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Intermittent Fasting Modulates Immune Response by Generating Tregs via TGF-β Dependent Mechanisms in Obese Mice with Allergic Contact Dermatitis

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Abstract

People with obesity maintain low levels of inflammation; therefore, their exposure to foreign antigens can trigger an excessive immune response. In people with obesity or allergic contact dermatitis (ACD), symptoms are exacerbated by a reduction in the number of regulatory T cells (Tregs) and IL-10/TGF-β–modified macrophages (M2 macrophages) at the inflammatory site. Benefits of intermittent fasting (IF) have been demonstrated for many diseases; however, the immune responses regulated by macrophages and $CD4^+$T cells in obese ACD animal models are poorly understood. Therefore, we investigated whether IF suppresses inflammatory responses and upregulates the generation of Tregs and M2 macrophages in experimental ACD animal models of obese mice. The IF regimen relieved various ACD symptoms in inflamed and adipose tissues. We showed that the IF regimen upregulates Treg generation in a TGF-β-dependent manner and induces $CD4^+$T cell hypo-responsiveness. IF-M2 macrophages, which strongly express TGF-β and inhibit $CD4^+$T cell proliferation, directly regulated Treg differentiation from $CD4^+$T cells. These results indicate that the IF regimen enhances the TGF-β-producing ability of M2 macrophages and that the development of Tregs keeps mice healthy against ACD exacerbated by obesity. Therefore, the IF regimen may ameliorate inflammatory immune disorders caused by obesity.

Key Words: Obesity, Inflammation, Intermittent fasting, Allergic contact dermatitis, Regulatory T cells, M2 macrophage

INTRODUCTION

Allergic contact dermatitis (ACD), an inflammatory disease of the skin, is common in children but occurs at any age and is associated with hypersensitivity to the skin, including redness and intense pruritus (Belloni Fortina *et al*., 2020; McSweeney *et al*., 2020). Allergens captured by antigen-presenting cells (APCs) cause the differentiation from normal T helper (Th0) cells to Th2 cells in the acute phase of ACD and induce the differentiation into Th1 cells during the chronic phase to secrete various inflammatory cytokines (Chen *et al*., 2004). In a normal state, the immune response is maintained by balancing the interaction between Th1 and Th2 cells. However, in ACD, the conversion of T lymphocytes to Th2 cells is promoted, resulting in increased levels of inflammatory cytokines and blood immunoglobulin E (IgE). In addition, the increased IL-4, which inhibits the activity of Th1 cells, decreases cell-mediated immunity and further promotes the inflammatory response (Chai *et al*., 2022).

$CD4^+$T cells activated by cell-to-cell contact with APCs are differentiated into various Th cells by specific cytokines. Th1 cells differentiate due to IFN-γ or IL-12 and release IFN-γ to regulate cell-mediated immune or inflammatory responses (Biedermann *et al*., 2004). Th2 cells differentiated due to IL-4 release IL-4 and IL-5 to regulate humoral immunity or allergy (Brandt and Sivaprasad, 2011). Th17 cells differentiated by IL-1β or IL-6 release IL-17 and IL-22 to regulate autoimmune responses (Noack and Miossec, 2014). Regulatory T cells (Tregs) differentiated by TGF-β express CD25 on the cell surface, have the transcription factor forkhead box P3 (Foxp3), and release IL-10 and TGF-β (Schmidt *et al*., 2012). In addition, Tregs maintain immune homeostasis by regulating vari-

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ous inflammatory reactions. In people with obesity or ACD, the number of Tregs is reduced at the inflammatory site, which further exacerbates the symptoms of the disease (Wagner *et al*., 2013; Balmert *et al*., 2017).

Obesity refers to a state in which excess energy is stored as fat because energy intake is higher than energy consumption. Obesity can cause diseases such as asthma, cardiovascular disease, and diabetes (Goran *et al*., 2003; Farhat *et al*., 2021). Adipose tissues (ATs) contain various immune cells, including T cells generated from the spleen or lymph node (LN), which modulate the immune response and, in turn, influence the metabolism in ATs (Kohlgruber *et al*., 2016). In white adipose tissues (WATs) in people with obese, M1 macrophages that can produce inflammatory cytokines are increased, and M2 macrophages or Tregs with the immunomodulatory ability are decreased (Zhuge *et al*., 2016).

Intermittent fasting (IF) periodically limits food intake, which may help prevent or treat chronic conditions such as metabolic and cardiovascular disease (Albosta and Bakke, 2021). IF includes various methods such as time-restricted feeding (TRF) and alternate-day fasting (ADF). TRF is a method of limiting energy intake during certain times of the day (Moro *et al*., 2016). In contrast, ADF, a more strict diet regimen often practiced in clinical settings, is a method of alternating eating and fasting days (Varady and Hellerstein, 2007). Recent studies have shown that IF alleviates symptoms of inflammatory bowel disease (IBD) by increasing intestinal Tregs and decreasing Th17 cells and alleviates cardiovascular disease by reducing plasma lipid levels and inflammatory responses (Cignarella *et al*., 2018; Rangan *et al*., 2019). In addition, IF reduced the prevalence of prostate and endometrial cancers through increased plasma adiponectin levels, decreased inflammatory cytokine expression, and regulation of reactive oxygen species (ROS) levels (Zeng *et al*., 2015). Although the beneficial effects of IF have been demonstrated in many diseases, the mechanisms regulating the inflammation and immunity directed by macrophages and $CD4^+$T cells in obese ACD animal models are poorly understood. Therefore, we investigated whether obesity exacerbates the symptoms of ACD. We also investigated whether the IF alleviated ACD symptoms in obese mice by increasing the number of $CD4^+Foxp3^+$Tregs and the anti-inflammatory efficacy of M2 macrophages.

MATERIALS AND METHODS

Please see supplemental data for more information.

Experimental animals

C57BL/6 mice (6 weeks old) were purchased from Samtako Bio (Osan, Korea) and were maintained under specific pathogen-free conditions in the animal facility of Jeju National University (Jeju, Korea). All animal experiments and animal care were approved by the Jeju National University Animal Care and Use Committee.

IF regimen and disease models

To induce experimental ACD in obese mice, they were fed either ad libitum ND (ND-AL; 18% fat, PMI Nutrition International, St. Louis, MO, USA) or 45% HFD (HFD-AL; Research Diets, NJ, USA) for 108 days. Mice in the IF group (HFD-IF) were subjected to a 2:1 IF regimen, comprising one-day fasting followed by two days of feeding on days 40 to 108 after 45% HFD intake. Eighty days after obesity induction, the mice were sensitized by treating their abdomen with 1% 2,4-Dinitrochlorobenzene (DNCB, 200 μL; Tokyo Kasei Kogyo Co. Ltd., Tokyo, Japan). After seven days, the mice were re-sensitized by applying 0.3% DNCB (50 μL) to the ears every other day for 21 days. The mice were sacrificed on Day 109 (Fig. 1A).

RESULTS

IF regimen inhibits the development of experimental allergic contact dermatitis (ACD) in obese mice

Among the various intermittent fasting (IF) regimens, the 2:1 IF regimen consists of repeated two-day meals and one-day fasts to offer sufficient time to solve the nutritional deficiencies caused by fasting. Therefore, many studies have shown that the 2:1 regimen is used as isocaloric IF to improve obesity (Kim *et al*., 2017). IgE, which activates mast cells, is a crucial target in the treatment of inflammatory diseases (Ettinger *et al*., 2017). Compared with the 45% HFD-fed (HFD-AL) group, the normal chow diet (ND-AL) and 45% HFD-fed IF (HFD-IF) groups showed significantly decreased serum IgE levels (Fig. 1B). The LNs of patients with atopic dermatitis develop swelling owing to excessive immune cell infiltration (Elentner *et al*., 2009). LNs from HFD-AL group mice were swollen, whereas those from ND-AL and HFD-IF mice were smaller (Fig. 1C). Compared with the HFD-AL group, the ND-AL and HFD-IF groups showed reduced immune cell infiltration and ear thickness (Fig. 1D, 1E). We also examined whether the IF regimen regulates the expression of genes related to Th cells or Tregs in the LNs. Compared with the HFD-AL group, ND-AL or HFD-IF group showed significantly reduced IL-2, TNF-α, IFN-γ, IL-17A, IL-4 and IL-5 mRNA levels. In addition, the IF regimen increased TGF-β and Foxp3 mRNA levels (Fig. 1F). We also analyzed Foxp3 protein expression in the ear and LN. Compared with the ND-AL and HFD-AL groups, the HFD-IF group exhibited an elevated Foxp3 expression (Fig. 1G, Supplementary Fig. 1A, 1B).

IF regimen inhibits the formation of white adipose tissue (WAT) and induces Treg development in WAT of obese mice with ACD

In obesity, WAT is over-formed, which exacerbates the occurrence of inflammation (Park *et al*., 2014). Therefore, we analyzed the weight and shape of WAT. Compared with the HFD-AL group, the HFD-IF group displayed decreased body weight, abdominal WAT weight (Fig. 2A-2C), and adipocyte size (Fig. 2D). Leptin, a hormone produced by adipocytes, plays a crucial role in the development of obesity and regulates the generation of inflammatory cytokines (Matarese *et al*., 2005). Adiponectin is secreted at low levels by adipose tissue (AT) in people with obesity. It also reduces insulin resistance and has anti-inflammatory properties (Villarreal-Molina and Antuna-Puente, 2012). The HFD-IF group showed lower leptin concentrations and higher adiponectin concentrations than the HFD-AL group (Fig. 2E). Peroxisome proliferator-activated receptor γ (PPAR-γ) induces AT accumulation by elevating adipocyte proliferation (Lehrke and Lazar, 2005). Acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) regulate lipid formation and adipogenesis in visceral adipose

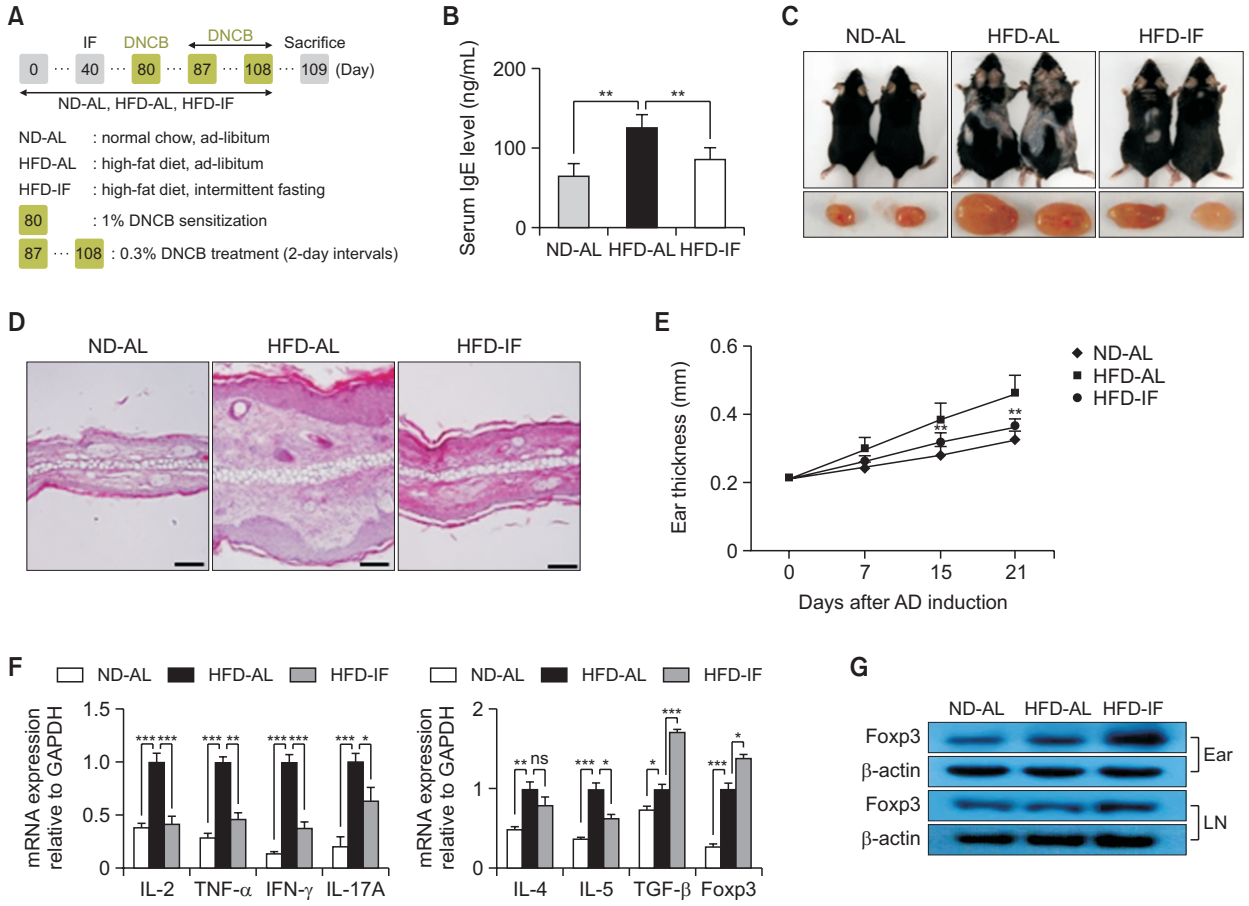

Fig. 1. IF regimen suppresses experimental ACD in obese mice. (A) Schematic for IF regimen and construction of mice disease models. (B) The IgE levels in serum were measured using ELISA. (C) Macroscopic views of the ears and LN, and (D) paraffin-embedded sections of ear tissue stained with H&E (Scale bar=0.1 mm). (E) Ear thickness was measured on Days 0, 7, 15, and 21. (F) Real-time PCR was used to measure the expression of mRNA for cytokines and transcription factors in LN. (G) The expression of Foxp3 was measured in ear and LN tissues using western blotting. (n=5 mice per group). Values represent the mean ± SD. *p<0.05; **p<0.01; and ***p<0.001. The full-length blots are presented in Supplementary Fig. 7.

tissue (Ussher and Lopaschuk, 2008; Wueest *et al*., 2010). Compared with the HFD-AL group, HFD-IF group showed significantly reduced PPAR-γ, ACC, FAS, IL-6, and TNF-α mRNA levels except for IL-1β and IFN-γ (Fig. 2F). In addition, western blot results revealed that the expression of Foxp3 was enormously increased in the HFD-IF group compared to that in the HFD-AL group (Fig. 2G, Supplementary Fig. 1C).

IF regimen induces the formation of brown adipose tissue (BAT) and upregulates Treg-related factors in BAT of obese mice with ACD

BAT is essential for preventing chronic diseases such as obesity and diabetes. BAT can modulate insulin sensitivity and susceptibility to obesity (Wang *et al*., 2015). Therefore, we analyzed the weight and shape of BAT. Compared with the HFD-AL group, the HFD-IF group showed a reduction in interscapular BAT weight (Fig. 3A, 3B) and suppressed lipid accumulation in BAT (Fig. 3C). In the HFD-AL group, the ratio of WAT among BAT increased, and the weight of BAT also increased. We also examined whether the IF regimen regulates the expression of genes related to Th cells or Tregs in the BAT. Compared with the HFD-AL group, the HFD-IF group showed reduced IL-2, IFN-γ, IL-5, IL-31, and IL-17A mRNA levels. In addition, the IF regimen elevated the mRNA levels of IL-10, TGF-β, Foxp3, cytotoxic T lymphocyte antigen-4 (CTLA-4), and granzyme B (GzmB) associated with Tregs (Fig. 3D). Additionally, confirming the Foxp3 expression in BAT by western blot, Foxp3 expression was enormously elevated in the HFD-IF group compared to that in the HFD-AL group (Fig. 3E, Supplementary Fig. 1D).

IF regimen facilitates the differentiation of $CD4^+Foxp3^+$T cells from $CD4^+$T cells in a TGF-β-dependent manner

T cells are augmented in the AT of individuals with obesity, and effector T cells, including $CD4^+$T cells, exacerbate obesity-related inflammation (Kaminski and Randall, 2010). Tregs can maintain immune homeostasis by regulating the inflammatory response of AT in obese mice; however, they are reduced in individuals with obesity (Panduro *et al*., 2016). We investigated whether the IF regimen regulates gene expression related to Th cells or Tregs from activated $CD4^+$T cells. Compared with the $CD4^+$T cells isolated from the HFD-AL group, $CD4^+$T cells isolated from the HFD-IF group showed reduced IL-2, IFN-γ, IL-4, IL-5, IL-31, and IL-17 mRNA levels and increased

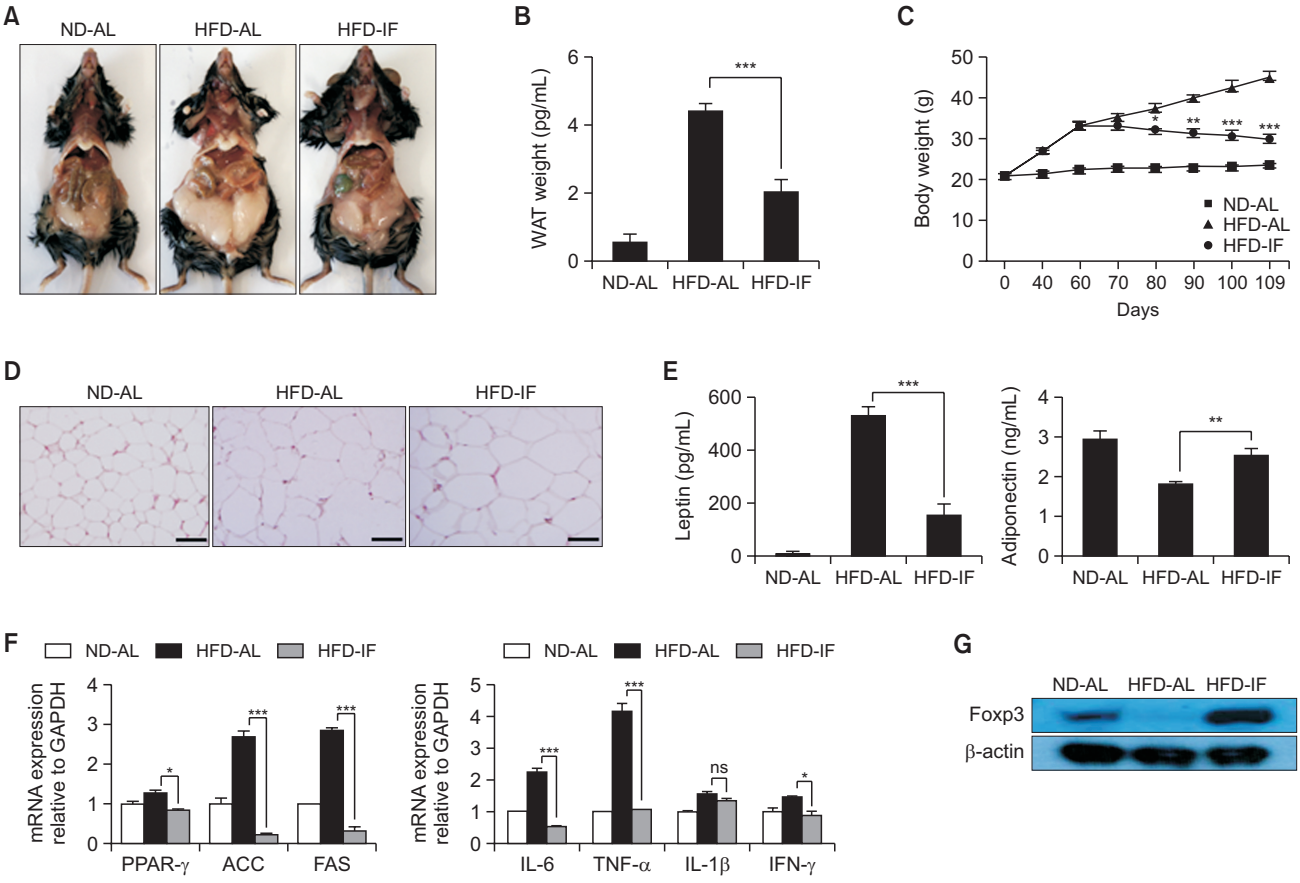

Fig. 2. IF regimen suppresses the WAT formation and induces Foxp3 expression in obese mice with ACD. (A) Macroscopic views of the visceral adipose tissues and (B) visceral adipose tissue weight (C) body weights were measured periodically or after the experiment. (D) Paraffin-embedded sections of visceral adipose tissues stained with H&E (Scale bar=0.1 mm). (E) The Leptin and adiponectin levels in serum were measured using ELISA. (F) Gene expression in visceral adipose tissues was measured using real-time PCR. (G) The expression of Foxp3 was measured in visceral adipose tissues using western blotting. (n=5 mice per group). Values represent the mean ± SD. *p<0.05; **p<0.01; and ***p<0.001. The full-length blots are presented in Supplementary Fig. 8A.

Foxp3, GzmB, TGF-β mRNA levels (Fig. 4A). Since TGF-β is known to promote differentiation from T cells to Tregs (Davidson *et al*., 2007), we investigated the mechanisms of the IF regimen and TGF-β in Treg differentiation. $CD4^+$T cells in the HFD-IF group represented significantly elevated TGF-β and Foxp3 mRNA levels by TGF-β stimulation (Fig. 4B). The TGF-β-treated $CD4^+$T cells of the HFD-AL and HFD-IF groups showed increased $CD4^+Foxp3^+$T cell populations than those without TGF-β (Fig. 4C, Supplementary Fig. 2A). In addition, compared to the HFD-AL group, $CD4^+Foxp3^+$T cell populations were significantly increased in a TGF-β-dependent manner in the HFD-IF group (p<0.01; 16.2 % *vs.* 26.3 %, Fig. 4C, Supplementary Fig. 2A). However, we recognized no differences in $CD4^+CD25^+$T cell populations between the two groups (Fig. 4D, Supplementary Fig. 2B). Furthermore, we compared $CD4^+$T cell proliferation between HFD-AL and HFD-IF groups. In the co-culture of $CD4^+CD25^+$Tregs (isolated from HFD-AL or HFD-IF groups) and normal $CD4^+$T cells (responder), the suppressive effect of Tregs on $CD4^+$T cell proliferation did not differ between the two groups (Fig. 4E, Supplementary Fig. 3A). In the co-culture of normal $CD4^+CD25^+$Tregs (suppressor) and $CD4^+$T cells (responder; isolated from HFD-AL or HFD-IF groups), no difference in $CD4^+$T cell proliferation was observed between the two groups (suppressors; Fig. 4F, Supplementary Fig. 3B, 3C). However, $CD4^+$T cells in the HFD-IF group were significantly more sensitive to suppression by Tregs than $CD4^+$T cells in the HFD-AL group in the presence of exogenous TGF-β (Fig. 4F, Supplementary Fig. 3D-3F).

IF regimen potently augments the ability of IL-10/TGF-β-modified M2 macrophages regulating Treg differentiation

We investigated the ability of IL-10/TGF-β-modified M2 macrophages by the IF regimen in the development of $CD4^+Foxp3^+$Tregs. MagniSort™ Mouse F4/80 Positive Selection Kit (Invitrogen Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. Spleens were removed aseptically from HFD-AL or HFD-IF mice and single-cell suspensions were prepared by removing red blood cells using 1x RBC lysis buffer. Splenocytes were harvested and the resulting cell suspension was filtered through 40 μm nylon mesh. Cells were incubated with MagniSort™ Positive Selection Antibody for 10 min followed by incubation in beads buffer for 10 min. For macrophage polarization, $F4/80^+$macrophages isolated from HFD-AL or HFD-IF mice were cultured in normal medium for 48 h to yield M0 macrophages, cultured with LPS (100 ng/mL) to yield M1 macrophages, or cultured with

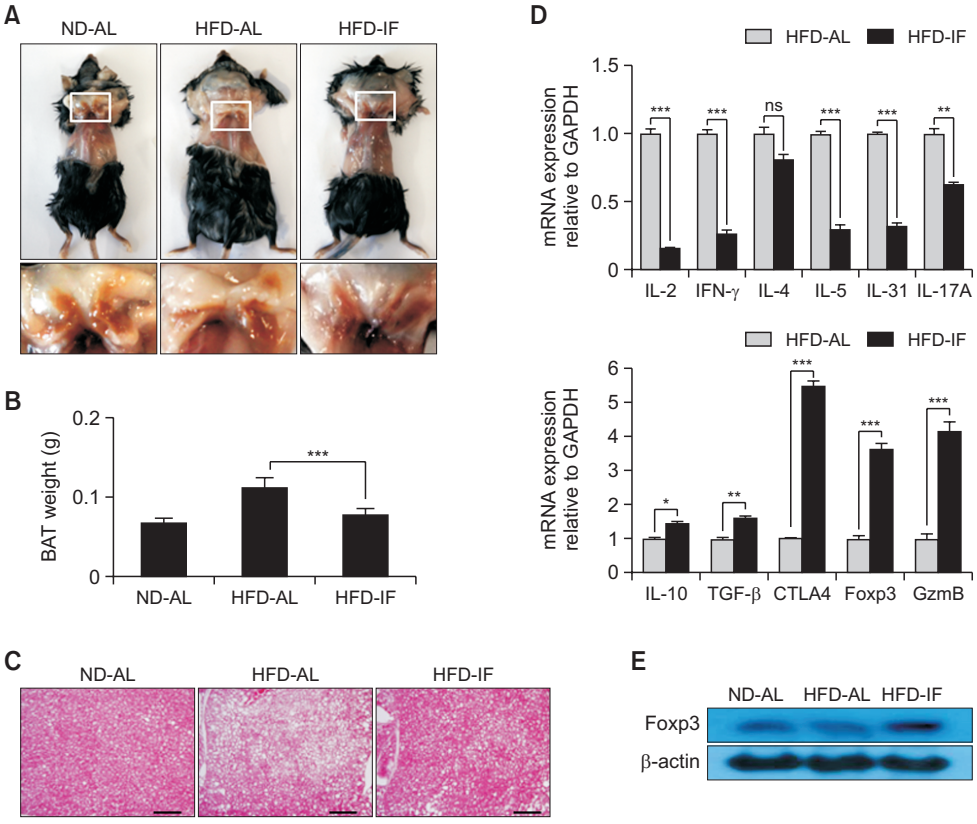

Fig. 3. IF regimen induces the BAT formation and upregulates Treg-related factors in obese mice with ACD. (A) Macroscopic views of the BAT and (B) BAT weight was measured after the experiment. (C) Paraffin-embedded sections of BAT stained with H&E (Scale bar=0.1 mm). (D) Gene expression in BAT was measured using real-time PCR. (E) The expression of Foxp3 was measured in BAT by western blotting. (n=5 mice per group). Values represent the mean ± SD. **p*<0.05; ***p*<0.01; and ****p*<0.001. The full-length blots are presented in Supplementary Fig. 8B.

IL-10/TGF-β (both at 10 ng/mL) to yield M2 macrophages. Cytokines expression in F4/80$^+$macrophages was analyzed using ELISA or real-time PCR. Compared with HFD-AL-M1 macrophages, HFD-IF-M1 macrophages suppressed the expression of IL-6 and TNF-α; however, there was no difference in other macrophages (Fig. 5A). In addition, HFD-IF-M1 or -M2 macrophages showed increased IL-10 or TGF-β mRNA levels than HFD-AL-M1 or HFD-AL-M2 macrophages (Fig. 5B). We then examined whether HFD-IF-M2 macrophages can regulate CD4$^+$Foxp3$^+$Treg differentiation. Macrophages isolated from HFD-AL or HFD-IF groups were polarized into M0, M1, and M2 macrophages and were co-cultured with CD4$^+$T cells in normal mice. Compared with HFD-AL-M0 macrophages, HFD-AL-M1 and -M2 macrophages significantly induced CD4$^+$Foxp3$^+$Treg differentiation (Fig. 5C, Supplementary Fig. 4A). In addition, except for HFD-IF-M1 macrophages, HFD-IF-M2 macrophages significantly induced CD4$^+$Foxp3$^+$Treg differentiation compared to HFD-IF-M0 macrophages (M2; p<0.05, Fig. 5C, Supplementary Fig. 4B), and HFD-IF-M2 macrophages influenced the generation of CD4$^+$Foxp3$^+$Tregs more strongly than HFD-AL-M2 macrophages (p<0.05, Supplementary Fig. 4E). In contrast, both groups of M0 macrophages and HFD-IF-M1 macrophages did not induce CD4$^+$T cell transformation into Tregs (Supplementary Fig. 4C, 4D). We evaluated whether the generation of CD4$^+$Foxp3$^+$Tregs by HFD-IF-M2 macrophages was caused by TGF-β or cell-to-cell contact. The neutralizing antibodies study showed that Foxp3 induced by HFD-IF-M2 macrophages was significantly reduced by neutralizing anti-IL-10 or neutralizing anti-TGF-β antibodies, and that anti-TGF-β antibody was more effective than anti-IL-10 antibody in foxp3 expression (Fig. 5D, Supplementary Fig. 5B). However, we recognized no differences in reducing Foxp3 expression by transwell assay (Fig. 5E, Supplementary Fig. 5A). We also investigated the role of polarized macrophages on normal CD4$^+$T cell proliferation. In both HFD-AL and HFD-IF groups, CD4$^+$T cell proliferation was strongly inhibited as the ratio of M2 macrophages increased (Fig. 5F, Supplementary Fig. 6A). CD4$^+$T cell proliferation increased as the proportion of M1 macrophages increased in both groups (Supplementary Fig. 6B, 6C), and decreased as the proportion of M2 macrophages increased (HFD-AL; ns, HFD-IF; p<0.001, Supplementary Fig. 6D, 6E). A comparison of M1 and M2 macrophages of two groups by the same ratio showed that HFD-IF-M1 or -M2 macrophages inhibited CD4$^+$T cell proliferation (Supplementary Fig. 6F-6H). In particular, at a ratio of 5:1, HFD-IF-M2 macrophages significantly suppressed CD4$^+$T cell proliferation than HFD-AL-M2 macrophages (Supplementary Fig. 6I). The inhibition of CD4$^+$T cell proliferation by HFD-IF-M2 macrophages may be due to the generation of Tregs and the expression of TGF-β.

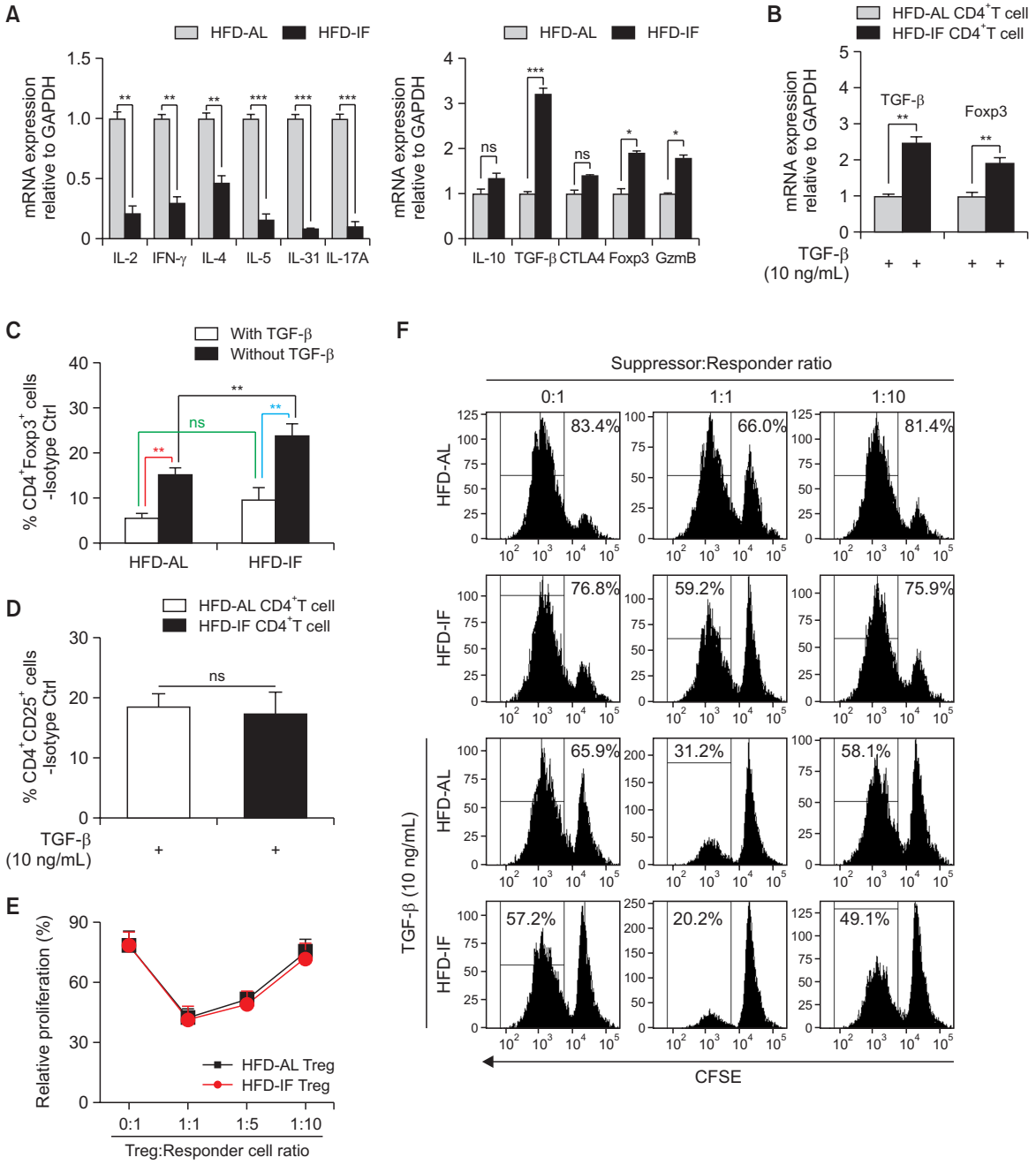

Fig. 4. IF regimen facilitates the differentiation of $CD4^+Foxp3^+$T cells from $CD4^+$T cells in a TGF-β-dependent manner. (A) Gene expression was measured using real-time PCR. (B-D) Cells were stimulated with anti-CD3/-CD28 antibodies in the presence or absence of TGF-β for 72 h. (E) $CD4^+CD25^+$ Tregs in each group were co-cultured with CFSE-labeled responder cells ($CD4^+CD25^-$T cells in normal mice) at different ratios. (F) The suppression sensitivity of CFSE-labeled $CD4^+CD25^-$T cells (responder; in each group) by $CD4^+CD25^+$ Tregs (suppressors; in normal mice) was measured by incubation with $CD4^+CD25^+$ Tregs at different ratios in the presence or absence of TGF-β for 72 h. (n=5 mice per group). Values represent the mean ± SD. **p*<0.05; ***p*<0.01; and ****p*<0.001.

DISCUSSION

Recent studies have consistently demonstrated the potent disease-preventing benefits of intermittent fasting (IF) regimens for various chronic diseases, including psoriasis, obesity, diabetes, and hypertension (Halagappa *et al*., 2007; Erdem *et al*., 2018). Nutrient excess increases the risk of developing early inflammation. In humans and rodents with obesity, inflammatory responses initiate in adipose tissues (ATs) and metastasize to immune tissues, triggering an inflammatory response (Chatzigeorgiou *et al*., 2012). Lipid accumulation in ATs in individuals with obesity promotes an immune response, leading to increased production of various inflammatory cytokines (Kojta *et al*., 2020). Here, we investigated how the im-

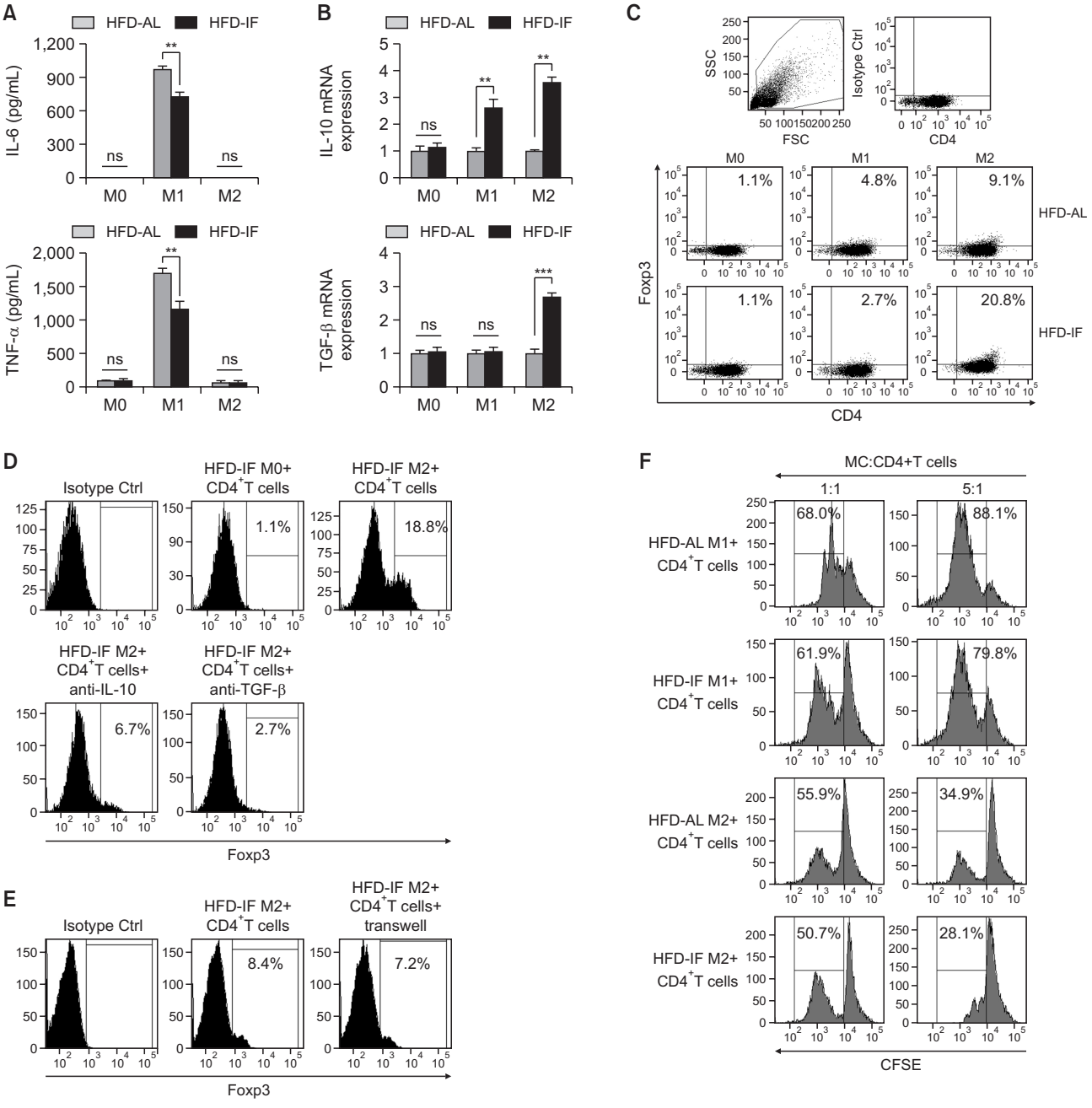

Fig. 5. IF regimen strongly augments the ability of IL-10/TGF-β-modified macrophages to induce the differentiation of $CD4^+Foxp3^+$ Tregs. (A, B) Cytokine expression was analyzed using ELISA or real-time PCR. (C) $CD4^+$T cells in normal mice were co-cultured with macrophages in each group and stimulated by anti-CD3 for seven days. (D, E) $CD4^+$T cells in normal mice and macrophages in HFD-IF mice were co-cultured in transwell chambers in the presence or absence of anti-IL-10 or anti-TGF-β-neutralizing antibodies for seven days. (F) CFSE-labeled $CD4^+$T cells were co-cultured different ratios with macrophages in each group and stimulated by anti-CD3/-CD28 for 72 h. (n=5 mice per group). Values represent the mean ± SD. Data are representative of three independent experiments. $^{**}p<0.01$; and $^{***}p<0.001$.

munomodulatory ability of the IF regimen affects the progression of experimental allergic contact dermatitis (ACD) in obese mice. Among the various mouse species, a suitable mouse for the animal model of ACD induced by DNCB stimulation is the BALB/c mouse, which is sensitive to the reactivity of Th2 cells. However, the C57BL/6 mice used in the experiment were susceptible to DNCB stimulation at low concentrations due to chronic low-grade inflammation caused by overnutrition by consuming a high-calorie diet over a long period. The IF regimen alleviated typical ACD symptoms, such as tissue hyperplasia, increased serum IgE levels, and overexpression of inflammatory cytokines. It increased the expression of Treg-related factors at the site of inflammation. In addition, the IF regimen is considered to be sensitive to immune responses

related to Th1 or Th17 cells because Th1/17 cytokines rather than Th2 cytokines are reduced in this model. Leptin, an adipocyte-derived hormone, secreted by ATs, exhibits high levels in individuals with obesity and can modulate immune cells (Horvath, 2005). It promotes the interaction between AT and immune cells. Leptin also promotes the expression of IFN-γ by inducing the differentiation of normal Th cells to Th1 cells rather than Th2 cells (Lord *et al*., 2002). According to a recent report, Tregs are accumulated preferentially in AT of lean people (Winer *et al*., 2009). However, in individuals with obesity, excessive leptin production adversely affects the anti-inflammatory function of Tregs (Wang *et al*., 2017). In this study, the IF regimen suppressed inflammatory cytokine production and increased Foxp3 expression in ATs (white adipose tissue; WAT and brown adipose tissue; BAT). These results may be due to decreased leptin concentration by the IF regimen. The IF regimen upregulated the number of Tregs in obesity ACD models, and the mitigating effect of the IF regimen was associated with overexpressed Tregs in the inflamed area. Tregs maintain immune homeostasis or suppress excessive immune responses (Campbell, 2015; Han *et al*., 2015). Recent studies in mice indicate an association of obesity with reduced Tregs in visceral AT (Feuerer *et al*., 2009). In this regard, the adoptive transfer of Treg into mice may alleviate adipose tissue inflammation and metabolic disease (Sakaguchi, 2005). The abundant Treg population increased by the IF regimen may inhibit $CD4^+$T cell proliferation in inflammatory sites and ATs, resulting in symptomatic relief in animal models of obesity-related ACD. TGF-β, an immunosuppressive cytokine, regulates excessive immune responses and induces differentiation of Tregs (Cook *et al*., 2021). The immuno-modulatory mechanism of the IF regimen increases the secretion of TGF-β and decreases inflammatory cytokine expression by immune cells. The IF regimen did not elevate $CD4^+CD25^+$Treg populations. However, it strongly upregulated the expression of Treg-related factors in $CD4^+$T cells and induced the differentiation of Tregs from $CD4^+$T cells in a TGF-β-dependent manner, thereby preventing further obesity and ACD. M2 macrophages induced due to the IF regimen regulate various immune factors, and there are many potential underlying mechanisms. Macrophages activate T cells by capturing antigens and then presenting them directly to T cells or secreting active substances such as cytokines (Friedl and Gunzer, 2001). M2 macrophages relieve chronic inflammatory kidney disease and regulate immune responses by remodeling the extracellular matrix (Wang *et al*., 2007). IL-10/TGF-β-modified M2 macrophages elevate Treg numbers in LNs or at sites of inflammation and boost Treg differentiation from $CD4^+$T cells (Savage *et al*., 2008). Therefore, we hypothesized that the IF regimen results in more potent Treg generation and inhibition of effector T cell proliferation by M2 macrophages. The IF regimen promoted M2 macrophages to express higher levels of IL-10 or TGF-β and induced M2 macrophages to generate higher numbers of Tregs. However, transwell assays showed that the IF regimen did not affect Treg generation via cell-to-cell contact between M2 macrophage and $CD4^+$T cell. Moreover, two findings were obtained regarding the suppression of $CD4^+$T cell proliferation by HFD-IF-M2 macrophages: first, HFD-IF-M2 macrophages suppress the proliferation of $CD4^+$T cells by generating Tregs from $CD4^+$T cells. Next, HFD-IF-M2 macrophages suppress the proliferation of $CD4^+$T cells by overexpressing TGF-β.

M2 macrophages are divided into M2a, M2b, and M2c subtypes, and we are currently trying to define the functions of various M2 macrophages in an obese ACD animal model. Because M2a macrophages activated by IL-4 or IL-13 produce chemokines that translocate immune cells to sites of inflammation (Tang *et al*., 2017), we aim to elucidate the role of M2a macrophages and the molecular mechanism of immunity between M2a macrophages and T cells in an ACD animal model exacerbated by obesity.

In conclusion, the IF regimen alleviated the symptoms of obesity and ACD aggravated by obesity by inhibiting the inflammatory response, regulating $CD4^+$T cell proliferation, and increasing Treg numbers by TGF-β-dependent or M2 macrophages. Therefore, the IF regimen may ameliorate inflammatory immune disorders caused by obesity.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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