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CTLA-4Ig immunotherapy of obesity-induced insulin resistance by manipulation of macrophage polarization in adipose tissues

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ABSTRACT

It has been established that obesity alters the metabolic and endocrine function of adipose tissue and, together with accumulation of adipose tissue macrophages, contributes to insulin resistance. Although numerous studies have reported that shifting the polarization of macrophages from M1 to M2 can alleviate adipose tissue inflammation, manipulation of macrophage polarization has not been considered as a specific therapy. Here, we determined whether cytotoxic T-lymphocyte-associated antigen-4IgG1 (CTLA-4Ig) can ameliorate insulin resistance by induction of macrophages from proinflammatory M1 to anti-inflammatory M2 polarization in the adipose tissues of high fat diet-induced insulin-resistant mice. CTLA4-Ig treatment prevented insulin resistance by changing gene expression to M2 polarization, which increased the levels of arginase 1. Furthermore, flow cytometric analysis confirmed the alteration of polarization from CD11c (M1)- to CD206 (M2)-positive cells. Concomitantly, CTLA-4Ig treatment resulted in weight reductions of epididymal and subcutaneous adipose tissues, which may be closely related to overexpression of apoptosis inhibitors in macrophages. Moreover, proinflammatory cytokine and chemokine levels decreased significantly. In contrast, CCAAT enhancer binding protein α, peroxisome proliferator-activated receptor γ, and adiponectin expression increased significantly in subcutaneous adipose tissue. This novel mechanism of CTLA-4Ig immunotherapy may lead to an ideal anti-obesity/inflammation/insulin resistance agent.

1. Introduction

Obesity has been considered as an independent risk factor for stroke, myocardial infarction, and type 2 diabetes [1]. Obesity-induced insulin resistance is closely associated with chronic low-grade tissue inflammation.

Many studies have demonstrated that macrophage infiltration is an essential step for metabolic disease pathogenesis [2,3], and secretion of cytokines and chemokines, such as TNF-α, IL-1, IL-6, and MCP-1, contributing to insulin resistance in adipocytes [4,5]. Polarization of macrophages has been classified as classically activated M1 macrophages and alternatively activated M2 macrophages [6,7]. Because reduced expression of genes associated with anti-inflammatory M2 macrophages and an increase of proinflammatory M1 macrophages occurs in obese states [8], it is important to control macrophage polarization as a therapy of obesity-induced insulin resistance. Moreover, such therapy would prevent type 2 diabetes and its subsequent complications, stroke and myocardial infarction. Therefore, the intimate mechanism of macrophage transformation should be confirmed to manipulate macrophage polarization as an effective treatment approach in clinical settings.

The fusion protein cytotoxic T lymphocyte-associated antigen-4IgG1 (CTLA-4Ig) has been applied to the treatment of rheumatoid arthritis (RA). CTLA-4Ig binds to CD28 ligands, CD80 and CD86

Abbreviations: AIM, apoptosis inhibitor of macrophage; AUC, area under the curve; CTLA-4Ig, cytotoxic T-lymphocyte-associated antigen-4IgG1; IPGT, intraperitoneal glucose tolerance test; ITT, insulin tolerance test; HFD, high fat diet; NCD, normal chow diet; MCP, monocyte chemotactic protein; RA, rheumatoid arthritis; SVF, stromal vascular fraction.

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(B7.1 and B7.2), on antigen-presenting cells such as macrophages, thereby blocking the engagement of CD28 on T cells and preventing T cell activation [9–12]. Although some studies have reported that the levels of IFN-γ, IL-6, IL-1β, and MMPs are reduced in the synovia of RA patients treated with CTLA-4Ig, the precise anti-inflammatory mechanism of CTLA-4Ig underlying the therapeutic response remains to be elucidated [13,14]. Furthermore, the possibility that CTLA-4Ig has direct and indirect effects on T cells has been presumed so far. Therefore, studies have focused on the immune system but excluded macrophages, and the possibility that the anti-inflammatory action of CTLA-4Ig is caused by alteration of macrophage polarization has not been explored.

In the present study, we found that CTLA-4Ig treatment significantly ameliorated insulin resistance in C57BL/6 mice fed a high fat diet (HFD). We hypothesized that CTLA-4Ig treatment affects the polarization of macrophages by favoring the M2 state that promotes resolution of inflammation.

2. Materials and methods

2.1. Animals

Five-week-old male C57BL/6 mice were purchased from Clea Japan Inc. (Tokyo, Japan). The average initial body weight was similar in each group of mice. Housing and diet are described in Supplementary methods. All protocols were approved by the Committee on the Ethics of Animal Experiments, Graduate School of Medical Science, Kyushu University.

2.2. Measurement of blood glucose, intraperitoneal glucose tolerance test (IPGTT), and insulin tolerance test (ITT)

Blood samples were obtained from the mouse tail vein. Plasma glucose and insulin concentrations were determined by the glucose oxidase method and an enzyme-linked immunosorbent assay, (Morinaga Institute of Biological Science, Yokohama, Japan), respectively. After 16 h of fasting, glucose tolerance was assessed by an IPGTT. Briefly, under anesthesia, a glucose bolus (5 mg/kg body weight) was injected intraperitoneally, and then blood samples were collected from the tail vein at 0, 15, 30, 60, 90, and 120 min. For the ITT, mice were injected with 2 U/kg human biosynthetic insulin (Novo Nordisk, NJ, USA), and then blood samples were collected at 0, 15, 30, 60, 90, and 120 min as described above.

2.3. Blood and urine analysis

Plasma concentrations of adiponectin, leptin, and non-esterified fatty acid (NEFA) were measured using commercially available kits (Wako, Osaka, Japan).

2.4. RNA extraction and quantitative RT-PCR

These procedures are described in Supplementary methods.

2.5. Morphometric study

Epididymal and subcutaneous adipose tissues were stained with hematoxylin-eosin (HE). Mouse adipose tissues were obtained at 12 weeks of age (after 6 weeks of treatment). To calculate the adipocyte area, sections were coded and analyzed by a blinded observer. In each animal of the four experimental groups, 2000 or more adipocytes in 24 randomly selected fields at 200-fold magnification of fluorescent microscopy (Model BZ-9000, Keyence, Osaka, Japan) were examined and averaged for morphometric analysis.

2.6. Isolation of stromal vascular fraction (SVF) cells and flow cytometric analysis

SVF isolation was performed as previously described with some modifications as detailed in the Supplementary methods.

3. Results

3.1. CTLA-4Ig treatment decreases adipose tissue weight and adipocyte size in HFD mice

At 6 weeks of CTLA-4Ig treatment, the body weights of CTLA-4Ig-treated HFD mice were significantly lower than those of untreated HFD mice (31.0 ± 0.83 g vs. 29.0 ± 0.59 g, $P < 0.05$; Fig. S1A), even though the consumed food quantities were similar among groups (Fig. S2). Additionally, there was no onset of any other organ disorders (Table S2). The weight of epididymal and subcutaneous adipose tissues in CTLA-4Ig-treated HFD mice was significantly lower than that in untreated HFD mice (Fig. 1A, B). CTLA-4Ig treatment decreased the size of adipocytes significantly in both epididymal adipose tissue (Figs. 1D, S5A) and subcutaneous adipose tissue (Figs. 1E, S5B).

3.2. CTLA-4Ig-treated mice are protected against impaired glucose homeostasis induced by a HFD

Next, we confirmed the effect of CTLA-4Ig treatment on impaired glucose homeostasis. In ITTs, although the blood glucose levels of untreated HFD mice rose again after 60 min, CTLA-4Ig-treated HFD mice maintained the glucose-lowering effect of insulin until the last minutes (Fig. 2A). The area under the curve (AUC) was also significantly lower in CTLA-4Ig-treated mice than that in untreated mice (AUC, $21 \times 10^3 \pm 1232.7$ vs. $13.0 \times 10^3 \pm 447.0$, $P < 0.001$, Fig. S6A). In IPGTTs, after a bolus injection of glucose, blood glucose levels were significantly lower at 15, 30, 90, and 120 min, and the AUC was significantly lower in CTLA-4Ig-treated HFD mice than that in untreated HFD mice (AUC, $45.6 \times 10^3 \pm 1725.1$ vs. $40.9 \times 10^3 \pm 703.5$, $P < 0.01$; Figs. 2B, S6B). Plasma insulin levels decreased by 65% (fasting state) and 36% (120 min) compared with those in untreated HFD mice (AUC, 111.9 ± 21.9 vs. 65.4 ± 4.4, $P < 0.01$) (Figs. 2C, S6C). Therefore, the homeostasis model assessment-insulin resistance (HOMA-IR) was markedly increased in untreated HFD mice and significantly reduced in CTLA-4Ig-treated HFD mice (4.8 ± 1.6 vs. 1.5 ± 0.2, $P < 0.01$; Fig. 2D).

3.3. CTLA-4Ig alters polarization of adipose tissue macrophages (ATMs) from proinflammatory M1 to anti-inflammatory M2

We next determined whether CTLA-4Ig alters macrophage polarization and is associated with the amelioration of insulin resistance in adipocytes. First, we analyzed the pan-macrophage marker, F4/80. HE staining and immunoflourescence staining showed an accumulation of macrophages as a crown-like structure in untreated HFD mice, whereas CTLA-4Ig treatment inhibited the infiltration of macrophages (Fig. S4A, C). This result was consistent with the total numbers of SVF cells in epididymal and subcutaneous adipose tissues. (Fig. S4D, E). In epididymal adipose tissue, mRNA expression of both iNOS and CD11c, M1 macrophage markers, increased significantly in untreated HFD mice, whereas CTLA-4Ig treatment decreased the mRNA expression of iNOS (iNOS, $235.2 \pm 39.1\%$ vs. $123.1 \pm 19.1\%$, $P < 0.05$; Fig. 3A). In addition, the mRNA expression of M2 macrophage markers arginase 1 (Arg1), CD206, and CD163 increased significantly by CTLA-4Ig treatment in both epididymal (Arg1, $163.6 \pm 45.5\%$ vs. $400.5 \pm 150.4\%$,

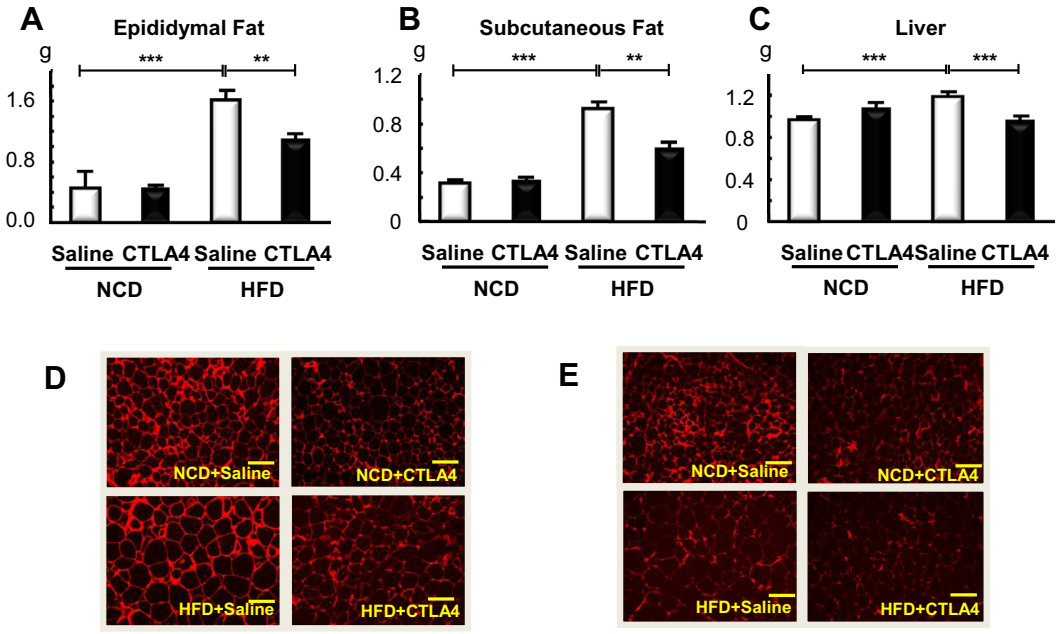

Fig. 1. CTLA-4Ig treatment decreases epididymal and subcutaneous adipose tissue weights, and adipocyte size in HFD mice. The weight of epididymal (A) and subcutaneous (B) adipose tissues; and the liver (C); Data are the means ± SE (n = 10 per group). $^{**}P < 0.01$; $^{***}P < 0.001$. Effect of CTLA-4Ig treatment on adipocyte size in epididymal (D) and subcutaneous (E) adipose tissues is shown; magnification 200×.

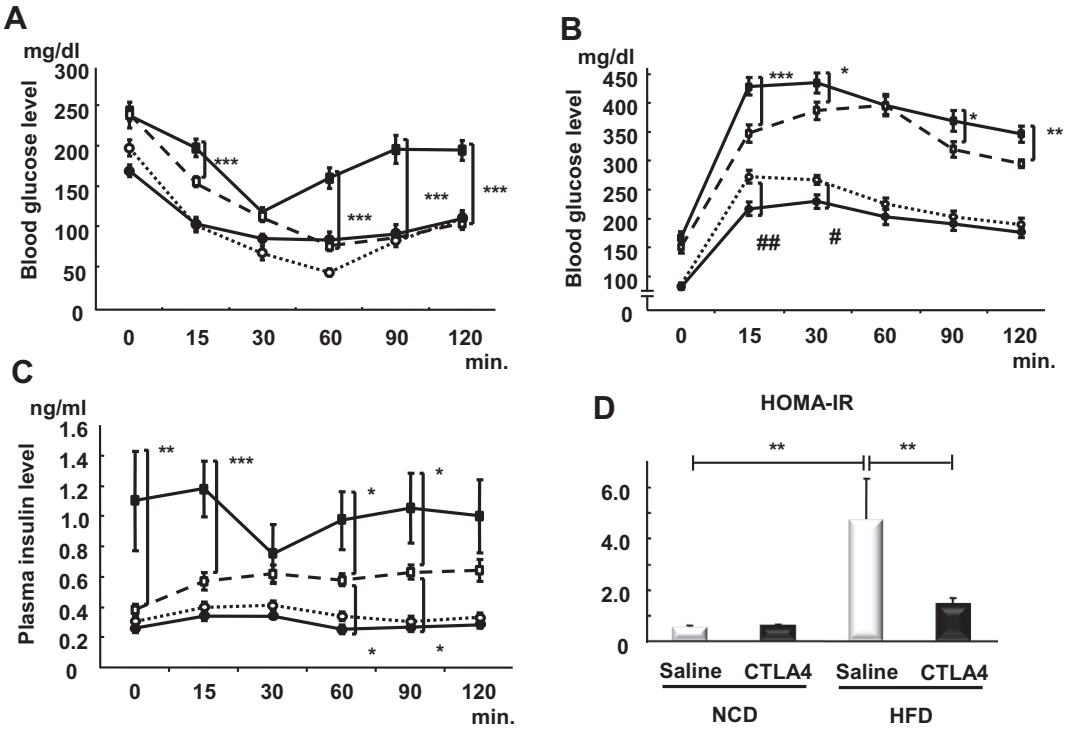

Fig. 2. CTLA-4Ig-treated mice are protected against impaired glucose homeostasis induced by a HFD. (A) Insulin sensitivity in untreated NCD mice (●), CTLA-4Ig-treated NCD mice (○), untreated HFD mice (■), and CTLA-4Ig-treated HFD mice (□). (B) Glucose tolerance was determined in 16 h-fasted mice after intraperitoneal injection of glucose (5 mg/kg body weight). Blood glucose levels were measured at the indicated times. (C) Insulin concentrations measured at the same time points as blood glucose levels in IPGTT. (D) HOMA-IR levels in untreated NCD mcie, CTLA-4Ig-treated NCD mice, untreated HFD mice, and CTLA-4Ig-treated HFD mice. Data are the means ± SE (n = 10 per group). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$.

$P < 0.05$; CD206, 184.6 ± 19.4% vs. 250.0 ± 19.2%, $P < 0.05$) and subcutaneous (Arg1, 379.5 ± 121.5% vs. 1135.6 ± 257.8%, $P < 0.01$; CD206, 71.3 ± 19.0% vs. 191.6 ± 36.5%, $P < 0.001$; CD163, 81.0 ± 21.6% vs. 273.8 ± 59.7%, $P < 0.01$) adipose tissues (Figs. 3B and S7B, C). Characteristically, mRNA expression of Arg1 was markedly increased more than that of the other M2 macrophage markers.

Next, we confirmed the shift of polarization of macrophages from M1 to M2 by CTLA-4Ig treatment using CD11c and CD206 as M1 and M2 markers, respectively. F4/80-positive SVF cells from

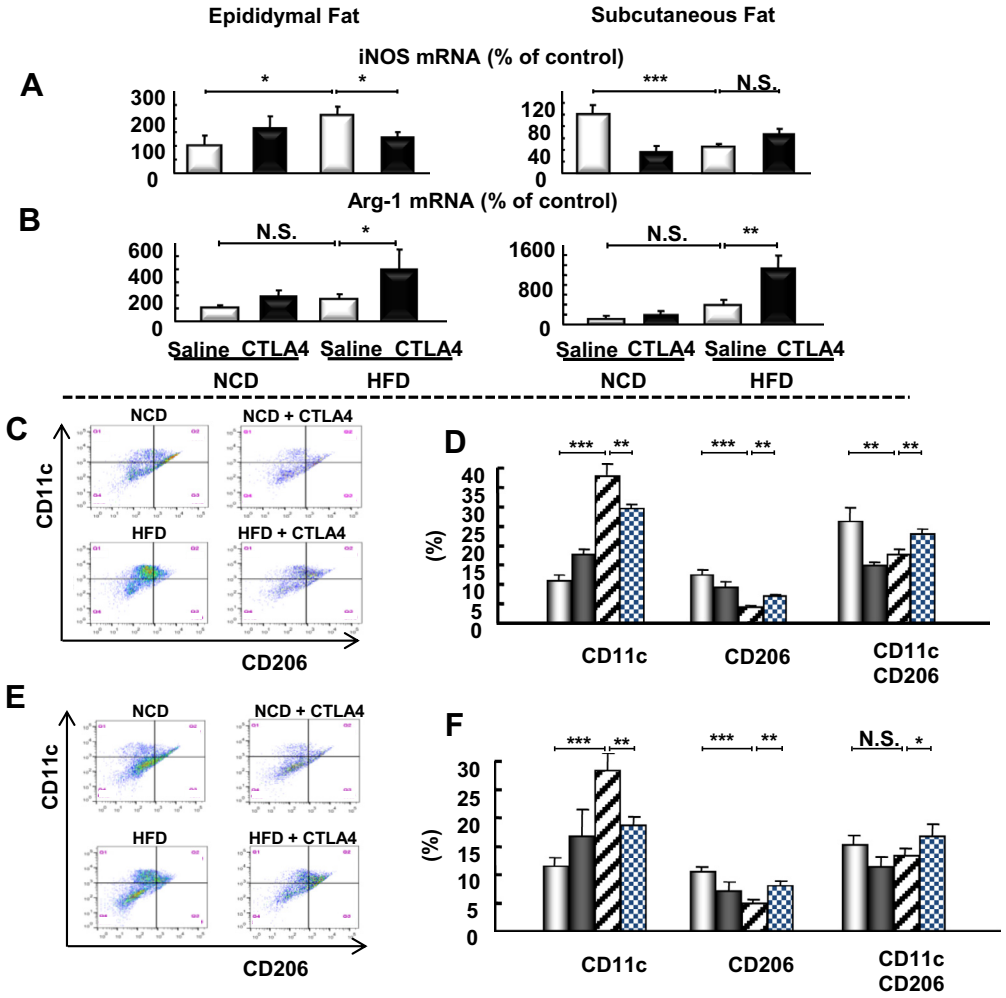

Fig. 3. CTLA-4Ig treatment induces macrophage polarization from proinflammatory M1 to anti-inflammatory M2. The mRNA levels of iNOS, a proinflammatory M1 macrophage marker (A) and Arg1, an anti-inflammatory M2 macrophage marker (B). Representative results of flow cytometric analyses of SVF cells in epididymal (C) and subcutaneous (E) adipose tissues are shown. Cell numbers in CD11c-positive, CD206/CD11c double-positive, and CD206-positive cell populations are shown according to the percentage of each population per total F4/80-positive SVF cells from epididymal (D) and subcutaneous (F) adipose tissues. Data are the means ± SE ($n = 10$ per group). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$, $n = 10$ per group. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; N.S., not significant.

epididymal and subcutaneous adipose tissues were analyzed by flow cytometry. Although the CD11c-positive cell population increased significantly in both the epididymal and subcutaneous adipose tissues of untreated HFD mice compared to that of NCD mice, CTLA-4Ig treatment decreased the population of CD11c-positive cells significantly (epididymal, 37.7 ± 2.8% vs. 29.5 ± 1.2%, $P < 0.01$; subcutaneous, 27.7 ± 2.9% vs. 18.7 ± 1.5%, $P < 0.01$; Fig. 3C–F) . In addition, CTLA-4Ig treatment increased the populations of CD206/CD11c double-positive cells (epididymal, 17.7 ± 1.3% vs. 24.2 ± 1.2%, $P < 0.01$; subcutaneous, 13.2 ± 1.4% vs. 18.7 ± 2.1%, $P < 0.05$) and CD206-positive cells (epididymal, 4.1 ± 0.5% vs. 6.9 ± 0.5%, $P < 0.01$; subcutaneous, 4.9 ± 0.6% vs. 8.1 ± 0.9 %, $P < 0.001$). Therefore, CTLA4-Ig treatment mainly shifted the macrophage population from CD11c positive to CD206/CD11c double positive or CD206 positive.

3.4. CTLA-4Ig induces overexpression of AIM and inhibits proinflammatory cytokine and chemokine expression in adiposetissues

CTLA-4Ig-treated HFD mice showed reductions of both epididymal and subcutaneous adipose tissue weights and adipocyte size. Therefore, we examined the possible association of CTLA-4Ig with AIM that directly inhibits fatty acid synthase in adipocytes, leading to lipolysis and a subsequent decrease in the size and number of lipid droplets. CTLA-4Ig-treated HFD mice showed a significant increase of AIM mRNA levels in both epididymal (168.5 ± 53.8% vs. 519.4 ± 185.9%, $P < 0.05$) and subcutaneous (42.4 ± 13.5% vs. 304.4 ± 100.0%, $P < 0.001$) adipose tissues (Fig. 4A). This result suggests the mechanism, at least in part, of adipose tissue reduction by CTLA-4Ig treatment in HFD mice.

TNF-α and IL-6 have been considered as an important mediators of insulin resistance in obesity [15,16]. In addition, CTLA4-Ig, which is upstream of TNF-α secretion, has been used for treatment of RA patients who are resistant to TNF-α blocking therapy to reduce the inflammatory status of the synovium [17]. In the present study, we confirmed the beneficial effect on the inflammatory status of epididymal adipose tissue of HFD mice by a reduction of mRNA levels of TNF-α (152.6 ± 31.3% vs. 76.6 ± 18.0%, $P < 0.05$, Fig. S8A) and IL-6 (154.5 ± 22.4% vs. 102.3 ± 11.7%, $P < 0.05$; Fig. S8B). Moreover, CTLA4-Ig treatment affected the expression of proinflammatory chemokines MCP-1 and -3. mRNA levels of both MCP-1 and-3 decreased significantly in epididymal (MCP-1, 810.1 ± 180.2% vs. 405.6 ± 94.2%, $P < 0.01$; MCP-3, 434.9 ± 97.7% vs. 213.9 ± 2 9.8%, $P < 0.01$) and subcutaneous (MCP-1, 435.9 ± 81.2% vs. 257.9 ± 50.7%, $P < 0.05$; MCP-3, 1004.3 ± 279.3% vs. 375.1 ± 34.0%, $P < 0.01$) adipose tissues (Fig. S8C, D).

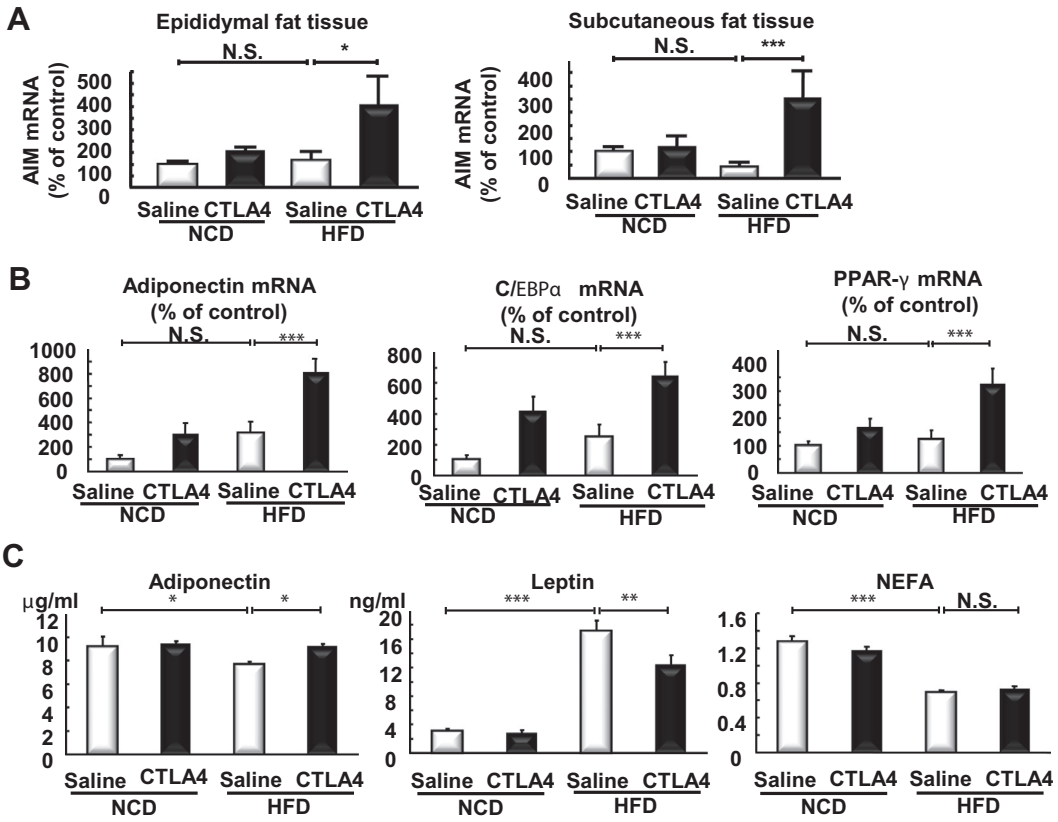

Fig. 4. CTLA-4Ig affects AIM mRNA expression, and adipocytokine mRNA expression and serum levels. (A) mRNA expression levels of AIM in each adipose tissue; (B) mRNA expression of the master regulators of adipogenesis, PPAR-γ and C/EBPα, and their product, adiponectin; (C) Circulating levels of adiponectin, leptin, and NEFA in 16 h-fasted state in untreated NCD mice, CTLA-4Ig-treated NCD mice, untreated HFD mice, and CTLA-4Ig-treated HFD mice. Data are the means ± SE ($n = 10$ per group). $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; N.S., not significant.

3.5. CTLA4-Ig treatment affects the expression of adipocytokines in subcutaneous adipose tissue

Finally, we examined the expression of adipocytokines and their upstream transcriptional factors. Interestingly, mRNA expression of both CCAAT enhancer binding protein α (C/EBPα) and peroxisome proliferator-activated receptor γ (PPAR-γ) showed significant increases in the subcutaneous adipose tissue of CTLA-4Ig-treated HFD mice (PPAR-γ, 123.2 ± 32.3% vs. 326.4 ± 55.2%, $P < 0.001$; C/EBPα, 247.2 ± 84.5% vs. 646.0 ± 89.3%, $P < 0.001$; Fig. 4B) but did not show any changes in epididymal adipose tissue (data not shown). Consistent with this finding, mRNA expression of adiponectin increased significantly in subcutaneous adipose tissue only (310.2 ± 97.4% vs. 808.6 ± 116.2%, $P < 0.001$). Furthermore, the serum levels of adiponectin and leptin were at optimal levels for insulin sensitivity (Fig. 4C).

4. Discussion

In the present study, we found that CTLA-4Ig completely alleviated HFD-induced insulin resistance. Moreover, CTLA-4Ig treatment showed reductions of epididymal and subcutaneous adipose tissue weights and adipocyte size, even though the dietary intake of each group was not influenced by CTLA-4Ig treatment. In parallel with these morphological changes, mRNA levels of inflammatory cytokines, IL-6 and TNF-α, and chemokines, MCP-1 and -3, decreased significantly in both adipose tissues. Moreover, expression of adipocytokines and their transcriptional factors, PPAR-γ and C/EBPα, increased significantly, especially in subcutaneous adipose tissue. The serum levels of general adipocytokines such as adiponectin and leptin were at optimal levels for insulin sensitivity. Therefore, CTLA-4Ig might be defined as an anti-obesity/inflammation/insulin resistance agent.

To evaluate the ameliorating mechanisms of CTLA4-Ig in the metabolic system, we focused on the ATMs that bind CTLA-4Ig. Numerous studies have reported that the numbers of ATMs are elevated in the adipose tissues of obese human subjects and rodents [18–20]. However, research focus has been on the relationship between CTLA-4Ig and its effect on T cell suppression, and not on the alterations of macrophages by CTLA-4Ig. We hypothesized that CTLA-4Ig not only transmits an inhibitory signal to T cells, but also induces a change in macrophage polarization to the M2 state. As expected, CTLA-4Ig treatment decreased the mRNA level of M1 marker iNOS, while significantly increased the mRNA levels of M2 markers Arg1, CD206, and CD163. Moreover, flow cytometric analysis confirmed the obvious shift from M1 to M2 macrophages. Untreated HFD mice showed a significant increase in their CD11c-positive cell populations, whereas CTLA-4Ig-treated HFD mice showed a significant increase in CD11c/CD206 double-positive and CD206-positive cell populations. These findings revealed that macrophages shift from M1 to M2, but not directly to single CD206-positive cells, but via intermediately CD11c/CD206 double-positive cells because of CTLA-4Ig engagement [21].

It has been strongly indicated that M2 macrophages show not only an anti-inflammatory effect, but also wound healing and anti-oxidative stress effects via Arg1 overexpression in adipose tissues. In the present study, it was confirmed that one of the crucial CTLA-4Ig actions is prominent Arg1 overexpression and iNOS downregulation in epididymal and subcutaneous ATMs. Adipose tissues in untreated HFD mice were characterized by infiltration

of M1 macrophages that accumulated around apoptotic adipocytes (referred to as crown-like structures) [1] and overexpression of iNOS, leading to tissue inflammation. Alternatively, M2 macrophages overexpressing Arg1 may show anti-inflammatory effects and restore homeostasis because of their wound healing effect. Furthermore, one of the causes of insulin resistance in obesity has been thought to be inflammation from oxidative stress. In a previous study, reactive oxygen species (ROS) such as O_{2-} increased selectively in the adipose tissue of obese mice, which was accompanied by augmented expression of NAD(P)H oxidase, a major source of ROS, and decreased expression of antioxidative enzymes [22]. Therefore, excess NO produced by iNOS reacts with O_{2-}, leading to production of $ONOO^-$ that can damage a wide array of molecules in cells, including DNA and proteins, and has a potent effect on NO^--mediated inflammation. For this reason, overexpression of Arg1 may play an important role, at least partially, in the antioxidative stress effect in adipose tissue. The anti-inflammatory effect from the antioxidative stress effect of CTLA-4Ig in adipose tissue should be clarified in more detail in future studies.

Interestingly, we found that CTLA-4Ig-treated HFD mice showed weight reductions of both epididymal and subcutaneous adipose tissues. Furthermore, the mRNA levels of AIM increased significantly in the adipose tissue of CTLA-4Ig-treated HFD mice. A previous report has shown that recombinant AIM significantly decreases the number of 3T3-L1 cells containing lipid droplets, adipocyte hypertrophy is less advanced, and the overall mass of visceral adipose and body weight are markedly reduced in $AIM^{+/+}$ mice than that in $AIM^{-/-}$ mice [23]. Additionally, an increase in lipolysis and a decrease in cell size were shown in 3T3-L1 adipocyte by overexpression of PPAR-γ [24]. Here, we found that CTLA-4Ig-treated HFD mice showed a significant decrease in the mRNA expression of chemokines, MCP-1 and -3, and overexpression of AIM and PPAR-γ mRNA, suggesting a dual effect of anti-inflammation and lipolysis by CTLA-4Ig. Therefore, it might lead to a promising and attractive therapeutic agent for treatment of obesity-induced insulin resistance. However, the factors regulating AIM gene expression in macrophages and the details of their pathway(s) after CTLA-4Ig engagement of CD80/86 require further investigation.

The anti-obesity effect of CTLA-4Ig contributed to the increased gene expression of adiponectin and its transcription factors in subcutaneous adipose tissue compared with that in epididymal adipose tissue. A human study has reported that weight loss induces a marked increase of adiponectin gene expression [25]. Zucker diabetic fatty rats show higher adiponectin gene expression, but the expression levels are lower compared with those in lean rats [26]. Notably, in both studies, adiponectin gene expression was higher in subcutaneous adipose tissue than that in visceral adipose tissue. Moreover, subcutaneous adipose tissue is thought to be more important for circulating adiponectin levels [27]. In the present study, our data also indicated a strong relationship between adipocytokine-affiliated gene expression in subcutaneous adipose tissue and circulating adiponectin levels. The anti-inflammatory effect of CTLA-4Ig and suppression of TNF-α and IL-6 expression eventually contribute to preventing strong suppression of adiponectin gene expression [28–31], which alleviates insulin resistance in a HFD-induced obese state.

In conclusion, we demonstrated that CTLA-4Ig ameliorates insulin resistance in HFD mice. Concomitantly, the weight of both epididymal and subcutaneous adipose tissues and the size of adipocytes decrease significantly. In the molecular mechanism, CTLA-4Ig alters the polarization of ATMs from inflammatory M1 to the anti-inflammatory M2 state. In particular, expression of the M2 marker Arg1 strongly increases, which inhibits inflammatory cytokine and chemokine expression. These favorable effects from the decrease of adipose tissue may be caused by overexpression of AIM and PPAR-γ by CTLA-4Ig treatment. Finally, there is optimal expression of adipocytokines for insulin sensitivity. CTLA-4Ig treatment fundamentally stops the vicious cycle of HFD-induced obesity, suggesting that CTLA-4Ig treatment may lead to a novel anti-obesity/inflammation/insulin resistance agent for manipulation of metabolic systems.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.07.034.

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