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Diet-induced obesity reduces the production of influenza vaccine-induced antibodies via impaired macrophage function

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Summary. – Obesity is a metabolic disease characterized by low-level chronic inflammation. Obese individuals are susceptible to infection by viruses, and vaccination against these pathogens is less effective than in nonobese individuals. Here, we sought to explore the immunological environment in a mouse model of obesity induced by a high-fat diet (HFD). HFD treatment increased the body weight and epididymal fat mass. The proportion of activated B cells, T cells, and macrophages was similar between mice in the HFD group and the regular-fat diet (RFD) group. The Th1 cell subpopulation in the HFD group was increased, whereas the proportion of Treg cells was reduced compared with the RFD group. Moreover, T-cell proliferation and cytokine production did not differ between the groups when cells were stimulated with anti-CD3 and anti-CD28 antibodies *in vitro*. In macrophages, phagocytic activity was higher in mice fed an HFD than in those fed an RFD, but expression levels of CD86 and MHC class II antigens were similar. When macrophages were cultured *in vitro*, the proportion of CD86-expressing macrophages was lower in those isolated from mice in the HFD group than in those isolated from the RFD group. Furthermore, lipopolysaccharide-induced interleukin 6 (IL-6) and tumor necrosis factor alpha secretions were significantly reduced in macrophages isolated from the HFD group. In addition, influenza vaccine-induced antibodies in the HFD group diminished more rapidly than in the RFD group. These results suggest that poor functionality of macrophages during obesity might contribute to a reduction in vaccine efficacy.

Keywords: high-fat diet; macrophage; obesity; vaccine efficacy

Introduction

Obesity is the accumulation of excessive body fat caused by hypertrophy and hyperplasia of adipocytes (Jo *et al.*, 2009). Adipose tissue is complex, composed of various cell types including adipocytes and immune cells, and it secretes adipokines to regulate nutrient homeostasis (Rosen and Spiegelman, 2006; Kanneganti and Dixit, 2012). In an obese environment, the population of immune cells in adipose tissue is increased, and more of them produce pro-inflammatory cytokines such as tumor necrosis factor alpha (TNF-α) and interleukin (IL) 6 than in the normal state, thereby inducing low-grade chronic inflammation (Hotamisligil *et al.*, 1995; Weisberg *et al.*, 2003). This inflammation is considered to be a cause of leptin and insulin resistance. Leptin is a multifunction cytokine that regulates immune cell behavior such as survival, proliferation, and differentiation (Loffreda *et al.*, 1998; Lord *et al.*, 1998; Mattioli *et al.*, 2005).

In mouse models of obesity such as those induced by diet, the leptin-deficiency genotype (*ob/ob*), and leptin receptor-deficiency genotype (*db/db*) are more susceptible to bacterial and viral infections (Webb *et al.*, 1976; Ikejima *et al.*, 2005;

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Abbreviations: CBV = cell-culture-based vaccine; CFSE = Carboxyfluorescein succinimidyl ester; DIO = diet-induced obesity; FITC = fluorescein isothiocyanate; HFD = high-fat diet; IL = interleukin; MHC = major histocompatibility; LPS = lipopolysaccharide; NP = nucleoprotein; PBSA = PBS bovine serum albumin; PBS = phosphate-buffered saline; RFD = regular-fat diet; TNF-α = tumor necrosis factor alpha

Smith *et al*., 2007). More obese people were hospitalized and died during the 2009 H1N1 influenza pandemic than did lean people (Cho and Nam, 2014). Moreover, vaccine-induced antibody production is reduced in obese mice compared with lean mice (Kim *et al*., 2009). Many studies have been conducted to determine the effect of obesity on immune responses. In obese mice, macrophage infiltration into adipose tissue is increased, and infiltrated macrophages are polarized to an M1 phenotype that produces pro-inflammatory cytokines causing systemic inflammation. In addition, macrophages isolated from obese rats showed lower phagocytic activity compared with those of normal rats (Plotkin and Paulson, 1996). The capacity for antigen presentation and allogeneic T-cell stimulation is reduced in dendritic cells from obese mice (Macia *et al*., 2006). However, these findings might not reflect the immune response capacity of obese humans because these genetic animal models of obesity showed disrupted leptin signaling.

Here we sought to analyze the overall functionality of immune cells in a diet-induced model of obesity. The activated states of macrophages, B and T cells, and the reactivity of T cells were similar in both normal and obese mice, whereas cytokine production by macrophages stimulated with lipopolysaccharide (LPS) was reduced in mice consuming a high-fat diet (HFD) compared with those consuming a regular-fat diet (RFD). Thus, it is possible that the reduced macrophage function might contribute to a reduction in vaccine efficacy.

Materials and Methods

Mice Four-week-old male C57BL/6 mice (Daehan Bio Link, Korea) were fed a 60% or 5% fat diet (Dooyeol Biotech, Korea) for 10 weeks. Mice (n = 3–5/group) were housed under specific-pathogen-free conditions and maintained according to protocols approved by the Institutional Animal Care and Use Committee, Sungsim Campus, Catholic University of Korea. Vaccination was conducted using 9 μg of cell-culture-based vaccine (CBV, SKYCellflu, SK Chemicals; Korea) by intramuscular injection after 10 weeks of the HFD. Body weights and food intake were checked, and serum samples were collected from a facial vein weekly.

Isolation and stimulation of peritoneal macrophages Peritoneal fluid was obtained by injecting cold sterile phosphate-buffered saline (PBS) into the peritoneal cavity using a 10 ml syringe, and then gently massaged to loosen cells. Peritoneal fluid was extracted on the opposite side of the mouse using an 18G needle, and the procedure was repeated three times. Peritoneal fluid was centrifuged at 800 × g for 5 min at 4°C, and the supernatant was discarded. The cell pellet was washed using PBS, incubated with red blood cell lysis buffer (Becton Dickinson (BD) Bioscience, USA) for 2 min, centrifuged at 1500 rpm for 5 min at 4°C, and washed twice with PBS. To determine the state of macrophage activation, cells were stained with fluorescent-labeled antibodies against F4/80, CD86, and MHC class II molecules (BD Bioscience), and measured by flow cytometry using a FACS Canto II analyzer (BD Bioscience) and FlowJo software (FlowJo, USA). Phagocytic activity was measured using a phagocytosis assay kit (IgG-FITC) from Cayman Chemicals (USA). Cells were cultured in RPMI 1640 medium (HyClone, USA) containing 10% fetal bovine serum (FBS; HyClone), antibiotic-antimycotic agents (Gibco, USA) (complete medium) with or without LPS (Sigma-Aldrich, USA) for 24 hr, and secreted cytokines in the supernatant were measured using specific ELISAs.

Culture of splenocytes Splenocytes were prepared as described (Socolovky *et al*., 2001). Cells were stained with fluorescent-labeled antibodies against CD19, CD69, CD4, CD8, IFN-γ, IL-4, CD25, and Foxp3 (BD Bioscience), and analyzed by flow cytometry. To measure the proportions of T-cell subsets, splenocytes were cultured in the presence of phorbol 12-myristate 13-acetate, ionomycin, and BD GolgiStop (BD Bioscience) for 4 hr. Cells were stained with CD4–FITC at 4°C for 30 min, washed with 3% FBS in PBS, fixed and permeabilized using a Cytofix/Cytoperm TM Fixation/Permeabilization Kit (BD Bioscience), and then intracellularly stained with prelabeled IL-4 or IFN-γ (BioLegend, USA). For T-cell stimulation, cells were incubated with antibodies against CD3 and CD28 or Influenza A virus (A/California/04/2009 (H1N1)) nucleoprotein (NP) peptide (ASNENVETM) for 48 hr (eBioscience, USA), and supernatants were used for quantifying cytokines by ELISA. Cells were stained with CFSE (eBioscience) and cultured in the presence of anti-CD3 and anti-CD28 antibodies for 4 days, and the proportion of mitotic cells was determined by flow cytometry.

Enzyme-linked immunosorbent cytokine assay Mouse IFN-γ, IL-4, IL-2, IL-6, and TNF-α ELISA kits were purchased from eBioscience, and cytokines in the culture supernatant were measured as described in the manufacturer's protocol. To titrate influenza-specific antibodies, sera were prepared weekly, and samples were prepared by serial dilution from 1:1,000 to 1:64,000 in PBS containing 1% bovine serum albumin (PBSA). Enzyme immunoassay 96-well plates (Corning Life Sciences, USA) were coated with 1 μg of CBV (SK Chemicals) at 4 ß°C overnight. Nonspecific binding sites were blocked with 1% PBSA for 1 h at room temperature (RT), and then 50 (l of an arbitrary standard was added (serum from one of the mice vaccinated against CBV). Samples were loaded into each well and incubated at 4°C for 24 hr. Plates were washed three times with PBS containing 0.05% Tween 20 and horseradish peroxidase-conjugated goat anti-mouse IgG was added (Bethyl Laboratories, USA) at RT for 1 hr. After washing five times, plates were developed using a chromogenic 3,3',5,5'-tetramethylbenzidine substrate (BD Bioscience), and the reaction was stopped with 2N H_2SO_4. Optical density was determined at 450 nm using a Multiskan EX spectrophotometer (Thermo Fisher Scientific, USA).

Statistical analysis Statistical analysis and graphic presentation were conducted using GraphPad Prism 5.01 software (GraphPad, USA). All data are presented as the mean ± standard error of the mean. Differences between means were determined using Student's *t* test and one-way analysis of variance (ANOVA); $P < 0.05$ was regarded as significant.

Results

The activation state of freshly isolated immune cells from obese mice was similar to those from non-obese mice

To generate a model of diet-induced obesity (DIO), we fed mice a 60% HFD and checked body weight and food intake weekly, with time 0 indicating the start point of feeding. Although overall food intake was lower in mice from the HFD group (data not shown), the mean body weight increased significantly compared with mice from the RFD group. After 10 weeks, epididymal fat mass in mice fed the HFD had increased four-fold compared with the RFD group, and the overall fat mass of mice fed the HFD was increased. The liver weights were similar between mice fed the HFD and RFD, but the HFD induced the accumulation of lipid droplets in hepatocytes (Fig. 1a,b; Supplemental Fig. S1). To compare the state of activation of immune cells between diets, we analyzed activated B cells, T cells, and macrophages from the mice of both groups. The percentage of cells expressing CD69, an early marker of activation in B and T cells, was similar in both groups (Fig. 1c). The population of Th1 cells in mice from the HFD group was higher, but the population of Th2 cells was not, compared with the RFD group. Incidentally, the population of Treg cells in mice from the HFD group was slightly reduced compared with the RFD group (Fig. 1d). As found for CD69 in T and B lymphocytes, the proportion of macrophages expressing CD86 was similar in mice from both groups (Fig. 1e).

T cells isolated from obese animals reacted normally to in vitro stimuli

T cells are important components of the cellular and humoral immune responses (Janeway *et al*., 2001). To analyze the activity of T cells in obese mice, we isolated splenocytes from mice fed either diet and investigated their capacity for proliferation and cytokine production in response to CD3 and CD28 stimulation *in vitro*. Carboxyfluorescein succinimidyl ester (CFSE)-labeled T cells from mice fed either diet were stimulated with CD3 and CD28

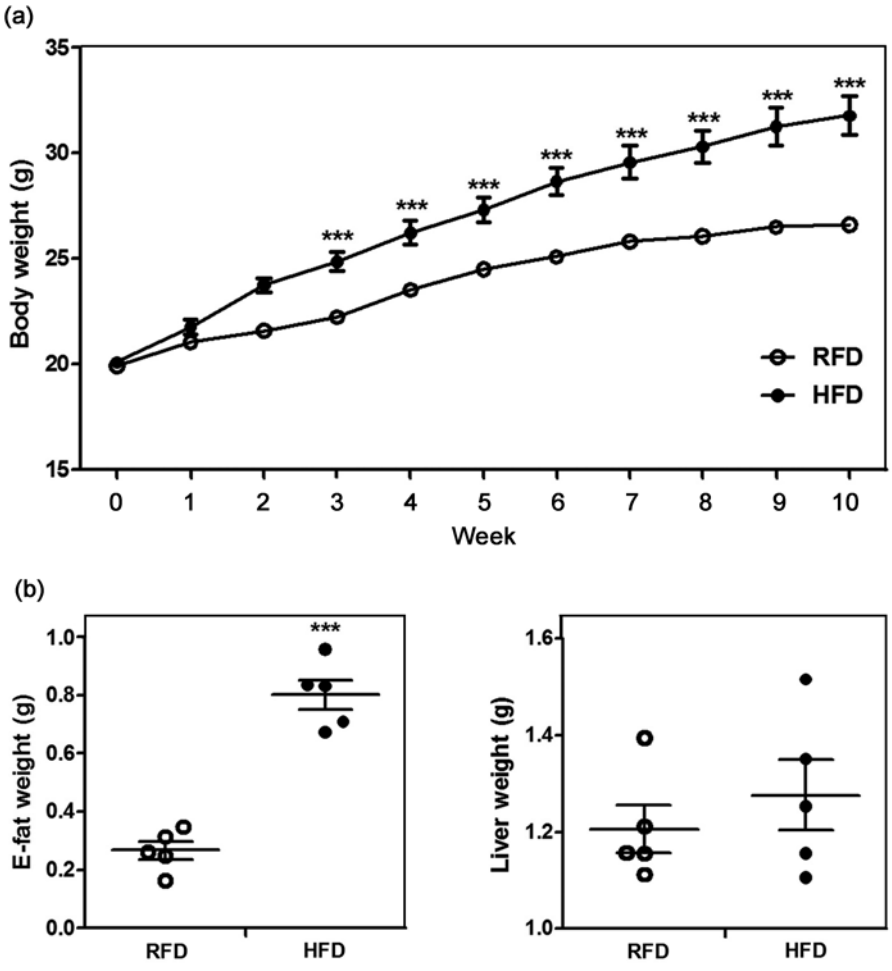

Fig. 1

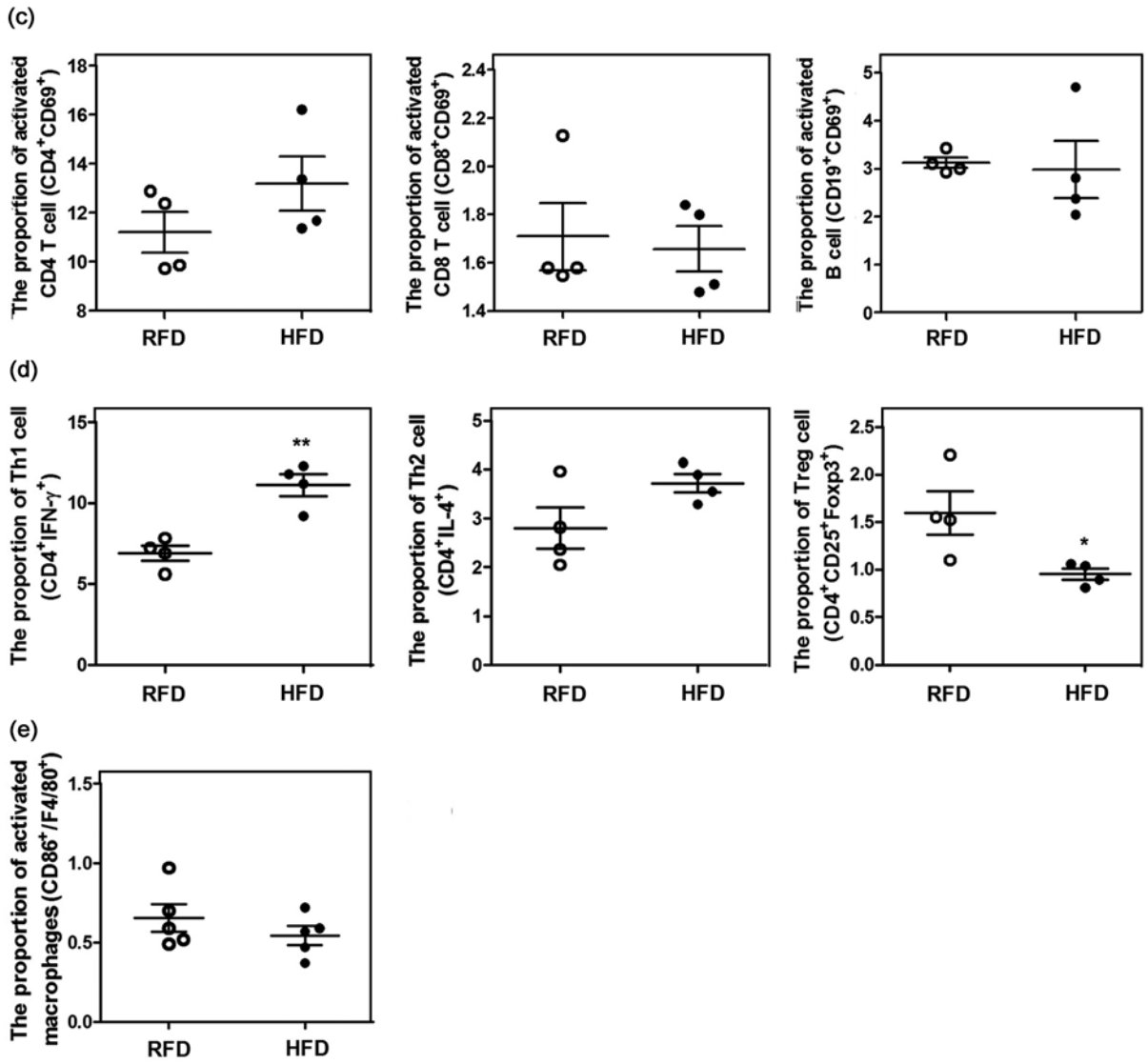

Fig. 1

The HFD increased both body and epididymal fat weights but had no effect on the steady state of immune cells

C57BL/6 mice were fed a 60% (HFD) or 5% (RFD) fat diet for 10 weeks. (a, b) Changes in body weight (a) and in the weights of epididymal fat and liver at 10 weeks (b) were measured (n = 5/group). (c) Freshly isolated splenocytes from both groups were stained with fluorescent-labeled antibodies against CD4, CD8, CD19, and CD69, and then analyzed by flow cytometry to determine the proportions of activated populations. (d) Splenocytes were labeled with antibodies against CD4, IL-4, IFN-γ, CD25, and Foxp3, and each subpopulation was analyzed as described in (c). (e) The proportions of CD86-expressing peritoneal macrophages freshly isolated from both groups of mice were measured by flow cytometry. The data were analyzed using Student's *t* tests; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

antibodies for 4 days, and the proportion of mitotic cells was compared. Fig. 2a shows that the proliferative capacity of CD4 T cells was similar for cells from mice in both groups. Moreover, the production of interferon gamma (IFN-γ), IL-2, and IL-4 wasß increased by stimulation but did not differ significantly between splenocytes from mice in either group (Fig. 2b).

Functionality of macrophages was diminished after diet-induced obesity

Macrophages are phagocytes and are at the forefront of the host's defense against infection (Janeway *et al.*, 2001). First, we compared the phagocytic activity of peritoneal macrophages isolated from mice in both groups. Unexpectedly, macrophages

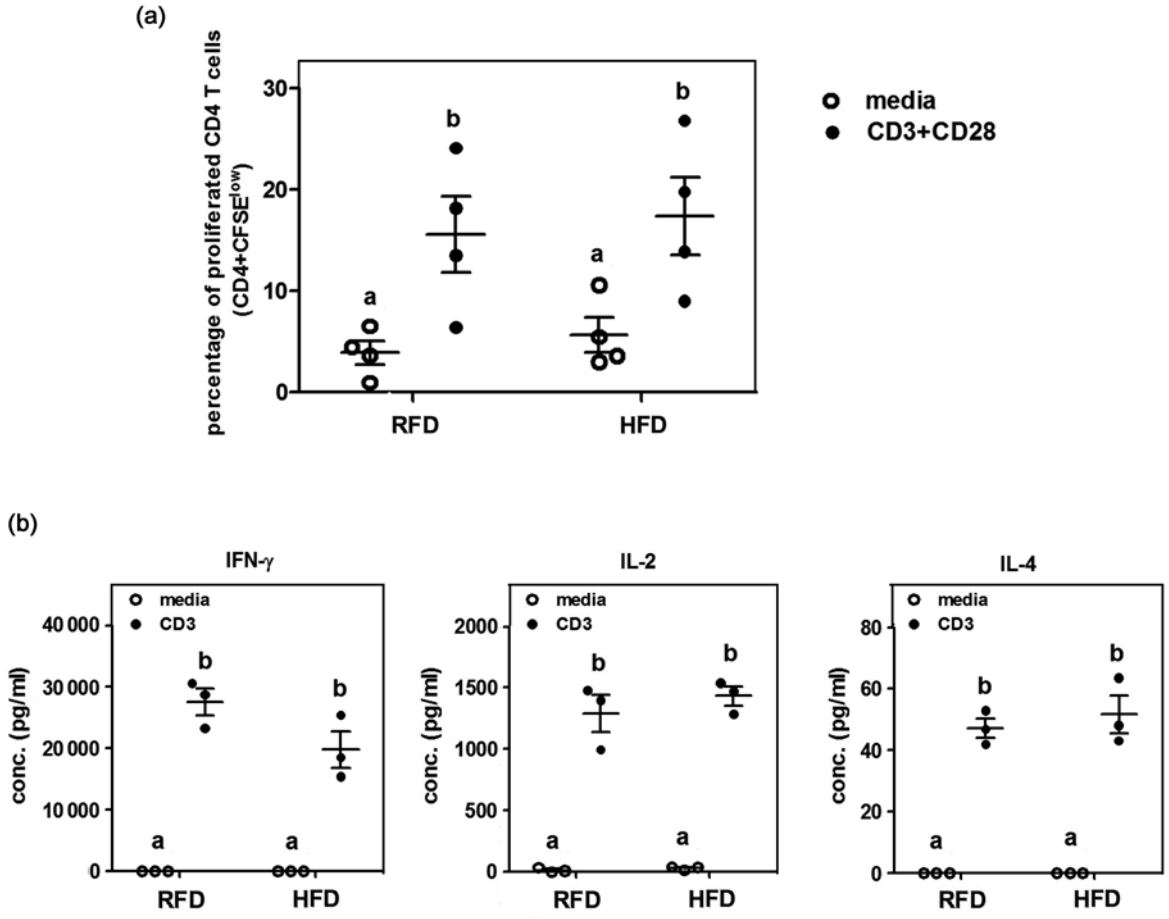

Fig. 2

The HFD had no effect on T-cell responsiveness to CD3 or CD28 stimulation

(a) CFSE-labeled splenocytes from HFD and RFD groups were cultured with anti-CD3 and anti-CD28 antibodies (1 μg/ml) for 4 days, and then the percentages of proliferative $CD4^+$ cells were analyzed using flow cytometry. (b) Splenocytes from the both groups were cultured with anti-CD3 and anti-CD28 antibodies (1 μg/ml) for 48 hr. The concentrations of IFN-γ, IL-2, or IL-4 in supernatants were measured using specific ELISAs.

from mice in the HFD group took up more latex beads coated with fluorescein isothiocyanate (FITC)-labeled rabbit IgG than those from the RFD group (Fig. 3a), suggesting that the phagocytic activity of macrophages from mice in the HFD group was higher than those from the RFD group. However, the level of expression of major histocompatibility (MHC) class II antigens on the macrophage surface and the proportion of macrophages expressing MHC class II molecules were similar in both groups (Fig. 3b). Interestingly, freshly isolated macrophages showed very low levels of expression of CD86 (Fig. 1e), but CD86 expression levels were increased when cells were cultured *in vitro*, and were further increased by lipopolysaccharide (LPS) stimulation (Fig. 3c). However, the basal and the LPS-induced CD86 levels in macrophages from mice in the HFD group were reduced compared with those in macrophages from the RFD group (Fig. 3c). In addition, cytokine production of macrophages induced by LPS also decreased in the HFD group (Fig. 3d). Although BALB/c mice fed the HFD had a slightly higher body weight than the C57BL/6 mice fed the HFD, it was not dramatically increased compared with C57BL/6 mice (Supplemental Fig. S2 and Fig. 1a). However, the effect of the HFD on the activity of macrophages from BALB/c mice was the same as in C57BL/6 mice (Fig. 3e), meaning that the functionality of macrophages may be abnormal in obese animals regardless of the mouse strain.

Vaccination-induced antibody production was decreased in obese animals

We measured the antibody level in DIO mice vaccinated with an inactivated influenza vaccine, made using a cell culture method (SKYCellflu, SK Chemicals; Seongnam, S. Korea). After 10 weeks on a HFD, we injected cell-culture-based vaccine (CBV) into the inner thighs of the hind legs of mice and

measured serum levels of vaccine-specific total IgG antibodies by enzyme-linked immunosorbent assay (ELISA) weekly for 8 weeks. Although the concentration of specific antibodies was slightly higher in mice from the HFD group at 1 week, this reversed gradually and by 3 weeks was lower than that in the RFD group, whereas the serum antibody level in mice from the RFD group continued to increase for 5 weeks after vaccination (Fig. 4a). We could not detect a vaccine-specific T cell response in the spleen (data not shown). However, responsiveness to polyclonal activation of splenic T cells remained intact in the HFD group (Fig. 4b).

Discussion

Obesity is a metabolic disease that is continuing to increase worldwide. Since the pandemic of H1N1 influenza virus in 2009, the relationship between obesity and the pathogenicity of viral infection has been investigated intensively, showing that the protective immune response is diminished in obesity (Kim *et al.*, 2009; Karlsson *et al.*, 2010a,b; Sheridan *et al.*, 2012; Park *et al.*, 2014). A previous study showed that mice fed an HFD showed lower levels of neutralizing antibody titers against influenza vaccine than did RFD mice because of obesity-induced chronic inflammation (Park *et al.*, 2014). Moreover, humans with a high body mass index showed quick declines in anti-influenza antibody titers (Sheridan *et al.*, 2012). These low titers might be caused by defective T-cell function (Karlsson *et al.*, 2010a,b). In the present study, we examined the activity of immune cells isolated from mice induced to be obese with an HFD to clarify the immunological environment of obesity.

Neutralizing antibodies are the best effectors of host defense against viral infections. They prevent infection of cells by blocking the binding of viruses to cell surface receptors (Janeway *et al.*, 2001). Vaccine-induced antibody production is lower in obese mice than it is in normal mice (Kim *et al.*, 2009; Park *et al.*, 2014). Moreover, Sheridan *et al.* (2012) also showed that antibody production was intact but maintenance was deficient in obese individuals. Here we found that the initial serum antibody concentration was similar in mice fed either diet, but the rate of increase was lower in mice from the HFD group and the rate of degradation was similar in both groups. These findings indicate that insufficient production of antibody significantly influenced the serum level of antibodies between mice fed either diet. Although B cells are the main producers of antibodies, many other immune cells are involved in antibody

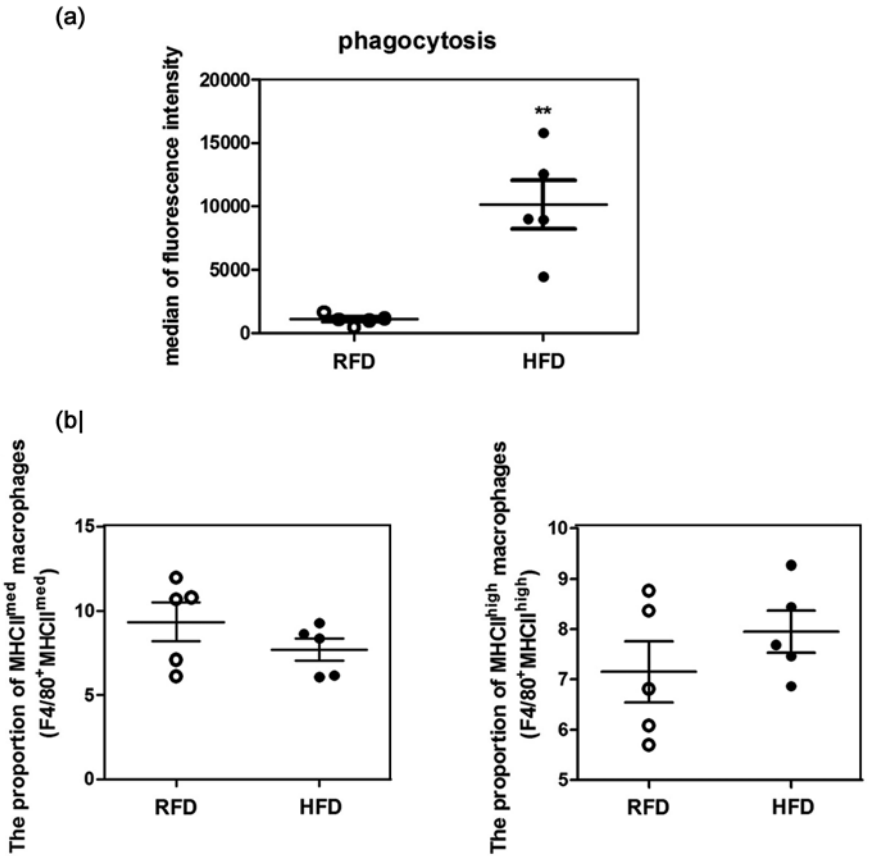

Fig. 3

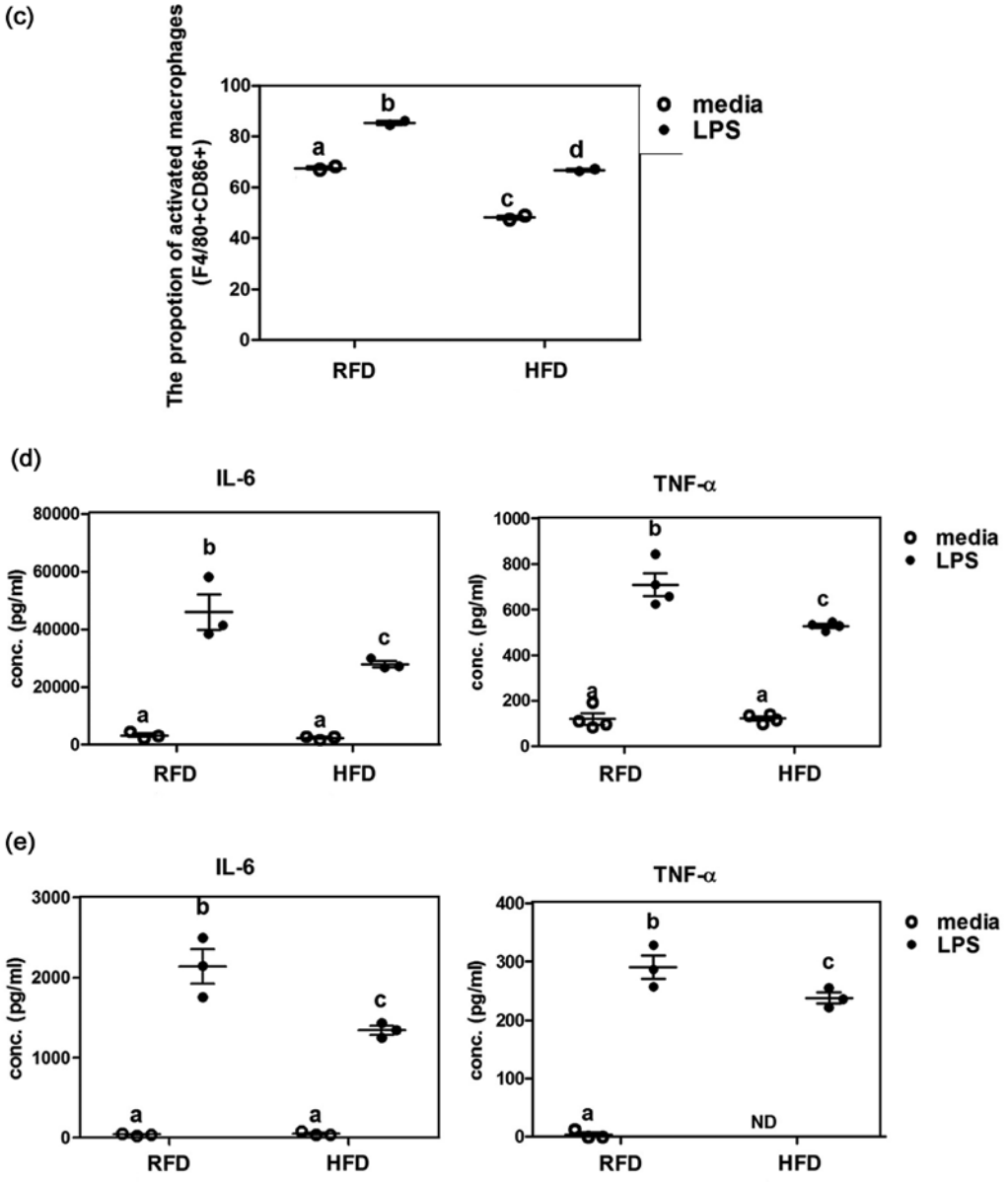

Fig. 3

Activation of macrophages by LPS and cytokine secretions were decreased in the HFD group

(a) Peritoneal macrophages from HFD and RFD groups were cultured in the presence of FITC-conjugated rabbit IgG for 24 hr, and phagocytic activities (FITC levels) were measured by flow cytometry. (b) Macrophages were stained with an anti-MHC class II (I-A/I-E) antibody, and the proportions of macrophages expressing MHC class II molecules were compared. (c, d) Macrophages were cultured with LPS (100 ng/ml) for 24 hr. The percentages of activated macrophages ($CD86^+$, c), pooled from five mice, were measured using flow cytometry, and the concentrations of IL-6 and TNF-α (d) in the supernatant were measured using specific ELISAs. (e) BALB/c mice were fed a 60% (HFD) or 5% (RFD) fat diet for 10 weeks. Cytokine production levels by LPS-stimulated macrophages were measured as in (d). The data were analyzed using Student *t* test; $^{**}P < 0.01$ and a one-way ANOVA followed by a Tukey post-hoc test; different letters indicate statistically significant differences between the groups.

production. Macrophages and dendritic cells take up antigen and produce peptides via antigen processing, and then present these peptides to T cells through an MHC-peptide complex. Activated T cells differentiate to form effector or memory cells and help other cells by secreting cytokines and costimulatory molecules, such as CD40. When a T cell encounters a cognate B cell, they help in the proliferation and differentiation of that B cell, which ultimately becomes a differentiated plasma cell producing antibodies (Janeway *et al.*, 2001). We found that the activation state of immune cells in obese mice did not differ

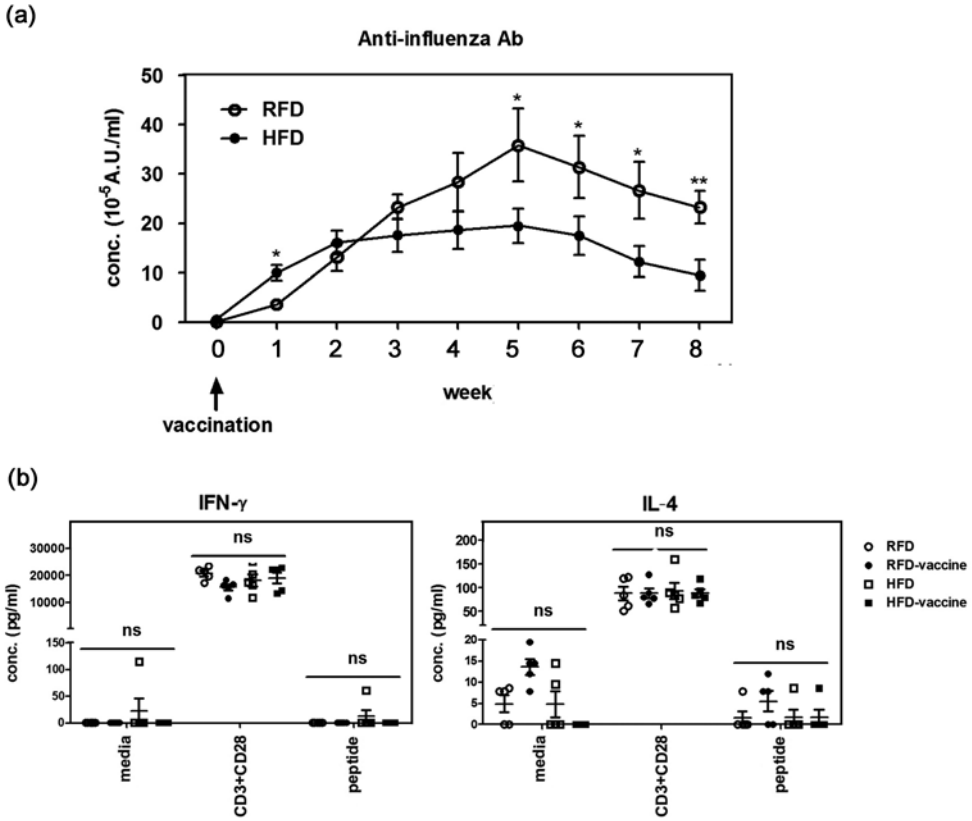

Fig. 4

Vaccine-induced antibody production was reduced in DIO mice

C57BL/6 mice were fed an HFD or an RFD for 10 weeks. (a) Antigen-specific total IgG was measured in sera from the both groups vaccinated with CBV at 10 weeks. (b) Reactivity to the anti-CD3 and anti-CD28 antibodies (1 μg/ml), or influenza NP peptide (1 μg) of T cells was analyzed by of IFN-γ, IL-4 ELISAs. The data were analyzed using Student *t* test; **P* <0.05; ***P* <0.01; ****P* <0.001.

significantly from those of lean mice and that the reactivity of T cells was similar in both groups. Even though we did not observe an antigen-specific response, we conclude that T cells in DIO mice have normal activity. The influenza-induced memory T cell response is reduced in DIO animals (Karlsson *et al*., 2010a,b). However, this might be the result of other influences – such as reduced antigen presentation or specificity of the antigen – rather than T-cell intrinsic activity.

Unlike T cells, macrophages from mice fed the HFD or RFD exhibited many functional differences. Macrophages are dedicated phagocytes and antigen-presenting cells, and play roles in the innate and adaptive immune systems. Following infection, macrophages not only take up and present antigens but also secrete proinflammatory cytokines including TNF-α and IL-1 to activate immune cells and kill pathogens (Murray and Wynn, 2011). Here, the population of activated macrophages *in vivo* seems to be unchanged between mice in the HFD and RFD groups. However, phagocytic activity *in vitro* was higher in macrophages from mice in the HFD group, whereas MHC levels were similar in both groups. These *in vivo* data do not seem to be matched by the *in vitro* data. It is a limitation of the study that macrophage phagocytic activity was not directly measured *in vivo*. Moreover, the population of activated macrophages does not directly represent phagocytic activity. Thus, this finding suggests that antigen presentation might be unchanged by obesity. However, when macrophages were stimulated with LPS, cytokine production was lower in mice in the HFD group compared with those from the RFD group. This indicates that although macrophage activity *in vivo* was seemingly unchanged, obesity-induced chronic inflammation might induce macrophage tolerance, leading to inactivation of macrophages despite the presence of an external stimulus such as LPS or vaccine. Moreover, the HFD group showed increased proportions of Th1 cells and reduced proportions of Treg cells, which might contribute to the induction of inflammation. Taken together, obesity could induce an abnormality in macrophages that can influence T cell behavior, thereby affecting antibody production. We could not obtain direct evidence of any reduced ability of macrophages from the HFD group in activated T cells. Further study is needed

to determine the exact mechanism of the inhibitory effect of obesity on the vaccine-induced humoral immune response.

In summary, we found that obesity induced by an HFD did not affect the phenotype of immune cells in a steady state, but it reduced CD86 expression and cytokine production of activated macrophages, which might contribute to a decreased immune response against vaccine. Therefore, recovery of macrophage functionality might help in increasing the efficacy of vaccination in obese individuals.

Supplementary information is available in the online version of the paper.

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