for 20 weeks) maintained high adiposity and metabolic disarrangement. Compared with NFD group, strikingly increased fat mass was observed in mice fed with HFD for two weeks, as examined in epididymal fat pads and perirenal fat, metabolic alterations in glucose, and lipid metabolism. A remarkable difference was observed in body weight and weight gain of mice fed with HFD for five or six weeks.

Several clinical and experimental observations support the relationship between inflammation and T lymphocytes of VATs (Exley et al., 2014). Balancing time-course changes in T cells is very important for initiation of inflammation. Our data showed higher proportions of CD3⁺ T, CD4⁺ T, and CD8⁺ T cells in epididymal fat pads of mice fed with HFD, with more significant increase in CD8⁺ T cells. Our results agree with those of other studies, which demonstrated that T lymphocytes may contribute to obesity-induced adipose tissue inflammation. CD8⁺ T cells are important components in initiation of inflammation in adipose tissues (Nishimura et al., 2009). CD8⁺ T cells increase
macrophage population, and their deletion results in decreased number of macrophages in adipose tissues and TNF-α and IL-6. Our data showed that after HFD feeding, CD8+ T cells significantly increased, indicating that such cells contribute to inflammation of adipose tissues.

Although CD4+ T cells are possibly essential in development of adipose tissue inflammation, Th cells playing dominant roles should still be determined (Cildir et al., 2013, Han and Levings, 2013, Watanabe et al., 2013). Therefore, we further investigated immune profiles of CD4+ T lymphocyte subsets in adipose tissues of obese mouse fed with HFD. We observed that HFD induced progressive Th1 and Th17 biases. These findings suggest that profiles of CD4+ T lymphocyte subsets may change from Th2 to Th1 and Th17 state with progression of obesity; such changes are favorable for inflammation development. Concordant with our results, some previous studies showed immune dysregulation by disruption of balance between Th1 and Th2 cells in white adipose tissue (WAT) (Kaminski and Randall, 2010, Rocha et al., 2008).

Th1 cells are suggested to be one of the important cells responsible for adipose inflammation and insulin resistance. Th17 secretes IL-17 and activates c-Jun N-terminal kinase, which is responsible for serine phosphorylation of insulin receptor substrate 1, leading to insulin resistance (Zuniga et al., 2010). Our data suggested that HFD induced progressive Th1 and Th17 biases in adipose tissues; such result may lead to phenotype switching of macrophages from anti-inflammatory to pro-inflammatory. In WAT, Th1 and Th17 cell biases may represent pathophysiological components of obesity, inflammation, and metabolic dysregulation (Winer and Winer, 2012).

In summary, our results support the hypothesis that in obese states, adipose-tissue-derived factors can disrupt...
Figure 3: Time-course changes in lipid and glucose metabolic components. (A) Changes in glucose of plasma of animals fed with HFD or NFD at different time points. (B) Changes in triglycerides in plasma of animals fed with HFD or NFD at different time points. (C) Changes in cholesterol in plasma of animals provided with HFD or NFD at different time points. (D) Changes in HDL-C in plasma of animals fed with HFD or NFD at different time points. (E) Changes in LDL-C in plasma of animals provided with HFD or NFD at different time points. Data are presented as means ± SEM of three experiments. *p < 0.05, **p < 0.01.
Figure 4: Time-course changes in T cells in epididymal fat pads of HFD and NFD mice. (A) Changes in CD3^+ T cells in epididymal fat pads of mice fed with HFD or NFD. (B) Changes in CD4^+ T cells in epididymal fat pads of mice fed with HFD or NFD. (C) Changes in CD8^+ T cells in epididymal fat pads of mice fed with HFD or NFD. (D) Changes in Th1 cells in epididymal fat pads of mice fed with HFD or NFD. (E) Changes in Th17 cells in epididymal fat pads of mice fed with HFD or NFD. (F) Changes in Th2 cells in epididymal fat pads of mice fed with HFD or NFD. Data are presented as means ± SEM of three experiments. *p < 0.05, **p < 0.01.
the balance of CD4+ T lymphocyte subsets, thus perpetuating inflammation within adipose tissues. Our data provide important information for research on inflammation in adipose tissues. Future studies are needed to investigate molecular mechanisms or environmental cues leading to Th1 and Th17 biases and to identify a way of modulating such processes for therapeutic strategies.

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