T cell costimulation protects obesity-induced adipose inflammation and insulin resistance

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ABSTRACT

A key pathophysiologic role for activated T cells in mediating adipose inflammation and insulin resistance has been recently postulated. However, mechanisms underlying their activation are poorly understood. In this study, we demonstrated a previously unrecognized homeostatic role for the costimulatory B7 molecules (CD80 and CD86) in preventing adipose inflammation. Instead of promoting inflammation which was found in many other disease conditions, B7 costimulation reduced adipose inflammation by maintaining regulatory T cells (Tregs) number in adipose tissue. In both humans and mice, expression of CD80 and CD86 was negatively correlated with the degree of insulin resistance and adipose tissue macrophage infiltration. Decreased B7 expression in obesity appeared to directly impair Treg proliferation and function that lead to excessive pro-inflammatory macrophages and the development of insulin resistance. CD80/CD86 double knockout (B7 KO) mice had enhanced adipose macrophage inflammation and insulin resistance under both high-fat and normal diet conditions, accompanied by reduced Treg development and proliferation. Adoptive transfer of Tregs reversed insulin resistance and adipose inflammation in B7 KO mice. Our results suggest an essential role for B7 in maintaining Tregs and adipose homeostasis, and may have important implications for therapies that target costimulation in type 2 diabetes.
INTRODUCTION

Inflammation is widely believed to play a key pathogenic role in the development of obesity-induced insulin resistance (IR) and type 2 diabetes (1-3). While innate immune activation typified by infiltrating macrophages are widely believed to represent important mediators of obesity-related complications, the role of adaptive immune responses is less well characterized. Recent findings suggest that T cells may play an important role in this process (4-6). Activated CD8$^+$ effector T cells have recently been shown to promote adipose inflammation by enhancing macrophage recruitment and activation (5; 6). The precise mechanisms by which T cells are activated in obesity remains poorly characterized.

Classically, T cell activation requires 2 different types of signals, both of which are delivered by antigen presenting cells (APCs) (7). The first signal derives from the interaction between T cell receptor and peptide-MHC complex, whereas the second signal (also called costimulatory signal) is provided by the binding of CD28 to the co-stimulatory molecules such as CD80 (B7-1) and CD86 (B7-2) (8-11). As the most classical costimulation, B7 involves in various inflammatory diseases (11-17). Nevertheless, whether B7 molecules play a role in regulation of adipose inflammation in IR is completely unclear. Prevention of B7-mediated costimulation has been suggested to result in impaired T-cell activation which may be protective in a number of experimental contexts such as experimental allergic encephalomyelitis (11; 14), allograft transplantation (12), arthritis (13; 16), hypertension (17), and type 1 diabetes (15). In this investigation, we elucidated a homeostatic role of B7-mediated costimulation in diet-induced obesity using CD80/CD86 double knockout (B7 KO) mice, and investigated the relevance of this process in humans with obesity and IR.
MATERIALS AND METHODS

Animal Models

All procedures of this study were approved by the Committees on Use and Care of Animals. B7 KO (B6.129S4-Cd80\textsuperscript{tm1Shr} Cd86\textsuperscript{tm2Shr}/J), Foxp3-GFP knockin (B6.Cg-Foxp3\textsuperscript{tm2(EGFP)Tch}/J) mice and C57BL/6 mice were purchased from Jackson Laboratory (Jax Labs, ME). Eight-week-old WT and B7 KO mice were randomized to a normal diet (ND) or a high fat diet (HF, 42% calories from fat, Harlan\textsuperscript{®} TD.88137) for 12 weeks. At sacrifice, mice were fasted overnight and serum was collected for insulin (Crystal Chem Inc., IL USA) and leptin (R&D Systems, Minneapolis, MN) ELISA. Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated as follows: \textit{HOMA-IR} = Fasting Serum Glucose (mg/dL) \times Fasting Plasma insulin (\mu U/mL) / 405.

Human Subjects

We enrolled prospective obese subjects (BMI > 30) as part of research protocol investigating the role of visceral adipose inflammation in obesity. Greater omental adipose tissue was obtained during endoscopic gastric bypass surgery. As controls, human visceral adipose tissue (VAT) was procured during endoscopic repair of hernias from lean controls (BMI<30). Human subcutaneous adipose tissue (SAT) was obtained from patients undergoing lipoaspiration/liposuction as part of a separate Institutional Review Board (IRB) approved protocol. The stromal vascular fraction (SVF) from visceral/subcutaneous adipose tissue was isolated by digesting it with 1 mg/mL collagenase type 2 from \textit{Clostridium histolyticum} (Sigma, St. Louis, MO) as described (18). All procedures of this study were approved by the Office of Responsible Research Practices, Human
IRB of the Ohio State University under OSU protocol #2008H0177. Human informed consent was obtained in writing and a copy was inserted in the patients' medical records.

**Visceral Adipose Assessment and Quantification by Magnetic Resonance Imaging (MRI)**

Magnetic resonance imaging (MRI) was performed to assess degree of adiposity. After 12 weeks of HF diet, MRI was performed on a 9.4 T Bruker BioSpin system equipped with ParaVision 4.0 software as previously described (19). A spin echo sequence (repetition time 920 ms; echo time 12 ms, in-plane resolution 256x256 µm; 2, 4 averages) was used to acquire 50 transversal, 1 mm thick slices that covered from the top of the kidneys to the hind legs. Fat quantification analysis was performed using OsiriX software (The Osirix Foundation, Geneva, Switzerland). Thresholding technique was applied to all images to separate fat and water signal.

**Induction of Bone Marrow-derived DCs (BMDCs) and Bone Marrow-derived Macrophages (BMMs)**

Bone marrow-derived DCs (BMDCs) were generated as previously described (20). For the induction of bone marrow-derived macrophages (BMMs), bone marrow cells were cultured in RPMI-1640 suspended with 10% FBS and 10ng/ml recombinant mouse M-CSF (R&D Systems, Minneapolis, MN). Media were replaced every 2 days. Adherent cells (BMMs) were collected for experiment at day 7.

**Intraperitoneal Glucose Tolerance Test (IPGTT) and Insulin Tolerance Test (ITT)**

IPGTT and ITT were performed following 12 weeks of diet feeding as detailed previously with minor modifications (21). For IPGTT, mice were weighed and baseline blood glucose was
determined after overnight fasting (16-18 h) with free access to water. Animals were then intraperitoneally (i.p.) injected with 2 g/kg D-glucose. Blood glucose was detected 30, 60, 90, and 120 min after the glucose injection by using Contour® blood glucose meter (Bayer, Pittsburgh, PA). For ITT, body weight and baseline blood glucose were measured after 4 h of fasting. Mice were then i.p. injected with 0.75U/kg insulin and blood glucose was detected at 30, 60, 90, and 120 min.

**Flow Cytometry**

All antibodies used in flow cytometry were purchased from BioLegend (San Diego, CA) or BD (San Jose, CA). Cells were stained with indicated antibodies as described (18) and then analyzed on a BD LSRII cytometer (BD, San Jose, CA). For intracellular staining of Foxp3, cells were incubated with antibodies against surface maker at 4°C for 15 min. After a wash with PBS, cells were then fixed and permeabilized using a Foxp3 staining kit (BioLegend, San Diego, CA) as instructed by the manufacturer.

**Realtime PCR**

Total RNA were isolated from epididymal fat using Trizol® Reagent (Life Technologies, Grand Island, NY). cDNA were synthesized from mRNA using a High Capacity cDNA Reverse Transcriptase Kit (Life Technologies, Grand Island, NY) according to the manufacturer’s instruction. Realtime PCR was performed using a LightCycler® 480 SYBR Green I Master kit (Roche, Indianapolis, IN). 18s rRNA was used as internal control. Primers are listed in supplemental table 1.
**ELISA**

Serum insulin level and leptin level were measured using a Ultra Sensitive Mouse Insulin ELISA kit (Crystal Chem Inc., Downers Grove, IL) and a Quantikine® Mouse Leptin Immunoassay kit (R&D Systems, Minneapolis, MN) as instructed by the manufacturer.

**Western Blot Detection**

Total proteins were prepared from adipose tissue using M-PER® Mammalian Protein Extraction Reagent (Thermo Scientific, Waltham, MA) supplemented with protease inhibitor cocktail (Sigma, St. Louis, MO). Western blot analysis was carried out as reported by probing the blots with an indicated primary Ab (Santa Cruz, CA) followed by an HRP-conjugated secondary antibody (Santa Cruz, CA). The reactive bands were visualized using an ECL Plus™ Western blot kit (PIERCE, Rockford, IL)

**Macrophage/T cell Coculture**

Treg was isolated from the lymph nodes and spleen of Foxp3-GFP knock-in mice by flow sorting. CD4\(^+\) GFP\(^+\) natural regulatory T cells (Treg) and CD4\(^+\) GFP\(^-\) naïve T cells (Tn) were cocultured with BMMs at the concentration of 1 x 10\(^6\)/mL for 5 days. Cells were then harvested for the detection of macrophage polarization and Treg proliferation. For cell proliferation assay, Tregs were labeled with CellTrace Violet stain (CellTrace™ Violet Cell Proliferation Kit, Life Technologies, Grand Island, NY) before coculture according to the manufacturer’s instruction. Fluorescence intensity was analyzed on a LSRII flow cytometer and proliferation was evaluated
by the percentage of divided cells (with lower fluorescence intensity). For the analysis of Treg development, thymocytes were isolated from Foxp3-GFP knock-in mice and cocultured with BMMs or BMDCs derived from WT or B7 KO mice. Treg percentage in thymocytes was evaluated after 5 days of coculture by flow cytometry.

**Treg Adoptive Transfer**

For the adoptive transfer study, natural Treg and Foxp3- naïve T cells were prepared from Foxp3-GFP knock-in mice by flow sorting. Treg or Tn (0.5 x 10^6/mouse) were then suspended in 200 µL 1 x PBS and adoptive transferred into B7 KO mice by intravenous injection. The day after first injection, mice were fed a HF for 12 weeks. The adoptive transfer was performed every 2 weeks until sacrifice.

**Statistical Analysis**

All data are presented as mean ± SEM. A $P$ value of <0.05 was considered statistically significant. Graphpad Prism 4 was used for statistical analysis using student’s t-test or one-way ANOVA and bonferroni’s post hoc test where appropriate.
RESULTS

Reduced CD80 and CD86 expression in adipose tissue macrophage (ATM) from obese humans and mice

To investigate a potential role for costimulatory molecules CD80 (B7-1) and CD86 (B7-2) in obesity-induced IR, visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) isolated from lean and obese humans were used for flow cytometric detection of CD80 and CD86. Supplemental Table 2 provides baseline metabolic data of patients recruited to the study. Expression of CD80 and CD86 was lower in monocyte/macrophage lineage cells (CD45⁺CD14⁺) and mature macrophages (defined as CD45⁺CD11b⁺) in VAT from obese patients compared with that of lean (body mass index [BMI]<30) controls (Figure 1A–1D). Similarly, CD80 and CD86 expression was lower in CD45⁺CD14⁺ and CD45⁺CD11b⁺ cells from SAT of obese subjects (BMI ≥ 30) compared to lean controls (BMI<30) (Figure 1E–1H). CD80 and CD86 expression on adipose tissue macrophage (ATM) in VAT was negatively correlated with Homeostatic model assessment of insulin resistance (HOMA-IR), suggesting a potential role of CD80 and CD86 in the pathogenesis of obesity-induced IR (Figure 1H & 1I). Consistent with human data, expression of CD80 and CD86 on ATM of mice fed a high-fat diet (HF) was lower compared with normal chow diet (ND)-fed mice (data not shown).

Deficiency of B7 exacerbates IR despite less adiposity

To investigate the involvement of costimulatory molecules CD80 and CD86 in obesity and IR, WT and B7 KO (CD80 and CD86 double knockout) mice were fed a high-fat (HF) or normal chow diet (ND) for a duration of 12 weeks. There were no significant differences in body
weight between WT and B7 KO fed ND. In response to HF diet, both WT and B7 KO mice significantly gained weight compared to ND fed animals, but the degree of body weight gain was identical in B7 KO and WT animals (48.9±1.2 g vs. 49.0±1.3 g, \( p > 0.05 \); Figure 2A). B7 KO mice had significantly lower epididymal fat after 12 weeks of HF diet feeding (1.31±0.04 g vs. 0.92±0.06 g for WT vs. B7 KO, \( p = 0.005 \), Figure 2B). MRI analysis of VAT content demonstrated similar findings. Abdominal fat analysis by MRI (visceral and subcutaneous) demonstrated a reduction in both compartments in B7 KO mice (Figure 2C). Liver weight was higher in B7 KO mice on HF diet (3.92±0.14 g vs. 4.38±0.08 g for WT vs. B7 KO, \( p = 0.02 \), Figure 2D). Hepatic triglyceride and cholesterol was significantly increased in response to HF but without significant differences between WT and B7 KO mice (Figure 2E).

Figure 2F & 2G depicts insulin and glucose homeostasis measures as evidenced by intraperitoneal glucose tolerance test (IPGTT) and insulin tolerance test (ITT). B7 KO mice exhibited significantly worse fasting glucose levels even under ND conditions. Fasting blood glucose levels significantly increased after 12 weeks of HF diet in both WT and B7 KO mice. Overnight fasting blood glucose levels in B7 KO mice fed a HF diet were higher than WT-HF fed animals (Figure 2H; ND: 78.4±1.5 mg/dL vs. 94±7.7 mg/dL, \( n=10, p = 0.041 \); HF: 107.6±4.7 mg/dL vs. 127±7.9 mg/dL, \( n=10, p = 0.042 \)). Fasting insulin levels were significantly higher in B7 KO mice on both ND and HF compared with WT mice on corresponding diets. (Figure 2I; ND: 0.46±0.13 ng/mL vs. 0.96±0.11 ng/mL, \( p = 0.024 \); HF: 1.27±0.10 ng/mL vs. 1.93±0.04 ng/mL, \( p = 0.027 \)). HOMA-IR values were significantly higher in the B7 KO mice on both ND and HF (Figure 2J). There was an increase in serum leptin level upon HF diet feeding. However no significant difference in leptin levels was observed between WT and B7 KO mice (Figure 2K).
Defective B7-mediated costimulation affects adipose inflammation and T cell activation in circulation

We next evaluated the contribution of B7 to inflammation in circulation and adipose tissue in diet induced obesity. We first confirmed the absence of CD80 and CD86 expression in splenic and peritoneal macrophages in B7 KO mice (data not shown). As depicted in Figure 3A & 3B, comparable amounts of macrophages (CD11b+ F4/80+) and total T cells (CD45+CD3+) were detected in the spleen of WT and B7 KO mice. However, B7 KO mice had reduced CD4+ T cells and increased CD8+ T cells in the spleen (Figure 3C). Further analysis demonstrated that B7 KO mice had decreased populations of activated CD4+ T cells (less expression of T cell activation marker CD44 and CD69) in the spleen compared with WT mice (Figure 3D-G). However, no significant differences were found in splenic CD8+ T cells (Figure 3D-G), suggesting a reduced circulating CD4 T cell activation in B7 KO mice. In contrast, innate adipose inflammation was enhanced in B7 KO mice. Increased CD11b+F4/80+ macrophages and expression of TNFα were observed in VAT of B7 KO mice (Figure 3H-3J). There was a ~2-fold increase of TNFα in the VAT of WT mice fed HF diet compared with ND, with a further increase seen in B7 deficient mice fed HF diet (Figure 3H & 3J). Monocyte chemotactic protein-1 (MCP-1) level in mice fed HF diet increased 1.9-fold compared with that of ND mice. However, no significance was observed between WT and B7 KO mice (Figure 3I & 3J). No significant difference in expression of IL-10, an anti-inflammatory cytokine, was observed (Figure 3K). The percentage of CD45+CD11b+ macrophages increased in VAT of HF WT mice compared with that of ND WT mice (increased from 27% to 36.9%, Figure 3J). Compared with WT mice, B7 KO mice had a higher level of macrophage infiltration under both ND and HF condition (Figure 3L). Consistent with these findings, there was an enhanced hepatic
inflammation in B7 KO mice as evidenced by increased TNFα expression and immune cell infiltration (data not shown).

**Loss of B7-mediated costimulation reduces regulatory T cells both systemically and locally in adipose tissue**

Prior studies have highlighted the importance of Tregs in obesity and IR with studies suggesting reduced local differentiation of Tregs as a critical determinant of heightened innate immune responses (4-6). Since CD28 and CTLA-4 have been implicated in Treg development, we reasoned that one mechanism for heightened innate immune activation in adipose tissue may involve Treg pathways. As shown in Figure 4A, CD3+ T cell infiltration in VAT increased in response to HF diet. However CD3+ T cells decreased in B7 KO mice in both ND and HF groups. CD4 significantly decreased in the visceral fat of B7 KO mice. CD8 also slightly decreased in B7 KO mice (data not shown). We next quantified Foxp3, a Treg-restricted transcription factor, in VAT of both WT and B7 KO mice in response to HF diet. Consistent with prior studies, Foxp3 expression decreased in the VAT of HF-fed WT mice compared to ND fed WT mice (Figure 4B). Foxp3 gene expression was however markedly decreased in the VAT of B7 KO mice compared with that of WT mice under both ND and HF diet conditions (Figure 4B). Flow cytometry confirmed the decrease of Treg in VAT of B7 KO mice (Figure 4C). Treg numbers were also decreased in the spleen, blood, and lymph nodes of B7 KO mice (Figure 4D), suggesting a systemic reduction of Tregs in B7 KO mice. To further confirm the relation between ATM B7 expression and Treg in human, human VAT samples were analyzed for the cytometric detection of Treg (CD4+Foxp3+CD25+) and B7-expressing ATMs.
As shown in Figure 4E, there was a positive correlation between B7 expression on ATM and Treg percentage in VAT ($P=0.01$).

Costimulation mediated by B7 molecules promotes proliferation of Treg

We next investigated the requirement of B7-mediated costimulation in Treg expansion. Treg cells were flow sorted from Foxp3-GFP knock-in mice and fluorescently labeled using a CellTrace™ Violet Cell Proliferation Kit. Labeled Treg cells were then cocultured with bone marrow-derived macrophages (BMMs) from WT or B7 KO mice for 5 days and proliferation of Treg was evaluated by the intensity of the fluorescence (Figure 5A). As demonstrated in Figure 5B & 5C, loss of B7-mediated costimulation significantly impaired the ability of macrophages to stimulate Treg proliferation (5.4±0.3% vs. 2.3±0.4%, $p<0.05$). To investigate the suppressive function of Treg cocultured with $B7^{-/-}$ macrophages, WT splenic T cells were cocultured with Treg cells harvested from the coculture system depicted in Figure 5A. After 5 days of coculture, T cells were collected for flow cytometric detection of T cell activation marker CD44 and CD69. As shown in Figure 5D & 5E, Treg cells cocultured with $B7^{-/-}$ macrophages were less effective in suppressing T cell activation.

Disruption of B7 impairs ability of DC/Macrophage to educate thymocytes and convert Foxp3+ T cells to Treg

Professional antigen presenting cells (APCs) including dendritic cells and macrophages are required for the development of Treg in thymus and peripheral tissues (22-25). To investigate the role of B7 molecules in Treg development, thymocytes from Foxp3-GFP knock-in mice were
cocultured with WT or B7−/− BMMs. After 5 days of coculture, CD4+ cells were gated for the detection of Treg (GFP+) population (Figure 6A). Smaller amounts of Treg cells were present in thymocytes cocultured with B7−/− BMMs (19.6±2.2% vs. 12.2±1.1% for WT vs. B7 KO, p<0.05; Figure 6B & 6C). Similarly, B7−/− bone marrow-derived dendritic cells (BMDCs) have a reduced ability to drive Treg development (Figure 6D & 6E). It has been shown that Foxp3− T cells can be converted into Foxp3+ Treg cells (26). To investigate whether B7 molecules play a role in the conversion of Foxp3− T cells to Treg, Foxp3− T cells were sorted from Foxp3-GFP knock-in mice and cocultured with WT or B7−/− BMDCs. As a result, B7−/− BMDCs were less potent in converting Foxp3− T cells to Foxp3+ Treg cells (11.1±0.3% vs. 5.9±1.2% for WT vs. B7 KO, p<0.05; Figure 6F & 6G).

Enhanced adipose inflammation in B7 KO mice is a result of reduced Treg

To confirm the involvement of Treg in preventing adipose inflammation, human VAT samples were used for flow cytometric detection of Treg (CD4+Foxp3+CD25+) and CD11c+CD11b+ cells. As depicted in Figure 7A, there was a negative association between Treg and CD11c+CD11b+ cells in VAT (R2=0.29, P=0.048). To further investigate whether reduced Treg in B7 KO mice is responsible for heightened adipose innate immune inflammation and IR, the effect of Treg on macrophage activation was examined. As depicted in Figure 7B & 7C, Treg reduced CD11c+CD11b+ cells (72.3±1.9% vs. 51.2±0.8% for WT vs. B7 KO, p<0.05), a subpopulation which plays an important role in adipose inflammation and IR (27). Consistently, higher percentages of CD11c+CD11b+ cells were observed in B7 KO mice on both ND and HF diet (Figure 7D).
To further confirm whether enhanced IR in B7 KO mice is dependent on the decrease of Tregs, CD4\(^+\)GFP\(^+\) Treg or CD4\(^+\)GFP\(^-\) naïve T (Tn) cells from Foxp3-GFP knock-in mice were adoptively transferred into B7 KO mice (once every other week) and fed a HF diet. After 12 weeks of HF diet feeding, transferred Treg (CD4\(^+\)GFP\(^+\) cells) presented in both circulation (data not shown) and VAT of mice injected with Treg (Figure 8A). No significant changes in body weight and visceral fat weight were observed (data not shown). There was a ~2.1-fold increase of Foxp3 expression in the VAT of mice transferred with Treg (Figure 8B). Macrophage infiltration and its classical activation were reduced by exogenous Treg transfer (Figure 8C & 8D). Adoptive transfer of Treg also restored insulin sensitivity in B7 KO mice as evidenced by improved IPGTT and ITT (Figure 8E & 8F).
DISCUSSION

In this paper we demonstrate a previously unrecognized homeostatic function of co-stimulation in preserving Treg numbers and modulating innate immune responses. The main novel findings of our work are as follows: 1) Expression of B7-1 and B7-2 (CD80 and CD86) on APCs in both VAT and SAT is reduced in diet-induced obesity in mice and human obesity; 2) Loss of B7-mediated costimulation exacerbates diet-induced IR despite lower visceral adiposity in both ND and HF diet contexts; 3) Contrary to the expected reduction in innate immune activation, genetic deletion of B7 promoted adipose inflammation; 4) B7-mediated costimulation is required for the development and expansion of Treg; 5) Adoptive transfer of Tregs restores insulin sensitivity in response to genetic B7 deficiency.

Recent studies have suggested a critical role of T cells in mediating obesity-induced adipose inflammation (4-6). Inflammed adipocytes and activated T cells in the VAT are widely believed to secrete a number of chemokines to recruit macrophages to adipose tissue. However, the underlying mechanisms for impaired T cell homeostasis in obesity is poorly understood.

B7/CD28-mediated costimulatory signal is required for activation and effector function of T cells (7). Typically, engagement of the TCR in the absence of co-stimulation leads to sub-optimal T-cell stimulation, anergy and apoptosis. CD28 is constitutively expressed by naive T cells and binds to the ligands CD80 (B7-1) and CD86 (B7-2), expressed on the surface of professional APCs. Whether B7 molecules are involved in obesity and IR is completely unclear. The contribution of costimulation has been widely studied in the context of autoimmune disease where in general it has been shown to play a facilitatory role in promoting inflammation. Approaches to decrease costimulation such as genetic or immune disruption of CD28, CTLA-4 or CD80 and/or CD86 have been shown to attenuate transplant rejection, autoimmune arthritis...
and experimental allergic encephalitis. The use of CTLA-4 Ig, which reduces costimulation by binding to CD80 and CD86, has been shown to be protective in humans with type 1 diabetes by slowing the rate of β cell loss when administered at an early stage disease. Unexpectedly, in this study we observed an enhanced adipose inflammation and IR in mice deficient for CD80/CD86 despite decreased T cell activation in circulation.

To investigate the role of costimulatory molecules CD80 and CD86 in obesity, human VAT and SAT derived from lean as well as obese/insulin resistant humans were used for the detection of CD80 and CD86 on APCs. The obese adipose samples were obtained from obese patients undergoing endoscopic gastric bypass surgery. Most of the obese patients were on antidiabetic medication due to morbid obesity and the blood glucose and HemA1c level was controlled in a relatively normal range. Despite of relatively normal metabolic parameters in those obese patients, a correlation between B7 expression and degree of insulin resistance was observed. Further studies are required to confirm the implication of B7 in insulin resistance in humans. Despite higher indices of innate and adaptive immune inflammation, macrophages expressing CD80+CD86+ were decreased in both VAT and SAT compartments of obese patients, as well as in the VAT of HF-fed mice. A trend towards a decrease in CD80 and CD86 single positive cells was also noted although no statistical significance was observed because of relatively limited sample size. Further study is required to address the significance of CD80, CD86 single positive cells. CD80/CD86 may be expressed in alternate cell types such as adipocytes and endothelial cells. The expression of CD80/CD86 on endothelial cells is typically low under unstimulated condition (28) and is insufficient to activate T cells (29). Since adipocytes constitute dominant cells in adipose, we investigated the expression in these cells and did not find a difference. Therefore, the expression of CD80/CD86 on macrophages, the
dominant antigen presenting cells in adipose tissue, may play an essential role in regulating costimulatory signaling of adipose tissue. Expression of CD80 and CD86 on macrophages in human VAT but not SAT was negatively correlated with HOMA-IR, suggesting a potential role of costimulation in IR. Consistent with our expectation that CD80/CD86 deletion would reduce T cell activation, we observed reduced T cell numbers and T cell activation in CD80 CD86 double knockout (B7 KO) mice (7; 10). Due to defective B7-mediated costimulation, T cell reduced in both spleen and adipose tissue. However, in contrast to our expectation, multiple measures of glucose/insulin homeostasis were impaired by deficiency of B7, despite lack of change in overall body weight and lower visceral adiposity. Since innate immune activation and inflammation are major drivers of IR and metabolic derangement (30-33), we observed an increased level of TNFα and MCP-1, as well as macrophage infiltration in VAT of both obese mice and humans.

Prior studies have implicated CD28 and CTLA-4 in Treg development (34-36). Blocking B7/CD28 axis by neutralizing antibodies or CTLA-4 Ig reduced Treg in C57BL/6 and non-obese diabetic (NOD) mice (34; 35). Using a CD80/CD86 double knockout mouse model, we provide direct evidence supporting the essential role of B7/CD28 axis in Treg development and proliferation in this study. Tregs in spleen, blood, lymph nodes, as well as VAT were dramatically reduced in B7 KO mice. More importantly, a positive correlation between B7 expression on ATM and adipose-tissue-resident Treg was noted in human VAT samples. During the development of obesity, it has been suggested that there is a shift to pro-inflammatory M1-type macrophages, which contributes to IR. Tregs play an important role in maintaining immune balance by interacting with inflammatory cells including T cells and APCs (37). By coculturing Treg or Tn with macrophages, we confirmed that Treg suppresses CD11c+ macrophage, a
proinflammatory subpopulation of macrophage contributing to obesity-induced complications including IR. In human VAT samples, we also observed a negative association between Treg and CD11c\(^+\)CD11b\(^+\) cells. Consistent with this, CD11c\(^+\)CD11b\(^+\) population significantly decreased in VAT of B7 KO mice. Furthermore, adoptive transfer of Treg to B7 KO mice abrogated the increase of both macrophage infiltration and macrophage classical activation in VAT.

We next demonstrated that B7 deficient APCs displayed a reduced ability to promote Treg proliferation, differentiation from thymocytes, and conversion from Foxp3\(^+\) T cells. Moreover, Treg cocultured with \(B7^{-/-}\) APCs has decreased suppressive functions, indicating that B7-mediated costimulation is essential for the differentiation, expansion, as well as function of Treg. Furthermore, Treg transfer restored insulin sensitivity and reduced macrophage-mediated adipose inflammation in B7 KO mice. It has been reported that Treg downregulates CD80 and CD86 on APCs through CTLA-4-mediated trans-endocytosis (38). On the basis of our observations, we propose a bi-directional control loop between Treg and B7 molecules on APCs in healthy individuals with prevention of excessive T cell activation (Figure 8G). In obese patients, it is likely that this feedback control loop between Treg and B7 molecules is impaired, leading to a progressive decrease of B7 and Treg which enhances adipose inflammation (Figure 8H). Our results do not provide insights on the primacy of each pathway or whether a decrease in CD80/CD86 occurs earlier in obesity than perturbations in Treg numbers. However our results in ND fed animals suggest that disruption of CD80/CD86 may alter Treg numbers/function even in the absence of HF diet.

Understanding the mechanisms underlying obesity-induced inflammation and IR is of critical importance in designing novel therapeutic approaches for the treatment of type 2 diabetes. In the current study, we demonstrate for the first time that CD80 and CD86 on ATM
are essential in sustaining immune balance in adipose tissue. Expression of CD80 and CD86 decreased during obesity and may represent an important mechanism impaired Treg homeostasis in obese patients. Our results may have implications for therapies that target costimulation for the treatment of type 2 diabetes and suggest that their application may need to consider potential adverse consequences on Tregs.
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AUTHOR CONTRIBUTIONS


DISCLOSURES

None
References

Figure Legends

**Figure 1 Reduced CD80 and CD86 expression on ATM of obese humans:** A-D, Stromal vascular fraction (SVF) was isolated from visceral adipose tissue (VAT) of lean humans undergoing inguinal herniorrhaphy BMI<30 and obese patients undergoing bariatric intervention (BMI≥30), followed by flow cytometric analysis. CD45+CD14+ monocyte/macrophage (A, representative images; B, statistical analysis) or CD45+CD11b+ mature macrophage (C, representative images; D, statistical analysis) were gated for the detection of CD80 and CD86. E-H, SVF was isolated from subcutaneous adipose tissue (SAT) of obese BMI<30 and lean (BMI≥30) humans undergoing lipoaspiration/liposuction followed by flow cytometric analysis. CD45+CD14+ monocyte/macrophage (E, representative images; F, statistical analysis) or CD45+CD11b+ mature macrophage (G, representative images; H, statistical analysis) were gated for the detection of CD80 and CD86. I&J, Associations of HOMA-IR with CD80+CD86+ monocyte/macrophage (I) or mature macrophage (J) in VAT was shown. N=10.

**Figure 2 B7 deficiency reduces visceral fat mass, but exacerbates diet-induced IR:** Wild-type (WT) and CD80−/−CD86−/− (B7 KO) mice were fed a normal chow diet (ND) or high-fat diet (HF). Body weight of the mice was measured after 12 weeks of diet feeding (A). The adiposity was evaluated by epididymal fat weight (B) and MRI analysis (C). Liver weight (D) and hepatic lipid levels (left, triglyceride; right, cholesterol) were examined. IPGTT (F) and ITT (G) on WT and B7 KO mice were performed after 12 weeks of diet feeding (N=8). Fasting blood glucose (H) was measured after 16 h of overnight fasting. Plasma was collected after 16 h fasting and used for ELISA detection of insulin and leptin. HOMA-IR was calculated as follows: $\text{HOMA-IR} = \frac{\text{Fasting Serum Glucose (mg/dL)} \times \text{Fasting Plasma insulin (µU/mL)}}{405}$. Fasting insulin (I),
HOMA-IR (J), and leptin (K) were shown. * p<0.05 compared with WT; # p<0.05 compared with ND; N=10-12/group.

**Figure 3 Lack of B7 in mice promotes adipose inflammation despite lower activation of T cells in circulation:** Splenocytes harvested from WT and CD80<sup>-/-</sup> CD86<sup>-/-</sup> (B7 KO) mice were used to analyze immune cell populations (n=12/group). A, CD45<sup>+</sup> inflammatory cells were gated for the analysis of macrophages. CD11b<sup>+</sup>F4/80<sup>+</sup> cells in WT and B7 KO mice were shown. B, CD45<sup>+</sup> inflammatory cells were gated and total T cells (CD3<sup>+</sup>) were analyzed. C, Splenic T cells (CD45<sup>+</sup>CD3<sup>+</sup>) were gated for helper T cell (CD4<sup>+</sup>) and cytotoxic T cells (CD8<sup>+</sup>). D-G, Splenic CD4<sup>+</sup> and CD8<sup>+</sup> T cells were then gated for the analysis of T cell activation marker CD69 (D, representative images; E, statistical analysis) and CD44 (F, representative images; G, statistical analysis). H-J, WT and B7 KO mice were fed a HF or ND for 12 weeks. Epididymal fat was isolated for the real-time PCR and western blot analysis of TNFα MCP-1, and IL-10 (H - K) expression. SVF isolated from epididymal fat was used for the analysis of macrophage (CD45<sup>+</sup> CD11b<sup>+</sup>) infiltration in adipose tissue (L; left, representative images; right, statistical analysis). Three independent experiments were performed and consistent results were obtained. * p<0.05 compared with WT; # p<0.05 compared with ND.

**Figure 4 Loss of B7 reduces Treg in mice:** A, WT and B7 KO mice were fed a HF or ND for 12 weeks. SVF was isolated from epididymal fat and used for the flow cytometric detection of T cells (CD45<sup>+</sup> CD3<sup>+</sup>; left, representative images; right, statistical analysis). B, Foxp3 expression in VAT was detected by real-time PCR. C, Epididymal fat from eight-week-old WT and B7 KO male mice (on ND) was used for the isolation of SVF. SVF was then used for flow cytometric
detection of Treg using a Foxp3 staining kit. CD4+ T cells were gated and Tregs (CD25+ Foxp3+) in SVF were examined. D, Tregs (CD4+Foxp3+) in spleen, blood, and lymph node (LN) were detected in WT and B7 KO mice. E, SVF isolated from human VAT was stained with anti-CD4, anti-CD25, anti-Foxp3, anti-CD11b, anti-CD80, and anti-CD86 antibodies, followed by flow cytometric analysis. CD4+ cells were gated for the analysis of Tregs (Foxp3+CD25+) and macrophages (CD11b+) were gated for the analysis of B7 expression (CD80+CD86+). Correlation between B7 expression on ATM and Treg percentage were examined. N=14. * p<0.05 compared with WT; # p<0.05 compared with ND.

**Figure 5 B7-mediated costimulation promotes Treg proliferation:** A - C, Tregs were isolated from Foxp3-GFP knock-in mice using flow sorting, labeled with CellTrace Violet, and then cocultured with WT or B7 KO BMMs. Cells were collected for detection of proliferation after 5 days. A, Gating strategy, leukocytes were gated according to FSC and SSC. Tregs (CD4+CellTrace+Foxp3+) were then gated for the analysis of CellTrace intensity. B, Proliferated Tregs with a lower fluorescence intensity were evaluated using flow cytometry. Representative images showing lower proliferation of Treg when cocultured with B7 KO BMMs. C, Statistical result of Treg proliferation. D & E, Tregs cocultured with WT or B7 KO BMMs were used for suppression assay. Tregs harvested from coculture system were then cultured with WT splenocytes for 5 days. Cells were collected for the analysis of T cell activation marker CD69 (D) and CD44 (E). Representative images (left panel) and statistical result (right panel) were shown. * p<0.05.
**Figure 6 Loss of B7 impairs the ability of APCs to induce Treg:** A-C, WT or B7 KO BMMs were cocultured with thymocytes isolated from Foxp3-GFP knock-in mice in the presence of 2 ng/mL TGF-β. Cells were collected for flow cytometric analysis after 5 days coculture. Gating strategy: cells were first gated for leukocytes according to FSC and SSC, and then CD4+ cells were gated for the analysis of Foxp3 expression (A). Foxp3+ Tregs in thymocytes cocultured with WT or B7 KO BMMs were shown (B, representative images; C, statistical analysis). D & E, WT or B7 KO BMDCs were cocultured with thymocytes isolated from Foxp3-GFP knock-in mice in the presence of 2 ng/mL TGF-β. Cells were collected for flow cytometric analysis of Treg. Thymocytes were first gated according to FSC and SSC. CD4+ cells were then gated for the analysis of Treg (D). Tregs differentiated from thymocytes cocultured with B7 KO BMDCs were shown (E). F & G, Foxp3− naïve T cells (Tn) were sorted from Foxp3-GFP knock-in splenocytes and cocultured with WT or B7 KO BMDCs in the presence of 2 ng/mL TGF-β. Tregs converted from Tn were shown (F, bar grap; G, representative images). Three independent experiments were performed and consistent results were obtained. * p<0.05. N=5-7/group.

**Figure 7 Treg suppresses classical activation of macrophages:** A, SVF isolated from human VAT was stained with anti-CD4, anti-CD25, anti-Foxp3, anti-CD11b, and anti-CD11c antibodies, followed by flow cytometric analysis. CD4+ cells were gated for the analysis of Tregs (Foxp3−CD25+) and macrophages (CD11b+) were gated for the analysis of CD11c+CD11b+ cells. Correlations between CD11c+ CD11b+ cells and Treg in VAT were examined. N=14. B & C, Treg or Tn were isolated from Foxp3-GFP knock-in mice using flow sorting. Treg or Tn were then cocultured with WT BMMs for 5 days, followed by flow cytometric analysis of CD11c+ CD11b+ cells (B, statistical analysis; C, representative images). D, SVF isolated from WT or B7
mice fed a normal chow or high-fat diet was used for flow cytometric analysis of CD11c⁺ CD11b⁺ cells. * \( p<0.05 \) compared with WT; # \( p<0.05 \) compared with ND.

**Figure 8 Adoptive transfer of Treg restore insulin sensitivity in B7 KO mice:** Treg or Tn isolated from Foxp3-GFP knock-in mice were adoptively transferred into HF-fed B7 KO mice every other week. Adipose inflammation and IR were evaluated after 12 weeks. A, Presence of transferred Treg (CD4⁺GFP⁺) in SVF of mice transferred with Tregs but not those transferred with Tn. B, Epididymal fat was used for the real-time PCR analysis of Foxp3 expression (B). C, Macrophage (CD11b⁺) infiltration in VAT of B7 KO mice transferred with Treg or Tn was evaluated by flow cytometry. D, Classical activation of ATM (CD11c⁺ CD11b⁺) was detected in VAT of B7 KO mice transferred with Treg of Tn. E, Levels of blood glucose were measured after i.p. challenge of glucose (IPGTT). F, Levels of blood glucose after i.p. challenge of insulin (ITT). N=5/group. G, Hypothetical model of negative feedback of B7 and Treg in healthy individuals. B7 on APCs promotes Treg proliferation and differentiation via interaction with CD28. CTLA-4 on Treg downregulates B7 expression on APCs. H, In lean humans, B7 expression on adipose resident APCs enhances Treg proliferation and differentiation. Treg subsequently suppresses CD11c⁺ CD11b⁺ cells and production of inflammatory cytokines such as TNFα and MCP-1. In obesity/IR, B7 reduced on adipose resident APCs, which results in reduced Treg and enhanced macrophage-mediated adipose inflammation. * \( p<0.05 \).
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8
### Supplemental Table 1 Real-time PCR primer sequence

<table>
<thead>
<tr>
<th>Target Gene</th>
<th>Primer Sequence (5’ to 3’)</th>
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| TNFα        | Forward: CAACGGCATGGATCTCAAAGAC  
             | Reverse: AGATAGCAAATCGGCTGACGGTG |
| MCP-1       | Forward: TCACTGCTGCTACTCATCCTCA  
             | Reverse: TACAGCTTCTTGGGACACCTGCT |
| Foxp3       | Forward: ACACCAGGAAAGACAGCAACC  
             | Reverse: CCTGAAGACCTTCTCAAAACC  
| IL-10       | Forward: TAGAGCTGCGGACTGCTTTCA  
             | Reverse: ATGCTCCTTTGATTTCTGCGCAT |
| 18s rRNA    | Forward: AACACAGGGATCGGACAAC  
             | Reverse: CAGTCATATTGCCAGTCCCAT |

### Supplemental Table 2 Baseline metabolic data of patients recruited in this study

<table>
<thead>
<tr>
<th></th>
<th>VAT Lean</th>
<th>VAT Obese</th>
<th>SAT Lean</th>
<th>SAT Obese</th>
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<tbody>
<tr>
<td>Number of Patients</td>
<td>9</td>
<td>18</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Age (year)</td>
<td>44.44 ± 6.20</td>
<td>46.19 ± 2.49</td>
<td>40.25 ± 4.55</td>
<td>42.40 ± 2.71</td>
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<tr>
<td>BMI</td>
<td>25.48 ± 0.73</td>
<td>53.01 ± 2.47</td>
<td>22.25 ± 0.48</td>
<td>32.0 ± 0.85</td>
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<tr>
<td>Blood Glucose (mg/dl)</td>
<td>99.78 ± 4.94</td>
<td>106.29 ± 9.67</td>
<td>88.5 ± 4.09</td>
<td>84.0 ± 2.79</td>
</tr>
<tr>
<td>Total Cholesterol (mg/dl)</td>
<td>159.44 ± 12.75</td>
<td>165.79 ± 10.12</td>
<td>166 ± 11.07</td>
<td>196.8 ± 19.10</td>
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<tr>
<td>Non-HDL Cholesterol (mg/dl)</td>
<td>105.00 ± 10.11</td>
<td>122.79 ± 10.09</td>
<td>137.25 ± 31.30</td>
<td>131.60 ± 10.38</td>
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<td>HemA1c (%)</td>
<td>5.80 ± 0.22% (40 ± 2.4 mmol/mol)</td>
<td>5.74 ± 0.29% (39 ± 3.2 mmol/mol)</td>
<td>5.18 ± 0.33% (33 ± 3.6 mmol/mol)</td>
<td>5.30 ± 0.09% (34 ± 1.0 mmol/mol)</td>
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<tr>
<td>CRP (mg/L)</td>
<td>5.83 ± 1.62</td>
<td>11.06 ± 3.03</td>
<td>1.52 ± 0.59</td>
<td>6.87 ± 5.15</td>
</tr>
</tbody>
</table>

Data are presented as Mean ± SEM. VAT Lean, visceral adipose tissue from lean patients; VAT Obese, visceral adipose tissue from obese patients; SAT Lean, subcutaneous adipose tissue from lean patients; SAT Obese, subcutaneous adipose tissue from obese patients; BMI, body mass index; CRP, C-reactive protein.