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Changes in the Adipose Tissue Expression of CD86 Costimulatory Ligand and CD163 Scavenger Receptor in Obesity and Type-2 Diabetes: Implication for Metabolic Disease

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Abstract

Background: The CD86 costimulatory ligand and CD163 scavenger receptor are expressed on monocytes/macrophages. However, modulations in the expression of these immune regulatory receptors in metabolic disease remain unclear. We, therefore, compared the adipose tissue expression of CD86/CD163 and signature inflammatory cytokines/chemokines in non-diabetic and diabetic individuals.

Methods: Subcutaneous adipose tissue biopsies were collected from 57 non-diabetics and 46 diabetics classified based on body mass index as obese, overweight and lean and the expression of CD86/CD163 was assessed by quantitative RT-PCR, immunohistochemistry/ confocal microscopy.

Results: The data show that CD86 and CD163 gene expression was elevated in non-diabetic obese individuals as compared with their lean counterparts ($P_{[CD86]}$ = 0.0035; $P_{[CD163]}$ = 0.0028). As expected, CD86 and CD163 protein expression was also found to be elevated in the adipose tissue samples of obese individuals. The CD86 gene expression in non-diabetic and diabetic individuals correlated positively (P<0.05) with that of TNF-α ($r_{[non-diabetic]}$ = 0.26; $r_{[diabetic]}$ = 0.49), IL-18 ($r_{[non-diabetic]}$ = 0.67; $r_{[diabetic]}$ = 0.63), and IL-8 ($r_{[non-diabetic]}$ = 0.38; $r_{[diabetic]}$ = 0.33). The CD86 gene expression correlated significantly with that of IL-23 and IP-10 in non-diabetic individuals. The CD86 gene expression also correlated with glycated hemoglobin ($r_{[diabetic]}$ = 0.38; P = 0.007). In parallel, CD163 gene expression also correlated positively (P<0.05) with TNF-α ($r_{[non-diabetic]}$ = 0.35; $r_{[diabetic]}$ = 0.38), IL-18 ($r_{[non-diabetic]}$ = 0.72; $r_{[diabetic]}$ = 0.71), IL-23 ($r_{[non-diabetic]}$ = 0.28), and IL-8 ($r_{[non-diabetic]}$ = 0.58; $r_{[diabetic]}$ = 0.35). The adipose tissue expression of IL-18 independently predicted the expression of CD86 and CD163 both in non-diabetic and diabetic individuals.

Conclusion: The adipose tissue expression of CD86 and CD163 is elevated in obesity and T2D which has consensus with inflammatory signatures and represents novel immune markers for metabolic inflammation.

Keywords: CD86; CD163; Obesity; Type-2 diabetes; Metabolic inflammation; Macrophage markers

Introduction

Obesity is an emerging pandemic and the adipose tissue imbalance or dysfunction contributes to obesity-induced chronic low-grade inflammation or metabolic inflammation which plays a key role in insulin resistance and type-2 diabetes (T2D). Adipose tissue macrophages (ATMs) are considered the major driver of metabolic inflammation due to higher expression of proinflammatory cytokines/ chemokines [1]. These inflammatory mediators act via autocrine/ paracrine mechanisms to induce local and systemic inflammation and impair insulin signaling and glucose uptake in the peripheral tissues. The CD86 costimulatory ligand is one of 4 known molecules in the CD28 system (CD28, CD152, CD80, CD86) and is expressed on monocytes and macrophages as well as other antigen presenting cells [2]. Monocytes and macrophages constitutively express only CD86, dendritic cells express both CD80 (B7-1/B7) and CD86 (B7-2/B70), and resting B cells have low expression of CD86 [3]. CD86/CD80 act as ligands for CD28 receptor on T cells and this interaction provides the potent costimulatory signal for T cell activation, clonal expansion, and optimal cytokine production [4,5]. On the contrary, CD86 binding with cytotoxic T lymphocyte-associated antigen (CTLA)-4 (CD152) negatively regulates T cell activation and suppresses immune response [6]. The CD163 is an acute phase-regulated scavenger receptor that belongs to cysteine-rich superfamily and is involved in clearance and endocytosis of hemoglobin-haptoglobin complexes by macrophages and may protect tissues from free hemoglobin-mediated oxidative damage [7].

CD86 is considered a proinflammatory and CD163 is considered an anti-inflammatory macrophage marker [8]. The perturbations in the expression of CD86 and CD163 markers have been related individually with certain disease conditions [9-11]. However, modulations in the adipose tissue expression of CD86 and CD163 in obesity and T2D remain poorly understood. We hypothesized that obesity/T2D might be a positive modulator of CD86/CD163 markers expression in the adipose tissue. Herein, we present the data showing that adipose tissue gene expression of CD86 and CD163 is upregulated in obesity/T2D and the changes are positively associated with tissue inflammatory state which may have significance as novel immune markers of metabolic inflammation.

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Materials and Methods

Study population

A total of 57 non-diabetic (32 men/25 women; aged 24-71 years) and 46 diabetic (26 men/20 women; aged 23-67 years) individuals were recruited in the study through outpatient clinics of Dasman Diabetes Institute, Kuwait. The study participants were classified based on body mass index (BMI) as lean ($BMI_{[non\text{-}diabetic]}$ = 22.611±2.397 kg/m^2; $BMI_{[diabetic]}$ = 24.827±1.144 kg/m^2), overweight ($BMI_{[non\text{-}diabetic]}$ = 28.300±1.144 kg/m^2; $BMI_{[diabetic]}$ = 28.829±0.909 kg/m^2), and obese ($BMI_{[non\text{-}diabetic]}$ = 34.701±3.227 kg/m^2; $BMI_{[diabetic]}$ = 33.693±2.566 kg/m^2). The diagnosis and confirmation of diabetes was performed by designated physician at the clinical services department of the institute. The most common comorbidities found in obese/T2D individuals included hyperlipidemia (5), kidney disease (4), and coronary artery disease (1). The demographic and clinico-therapeutic characteristics of study participants are summarized in **Table 1**. All participants gave written informed consent and study was approved by the institutional ethics committee.

Anthropometric and physio-clinical measurements

Anthropometric and physical measurements included body weight, height, waist circumference and blood pressure. Height and weight were measured with barefoot participants wearing light indoor clothing using calibrated portable electronic weighing scales and portable inflexible height measuring bars. The waist circumference at the highest point of the iliac crest and the mid-axillary line was measured using constant tension tape at the end of a normal expiration with arms relaxed at sides and waist-to-hip ratios were calculated. The whole-body composition including percent body fat (PBF), soft lean mass and total body water were measured using IOI 353 body composition analyzer (Jawon Medical, South Korea). Blood pressure, an average of 3 readings taken at 5-10 resting minutes apart, was measured using Omron HEM-907XL digital automatic sphygmomanometer (Omron Healthcare Inc. IL, USA). BMI was calculated using standard formula: body weight (kg)/height (m^2). For clinical laboratory measurements, peripheral blood was collected by venipuncture from overnight-fasted (10 hrs minimum) individuals and samples were analyzed for fasting blood glucose, glycated hemoglobin (HbA1c), fasting insulin, and lipid profile. Glucose and lipid profiles were measured by using Siemens dimension RXL chemistry analyzer (Diamond Diagnostics, Holliston, MA, USA) and HbA1c was measured by using Variant™ device (BioRad, Hercules, CA, USA).

Collection of subcutaneous adipose tissue samples

Adipose tissue samples (~0.5g) were collected via abdominal subcutaneous fat pad biopsy lateral to the umbilicus using standard surgical method. Briefly, the periumbilical area was sterilized by alcohol swab and locally anesthetized by 2% lidocaine (2ml). Fat tissue was collected through a superficial skin incision (0.5cm), further into small pieces, rinsed in cold phosphate buffered saline (PBS), fixed in 4% paraformaldehyde for 24hr and was embedded in paraffin for further use. At the same time, freshly-collected adipose tissue samples (~50-100 mg) were preserved in RNAlater or optimal cutting temperature (OCT) medium and stored at -80°C until use.

Quantitative real-time PCR

Total RNA was purified using RNeasy kit (Qiagen, Valencia, CA, USA) as per manufacturer's instructions. Briefly, adipose tissue samples in RNAlater or OCT were thawed and homogenized 33,000 rpm for 40sec) in Qiazol lysis solution (Qiagen, Valencia, CA, USA) using TissueRuptor (Qiagen, Hildon, Germany), treated with chloroform and centrifuged at 12,000 ×g for 15min at 4°C. The upper aqueous RNA phase was collected, 70% ethanol was added, applied to an RNeasy spin column, and total RNA was eluted in RNase-free water. RNA quantity was measured using Epoch™ Spectrophotometer (BioTek, Winooski, USA) and quality was assessed by formaldehyde-agarose gel electrophoresis. RNA samples (1µg each) were reverse transcribed to cDNA using random hexamer primers and TaqMan reverse transcription reagents (High Capacity cDNA Reverse Transcription Kit, Applied Biosystems, CA, USA). The cDNA samples (50ng each) were amplified using TaqMan® gene expression MasterMix

Table 1 Patients' demographic and clinico-therapeutic data.

Parameter	Non-diabetic			Type-2 diabetic		
	Lean	Overweight	Obese	Lean	Overweight	Obese
Total number (N)	9	21	27	3	11	32
Male (N)	6	13	13	2	6	18
Female (N)	3	8	14	1	5	14
Age (Yrs.)	25-53	29-71	24-66	48-58	45-59	23-67
Body mass index (kg/m^2)	22.611±2.397	28.300±1.144	34.701±3.227	24.827±1.144	28.829±0.909	33.693±2.566
Percentage of body fat (PBF)	27.300±2.037	32.520±1.182	39.280±0.849	32.100±4.400	31.840±1.823	36.440±1.018
Glucose (mmol/L)	4.948±0.224	5.540±0.326	5.387±0.150	5.800±0.3000	8.727±0.707	8.708±0.437
Cholesterol (mmol/L)	5.304±0.394	4.913±0.160	5.106±0.213	5.400±1.800	4.695±0.511	5.160±0.241
High-density lipoprotein (mmol/L)	1.630±0.498	1.261±0.289	1.174±0.275	1.050±0.181	1.138±0.372	1.199±0.285
Low-density lipoprotein (mmol/L)	3.378±0.964	3.133±0.671	3.374±0.937	3.800±1.510	2.582±1.333	2.946±1.092
Triglycerides (mmol/L)	0.616±0.244	1.179±0.626	1.200±0.742	1.470±0.967	1.791±0.891	1.874±1.494
HbA1c (%)	5.689±0.483	5.845±1.580	5.766±0.589	7.733±3.009	7.373±1.547	8.206±1.479
Hyperlipidemia (N)	0	0	3	0	3	5
Coronary artery disease (N)	0	0	1	0	1	1
Lung disease (N)	0	0	1	0	0	0
Kidney disease (N)	0	0	0	0	0	4
Therapy		Zocor, Aspirin	Concor, Lipitor, Aspirin	Metfornin, Aspirin	Glucophage, Zocor, Januvia, Metformin, Aspirin, Zestril	NovoRapid, Insulin, Aspirin, Zocor, Lipitor, Glucophage, Metformin, Zestril, Januvia

(Applied Biosystems, CA, USA) and gene-specific 20× TaqMan assays for CD86:Hs01567026_m1, CD163:Hs00174705_m1, TNF-α:Hs01113624_g1, IL-18:Hs01038788_m1, IL-23a:Hs00900828_g1, IL-8:Hs00174103_m1, IP-10:Hs01124251_g1, and GAPDH:Hs03929097_g1 (Applied Biosystems, CA, USA) containing forward and reverse primers and a target-specific TaqMan® minor groove binder (MGB) probe labeled with 6-fluorescein amidite (FAM) dye at the 5' end and non-fluorescent quencher (NFQ)-MGB at the 3' end of the probe, and 40 cycles of PCR reaction using a 7500 Fast Real-Time PCR System (Applied Biosystems, CA, USA). Each cycle included denaturation for 15sec at 95°C, annealing/extension for 1min at 60°C following uracil DNA glycosylase (UDG) activation (50°C for 2min) and AmpliTaq Gold enzyme activation (95°C for 10min). The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression was used as internal control to normalize the differences in individual samples. Relative mRNA expression (fold change) of CD86, CD163, TNF-α, IL-18, IL-8, IL-23, and IP-10 with regard to average control expression (lean subjects) taken as 1 was calculated using Ct method ($2^{-\Delta\Delta Ct}$).

Immunohistochemistry

Subcutaneous adipose tissue paraffin-embedded sections (4μm) were deparaffinized in xylene and rehydrated serially through ethanol (100%, 95%, & 75%) to water. For antigen retrieval, slides were placed in target retrieval solution (pH6.0; Dako, Glostrup, Denmark) under pressure cooker boiling for 8min and cooled for 15min. After PBS wash, endogenous peroxidase activity was blocked with 3% H_2O_2 for 30min and non-specific antibody binding was blocked with 5% nonfat milk for 1hr, followed by 1% BSA solution for 1hr. Samples were incubated overnight at room temperature with rabbit polyclonal anti-human CD86 (1: 100 dilution, Abcam® ab53004) and CD163 (1:800 dilution, Abcam® ab87099) primary antibodies. After two washes (PBS with 0.5% Tween), slides were incubated for 1hr with secondary antibody (goat anti-rabbit conjugated with horse radish peroxidase (HRP) polymer chain; EnVision™ Kit from Dako, Glostrup, Denmark) and color was developed using 3,3'-diaminobenzidine (DAB) substrate. Specimens were washed in running tap water, lightly counterstained with Harris hematoxylin, dehydrated through ascending grades of ethanol (75%, 95%, & 100%), cleared in xylene, and finally mounted in dibutyl phthalate xylene (DPX). For analysis, digital photomicrographs of entire adipose tissue sections (100×; Panoramic Scan, 3D-HISTECH, Hungary) were used to quantify the immunohistochemical staining in three different regions to assess the regional heterogeneity in tissue samples and regions were outlined using Aperio ImageScope software (Aperio Vista, CA, USA). Aperio-positive pixel count algorithm (version 9) was used to quantify the amount of specific staining in the region. The number of positive pixels was normalized to the number of total (positive and negative) pixels to account for variations in the size of the region sampled. Color and intensity thresholds were established to detect the immunostaining as positive and background staining as negative pixels. Once the conditions were established, all slides were analyzed using the same parameters. The resulting color markup of the analysis was confirmed for each slide.

Confocal microscopy

Subcutaneous adipose tissue formalin-fixed and paraffin-embedded sections (8μm) were processed for immunofluorescent labeling using similar protocol for antigen retrieval and blocking as described for immunohistochemistry. Samples were incubated overnight at room temperature with rabbit polyclonal anti-human CD86 (1:100 dilution, abcam® ab53004) and CD163 (1:400 dilution, abcam® ab87099) primary antibodies. After two washes with PBS-Tween, slides were incubated for 1hr with secondary antibody (1:400 dilution of goat anti-rabbit conjugated with Alexa Fluor® 488, Abcam® ab150077) and washed in PBS at least thrice. Samples were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Vectashield, Vectorlab, H1500) and cover slip mounted. Confocal images were collected using inverted Zeiss LSM710 Spectral confocal microscope (Carl Zeiss, Gottingen, Germany) and EC Plan-Neofluar 40×/1.30 oil DIC M27 objective lens. After sample excitation using a 488nm diode-pumped solid-state laser and 405nm line of an argon ion laser, optimized emission detection bandwidths were configured using Zeiss Zen 2010 control software.

Statistical Analysis

The data obtained were expressed as mean ± SEM values. Group means were compared using unpaired *t*-test and linear dependence between two variables was assessed by Pearson's correlation coefficient (r) values. GraphPad Prism software (version 6.05; San Diego, CA, USA) was used for statistical analysis and graphical representation. Multiple linear regression (SPSS-22.0 statistical software, IBM, USA) was used to determine independent correlations of CD86 and CD163 with other inflammatory markers. All *P*-values ≤ 0.05 were considered statistical significant.

Results

Adipose tissue CD86 and CD163 gene expression is significantly upregulated in non-diabetic obese individuals

CD86 is a costimulatory ligand expressed mainly by monocytes/macrophages. The changes and significance of its modulated expression in the adipose tissue in obesity and/or T2D remain unclear. Our data show that in non-diabetic obese individuals, the adipose tissue gene expression of CD86 was upregulated as compared with lean counterparts and the difference was statistically significant ($P = 0.0035$) (Figure 1A). CD86 mRNA expression correlated with BMI, however, the association did not reach the exact level of statistical significance ($r = 0.25$, $P = 0.08$) (Figure 1B). CD163 is a scavenger receptor for hemoglobin-haptoglobin complexes and CD163+ macrophages play an anti-inflammatory role in free hemoglobin-mediated oxidative damage. We also assessed whether the adipose tissue expression of CD163 was modulated by metabolic inflammation associated with obesity or T2D. To this end, the data show that CD163 gene expression in non-diabetic obese individuals was significantly higher than in lean counterparts ($P = 0.0028$) (Figure 1C) and CD163 mRNA expression correlated with BMI ($r = 0.255$, $P = 0.049$) (Figure 1D). In the diabetic cohort, however, mean CD86 and CD163 gene expression in obese individuals was relatively higher than lean subjects and the correlation between CD86/CD163 gene expression and BMI was non-significant (Figure S1).

Elevated adipose tissue CD86 and CD163 protein expression in diabetic and non-diabetic obese individuals

We next asked whether the protein expression of these macrophage regulatory markers was also increased in the adipose tissue in obesity or T2D. To this effect, as expected and consistent with the upregulated gene expression, CD86 (Figure 2) and CD163 (Figure 3) protein expression was also found to be remarkably higher in obese adipose tissue compared with overweight or lean tissue samples as determined by immunohistochemistry and/or confocal microscopy.

CD86/CD163 gene expression in the adipose tissue relates with the local inflammatory signatures

We asked whether the increased adipose tissue gene expression of CD86 and CD163 was associated with the state of metabolic

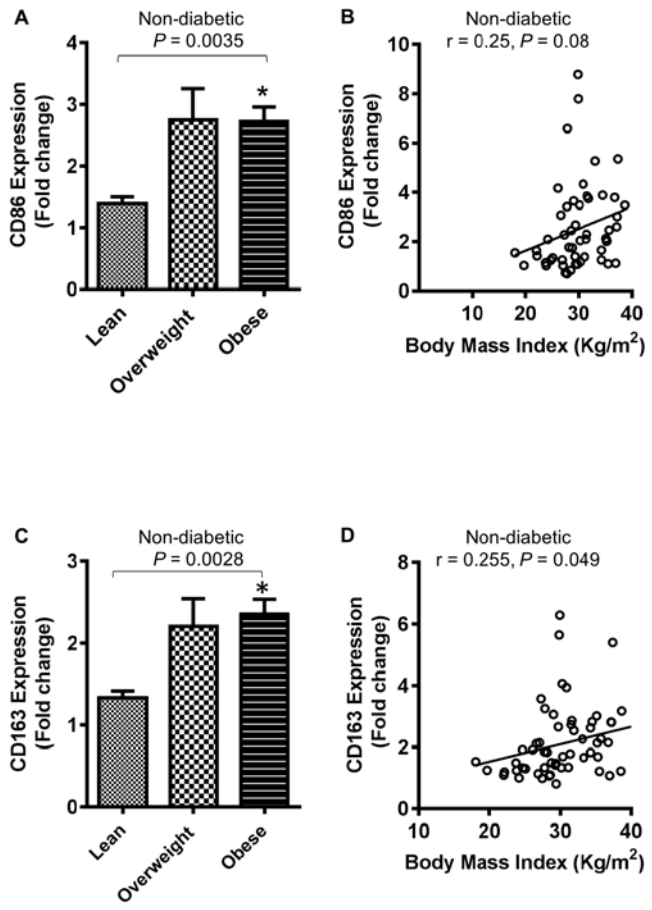

Figure 1 Upregulated CD86 and CD163 gene expression in the adipose tissues from non-diabetic obese individuals.

The subcutaneous adipose tissue gene expression of CD86 and CD163 was assessed by quantitative real-time PCR in 57 non-diabetic individuals classified based on body mass index (BMI) as lean, overweight and obese as described in Methods. CD86 mRNA expression was found to be significantly higher in obese as compared with lean subjects (P = 0.0035) (**A**); however, the positive correlation with BMI (r = 0.25) did not reach statistical significance (P = 0.08) (**B**). Similarly, CD163 mRNA expression was also found to be higher in obese as compared with lean subjects (P = 0.0028) (**C**), which correlated positively with BMI (r = 0.255 P = 0.049) (**D**).

inflammation in obesity/T2D. Since a good agreement was found between gene (qPCR data) and protein (immunohistochemistry data) expression (r = 0.69, P = 0.0002) (Figure S2), we herein show the correlation between CD86/CD163 mRNA expression and gene expression of various inflammatory mediators or markers of metabolic inflammation. To this end, the data show that CD86 mRNA expression (Figure 4) correlated positively with that of TNF-α ($r_{[non-diabetic]}$ = 0.26 P = 0.05; $r_{[diabetic]}$ = 0.49 P = 0.0005), IL-18 ($r_{[non-diabetic]}$ = 0.67 P < 0.0001; $r_{[diabetic]}$ = 0.63 P < 0.0001), IL-23 ($r_{[non-diabetic]}$ = 0.31 P = 0.02), IL-8 ($r_{[non-diabetic]}$ = 0.38 P = 0.008; $r_{[diabetic]}$ = 0.33 P = 0.02), and IP-10 ($r_{[non-diabetic]}$ = 0.34 P = 0.01). Moreover, CD86 gene expression in the diabetic cohort correlated with HbA1c (r = 0.38 P = 0.007). As shown in **Figure 5**, CD163 mRNA expression also correlated positively with that of TNF-α ($r_{[non-diabetic]}$ = 0.35 P = 0.008; $r_{[diabetic]}$ = 0.38 P = 0.009), IL-18 ($r_{[non-diabetic]}$ = 0.72 P < 0.0001; $r_{[diabetic]}$ = 0.71 P < 0.0001), IL-23 ($r_{[non-diabetic]}$ = 0.28 P = 0.03), and IL-8 ($r_{[non-diabetic]}$ = 0.58 P < 0.0001; $r_{[diabetic]}$ = 0.35 P = 0.01).

Multivariate regression analysis revealed that in non-diabetic population, CD86 was independently predicted by IL-18 and IP-10 ($F_{(2, 38)}$ = 19.94 P < 0.001) while in diabetic individuals, CD86 was predicted by TNF-α, IL-18, and HbA1c ($F_{(3, 40)}$ = 16.428 P < 0.001). The CD163 gene expression in non-diabetics was independently predicted by IL-18 and IL-8 ($F_{(2, 42)}$ = 34.01 P < 0.001) and in diabetics was predicted by IL-18 ($F_{(1, 43)}$ = 45.52 P < 0.001). Overall, IL-18 independently predicted both CD86 and CD163 in non-diabetic and diabetic individuals (Table 2).

Discussion

This is the first report, to our knowledge, that evaluated changes in the adipose tissue expression of CD86 costimulatory ligand and CD163 scavenger receptor in obesity and/or T2D. To assess the relationship of these changes with metabolic inflammation, we determined the adipose tissue gene expression of signature inflammatory markers including TNF-α, IL-18, IL-23, IL-8, and IP-10. Our data show elevated CD86 gene expression in the adipose tissue samples from obese individuals with or without T2D as compared with lean counterparts. In diabetic individuals, however, CD86 gene expression differed non-significantly between obese and lean subjects. In parallel with upregulated CD86

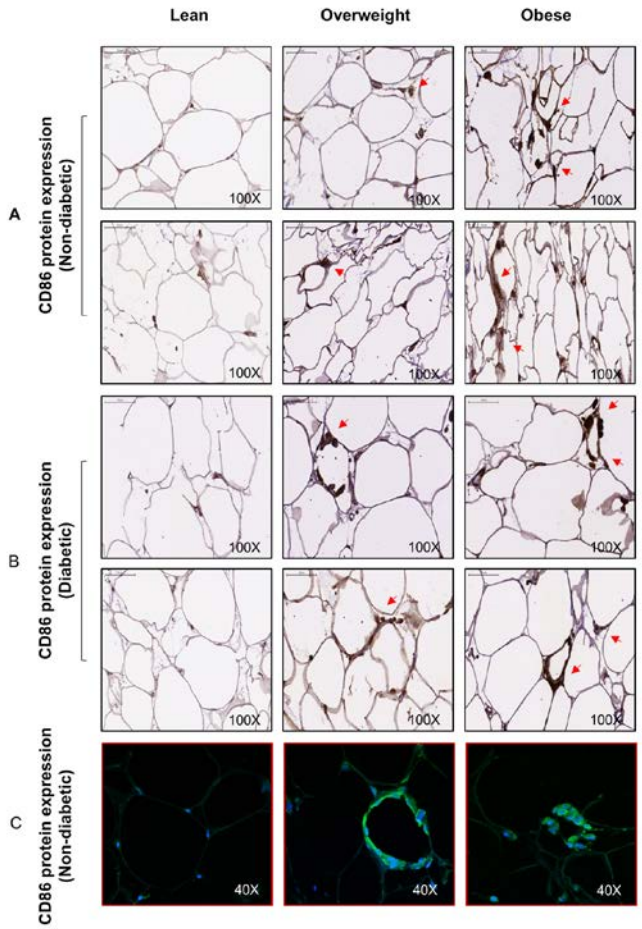

Figure 2 Higher CD86 protein expression in the adipose tissues from obese individuals with or without type-2 diabetes.

CD86 protein expression in the adipose tissues of non-diabetic and diabetic individuals was determined by immunohistochemistry (IHC) and/or confocal microscopy. The representative IHC photomicrographs show CD86 protein expression (arrows) in lean, overweight, and obese individuals, 2 each from 5 independent stainings in non-diabetics (**A**) and diabetics (**B**). The elevated CD86 protein expression was also confirmed by confocal microscopy in randomly selected lean, overweight, and obese samples from non-diabetic individuals; shown 1 each from 3 independent stainings (**C**).

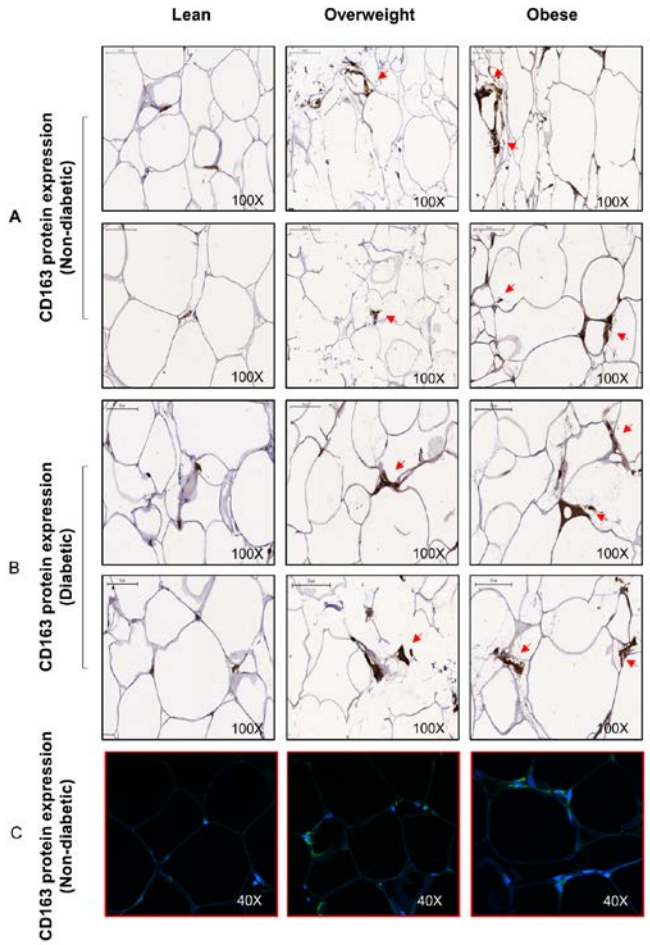

Figure 3 Elevated CD163 protein expression in the adipose tissues from non-diabetic and type-2 diabetic obese individuals.

The adipose tissue CD163 protein expression in non-diabetic/diabetic individuals was determined by immunohistochemistry (IHC) and/or confocal microscopy. The representative IHC photomicrographs show CD163 protein expression (arrows) in lean, overweight, and obese individuals, 2 each from 5 independent stainings in non-diabetics (**A**) and diabetics (**B**). The increased CD163 protein expression was also confirmed by confocal microscopy in randomly selected lean, overweight, and obese samples from non-diabetic individuals; shown 1 each from 3 independent stainings (**C**).

mRNA expression, the CD86 protein expression as expected, was also found to be elevated in obese individuals whether or not diabetic. The CD86 costimulatory ligand is expressed constitutively on monocytes/macrophages and is a key molecule involved in the early immune response [12]. Since CD86 is induced earlier than CD80, it is considered to be a more sensitive inflammatory marker [13]. On the other hand, CD86 was also reported to play a regulatory role in IL-10-mediated response to sepsis by tissue macrophages [14]. From this perspective, the increased CD86 expression may have immunophysiologic significance in maintaining the adipose tissue homeostasis in metabolic inflammation. In agreement with argument, a previous study reported that B7 knockout mice fed on high fat diet showed enhanced adipose tissue inflammation, insulin resistance, and suppressed regulatory T cell development and proliferation [15]. A similar role of costimulatory molecules was reported in certain morbid conditions including infections with intracellular pathogens, myasthenia gravis, murine polymicrobial sepsis, and systemic lupus erythematosus [16-20]. It is important to study changes in the expression of CD86 costimulatory ligand to assess an immune response to a morbid condition as its interaction with CD28 receptor leads to autoregulation whereas its engagement with CTLA4 molecule attenuates the regulatory T cell responses [21]. Our data further show that in non-diabetic individuals, CD86 mRNA expression in the adipose tissue associated positively with local expression of TNF-α, IL-18, IL-23, IL-8, and IP-10. In diabetics, CD86 expression correlated with TNF-α, IL-18, and IL-8. In obesity and T2D, ATMs largely secrete proinflammatory cytokines and chemokines that may function via autocrine/paracrine mechanisms and lead to metabolic inflammation. Our data showing the increased expression of signature inflammatory mediators in obesity/T2D are in agreement with the findings of previous studies [22-24]. In diabetic patients, CD86 expression was also found to correlate positively with HbA1c levels which suggests that its expression may be influenced by levels of glycemia in these individuals. In concordance with this argument, a previous group reported that hyperglycemic conditions might induce the upregulation of several costimulatory receptors including the CD86 expression on monocytes/macrophages and mature dendritic cells [25]. Costimulation via the interaction of CD86 ligand with its cognate receptor CD28 leads to efficient T-cell stimulation

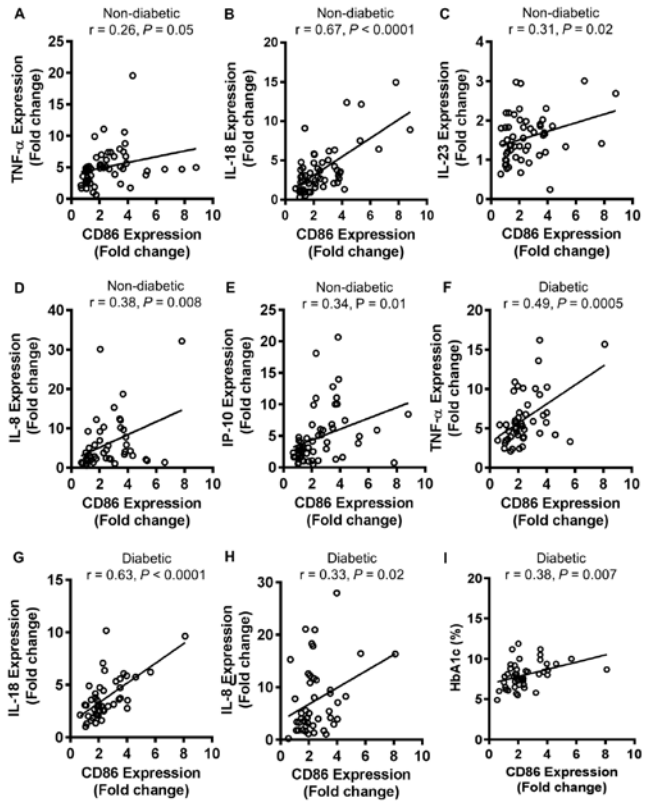

Figure 4 Adipose tissue CD86 gene expression correlates with tissue inflammatory markers.

The adipose tissue gene expression of CD86 and signature inflammatory cyto-/chemokines (TNF-α, IL-18, IL-23, IL-8, and IP-10) was determined by quantitative real-time PCR as described in Methods. The mRNA expression of target gene with regard to average control expression (lean subjects) taken as 1 (fold change) was calculated by using Ct ($2^{-\Delta\Delta Ct}$) method. The adipose tissue CD86 gene expression in non-diabetic individuals correlated positively with TNF-α (r = 0.26 P = 0.05) (**A**), IL-18 (r = 0.67 P < 0.0001) (**B**), IL-23 (r = 0.31 P = 0.02) (**C**), IL-8 (r = 0.38 P = 0.008) (**D**), and IP-10 (r = 0.34 P = 0.01) (**E**). CD86 gene expression in diabetic subjects correlated positively with TNF-α (r = 0.49 P = 0.0005) (**F**), IL-18 (r = 0.63 P < 0.0001) (**G**), IL-8 (r = 0.33 P = 0.02) (**H**), and glycated hemoglobin (HbA1c) (r = 0.38 P = 0.007) (**I**).

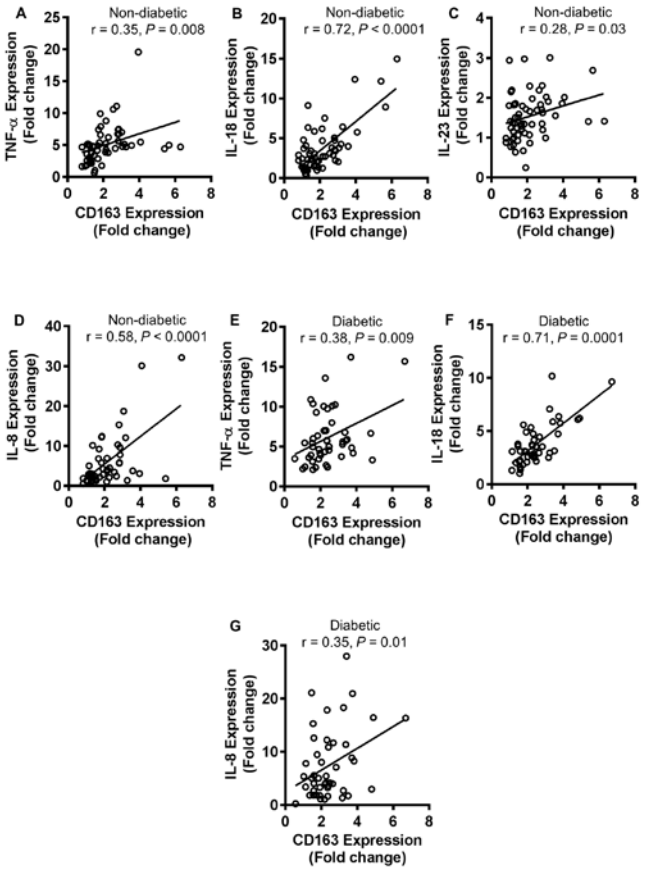

Figure 5 Adipose tissue CD163 gene expression also relates with tissue inflammatory state.

The adipose tissue gene expression of CD163 and signature inflammatory mediators was determined by quantitative real-time PCR as described in Methods. The relative mRNA expression of target gene was expressed as fold change over average expression in controls (lean subjects) taken as 1. The adipose tissue CD163 gene expression in non-diabetic population correlated positively with TNF-α (r = 0.35 *P* = 0.008) (**A**), IL-18 (r = 0.72 *P* < 0.0001) (**B**), IL-23 (r = 0.28 *P* = 0.03) (**C**), and IL-8 (r = 0.58 *P* < 0.0001) (**D**). CD163 gene expression in diabetic population correlated positively with TNF-α (r = 0.38 *P* = 0.009) (**E**), IL-18 (r = 0.71 *P* = 0.0001) (**F**), and IL-8 (r = 0.35 *P* = 0.01) (**G**).

and cytokine expression. A previous study reported the higher CD86 expression on dendritic cells in diabetic patients with unstable angina pectoris (UAP) than non-diabetic patients with UAP [26]. Another study by Spencer *et al.* reported that the adipose tissue macrophages found in crown-like structures (CLS) were predominantly $CD86^+$ (M1-type) while the non-CLS interstitial macrophages were mostly $CD206^+$ (M2-type) and thus the CD86 expression on $CD68^+$ macrophages was used as an inflammatory marker for monocyte/macrophage infiltration observed in the adipose tissue of obese insulin-resistant subjects as compared with lean insulin-sensitive individuals [27]. Our data further show that CD86 gene expression in diabetics, unlike non-diabetics, did not associate with IL-23 and IP-10 expression which might be due to immune metabolic mechanisms that affect the expression of cytokine-chemokine spectrum differentially in diabetics and non-diabetics, while plausible effects of anti-diabetic therapy regarding this discrepancy between diabetic and non-diabetic subjects may not be excluded as well. Interestingly, a previous study showed that unlike other inflammatory cytokines, IL-23 levels varied non-significantly between diabetic and non-diabetic individuals [28]. Overall, the increased costimulatory CD86 ligand expression in adipose tissue may represent an immune marker of metabolic inflammation.

Likewise, CD163 expression was also found to be elevated in individuals with obesity or T2D. The CD163 gene expression correlated positively/significantly with TNF-α, IL-18, and IL-8, while it associated with IL-23 and IP-10 only in non-diabetic individuals. CD163 scavenger receptor plays a regulatory role in inflammatory immune response [7, 29]. The clearance and endocytosis of hemoglobin-haptoglobin complexes by $CD163^{hi}$ macrophages is an important mechanism to counteract free hemoglobin-mediated oxidative damage [30,31]. Hemoglobin-hapatoglobin binding of CD163 in monocytes/macrophages was reported to induce anti-inflammatory effects via IL-10 production [32]. The circulatory levels of soluble CD163 (sCD163) in individuals with obesity/T2D were found to be a predictive biomarker for insulin resistance [33,34]. Our data showing the elevated CD163 expression in obesity/T2D are supported, in part, by a previous study reporting increased numbers of $CD163^+/CD206^+$ macrophages in the adipose tissue of diabetic individuals [35]. The elevated CD163 gene expression in the adipose tissue in obesity or T2D correlated positively/significantly with that of TNF-α, IL-18, and IL-8. The positive association between CD163 and proinflammatory cyto-/chemokines in obesity or T2D indicates that its modulated expression may be relevant as a marker for metabolic inflammation. Notably, a previous study demonstrated that the human ATMs with $CD163^{hi}$ phenotype could produce excessive amounts of proinflammatory mediators [36]. The multivariate regression analysis of our data revealed that in non-diabetic individuals, IL-18 and IP-10 predicted CD86 while IL-18 and IL-8 predicted CD163 expression in the adipose tissue. In diabetics, TNF-α, IL-18, and HbA1c predicted CD86 while only the IL-18 predicted CD163 expression. Thus, IL-18 predicted expression of both CD86 and CD163 in individuals with obesity or T2D.

These data represent obesity- or T2D-related changes in the expression of CD86 and CD163 in the subcutaneous adipose tissue which is easily accessible by transcutaneous biopsy for clinical studies. Regarding the concern of suitability of subcutaneous and visceral fat samples for studying inflammatory changes, a previous report demonstrated that both subcutaneous and visceral fat tissues comparably represented inflammatory changes associated with insulin resistance in obesity [37]. On the other hand, Samaras et al. reported that T2D inflammatory changes were more pronounced in the visceral than subcutaneous adipose tissue [38]. In any case, further work including visceral adipose tissue samples will be valuable. Besides, we determined the expression of CD86 in non-fractionated adipose tissue samples and since CD86 surface marker is expressed on monocytes/macrophages as well as dendritic and B cells, the increased expression of CD86 in the adipose tissue may not be attributed solely to former

Table 2 Multivariate regression analysis of the data.

Immune marker	Population Predictor	Value	
		β	*P*
CD86	Non-diabetic		
	IL-18	0.325	< 0.001
	IP-10	0.085	0.033
	Diabetic		
	TNF-α	0.245	0.013
	IL-18	0.335	< 0.001
	HbA1c	0.115	0.017
CD163	Non-diabetic		
	IL-18	0.218	< 0.001
	IL-8	0.061	0.001
	Diabetic		
	IL-18	0.393	< 0.001

immune cell population as though the macrophage numbers in obese adipose tissue happen to be far more higher than other types of antigen presenting cells in this compartment. We warrant caution while interpreting these data. In addition, the increased expression of CD86 costimulatory ligand in obesity or T2D may lead to activation of regulatory T cell responses which also needs to be addressed in future studies by assessment of forkhead box P3 ($FOXP3^+$) expression or cell numbers in the obese adipose tissue.

Conclusion

Taken together, our data show the significantly elevated expression of CD86 and CD163 in the subcutaneous adipose tissues of individuals with obesity/T2D. Based on consensus of the altered adipose tissue expression of CD86 and CD163 with local inflammatory signatures, these changes may represent as potential immune markers for metabolic inflammation.

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Author Contributions

SS guided experiments, performed the data analysis and interpretation, and wrote the manuscript. RT, SK, EA, AH, and MA collected samples, carried out the experiments, and collected data. KB provided materials and edited the manuscript. RA conceived of the study, participated in its design and coordination, prepared graphs and helped to draft the manuscript, and procured funds. All authors read and approved the final manuscript.

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