Increased CD19$^+$CD24$^+$CD27$^+$ B regulatory cells are associated with insulin resistance in patients with type I Hashimoto's thyroiditis

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Abstract. Hashimoto's thyroiditis (HT) is characterized by dysregulated immune responses and is commonly associated with insulin resistance. However, the mechanism of insulin resistance in HT remains to be fully elucidated. The aim of the present study was to investigate the correlation between the percentage of B regulatory lymphocytes (Bregs) and insulin resistance in patients with HT but with normal thyroid function (type I). A total of 59 patients with type I HT and 38 healthy volunteers were enrolled in the study. An oral glucose tolerance test was performed to measure insulin secretion and assess β-cell functions. Flow cytometry was performed to examine the percentages of lymphocyte populations. The patients with HT exhibited normal fasting and postprandial glucose and fasting insulin secretion, but increased secretion of early-phase and total insulin. The patients with HT also had insufficient β-cell compensation for insulin resistance, indicated by a reduced disposition index, in the fasting state. An elevation in the percentage of CD19$^+$CD24$^+$CD27$^+$ Bregs was also observed, which correlated positively with insulin secretion and insulin resistance in the fasting state. The patients with type I HT had postprandial insulin resistance and insufficient β-cell compensation for fasting insulin resistance. Therefore, the increase in CD19$^+$CD24$^+$CD27$^+$ Bregs was closely associated with fasting insulin secretion. These results provide novel insight into the mechanism of insulin resistance in HT.

Introduction

Hashimoto's thyroiditis (HT) is as an autoimmune disorder and is often a common cause of hypothyroidism. It accounts for ~7.3-20.5% of all thyroid diseases, and its frequency of occurrence is ~7-fold higher in women, compared with men, often during middle age (1,2). Patients with HT, particularly clinical hypothyroidism, are particularly susceptible to other associated medical conditions, including insulin resistance and metabolic syndromes (1-5).

Insulin resistance and metabolic disorders are common complications of HT. Accumulating evidence from previous investigations has demonstrated associations between hypothyroidism and disordered glucose and insulin metabolism. Increased insulin resistance has been observed in patients diagnosed with clinical and subclinical hypothyroidism (4,6-9). To date, reports on the underlying mechanisms of insulin resistance in HT remain inconclusive. Dimitriadis *et al* reported that decreased glucose uptake in muscle and adipose tissues may be due to reduced blood flow, resulting in impaired mitochondrial oxidation in patients with hypothyroidism (4). However, few studies have been performed, which link dysfunctions in the immune response with HT and insulin resistance.

Immune dysregulation leading to chronic inflammation and autoimmunity has been implicated in insulin resistance and the pathogenesis of diabetes mellitus (DM) (10). Immunotherapeutic agents have been introduced to treat type 1 and type 2 DM. For example, anti-CD3 antibody therapy, which targets the clearance of effector T cells and promotes T cell tolerance, has been validated studies in several clinical trials to be an effective therapeutic agent in autoimmune diseases (11-13). Rituximab, a monoclonal antibody targeting CD20-expressing B cells, has been used to treat type 1 DM through its protective effect on insulin-secreting βcells (14). Additionally, in a study using type 2 DM mouse models, B cell depletion by anti-CD20 antibodies prevented disease onset and inhibited insulin resistance by eliminating autoantibody production (15). As dysregulated immune responses and autoimmunity are important contributors to HT and insulin resistance, the present study aimed to examine the immune components shared between the two conditions.

To modulate immune responses and prevent the hyperactivation of immune components and incidence of autoimmune disorders, including DM, a subset of T cells has evolved to function as regulatory cells. These regulatory T cells (Tregs) control the scope of inflammation and

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suppresses autoimmunity, traditionally through the secretion of suppressor cytokines, including interleukin (IL)-10. Furthermore, a $CD8^+CD28^-$ suppressor T cell subset, which functions to inhibit the cytotoxic activity of $CD8^+$ cytotoxic T lymphocytes, has been defined (16). It is well established that the activation of cytotoxic T lymphocytes is among the earliest events leading to the destruction of thyrocytes, and the development of HT and other autoimmune conditions. Whether the newly identified $CD8^+CD28^-$ suppressor T cells are involved in the pathophysiologic process remains to be fully elucidated. Similarly, the concept of regulatory B cells (Bregs) has been suggested with intrinsic immunoregulatory properties similar to Tregs (17,18). Bregs are reported to comprise two major populations: $CD19^+CD24^{hi}CD38^{hi}$ and $CD19^+CD24^+CD27^+$, which are capable of producing IL-10 upon stimulation (19). IL-10 is an essential anti-inflammatory cytokine, and IL-10-producing Bregs are sometimes referred to as B10 cells (20). The contribution of Bregs in autoimmune thyroiditis was reported in a previous study using mouse models (21). However, evidence of the contribution of Bregs in a human study of HT is not currently available. Therefore, in the present study, the Breg and $CD8^+CD28^-$ suppressor T cell populations were examined in patients with HT, and the association between HT and insulin resistance was analyzed in those patients. In order to eliminate the effect of thyroid hormones on insulin signaling and glucose regulation, which may confound the results (22), only patients with type I HT who had normal thyroid functions and were not on hormone treatment were included in the study.

Materials and methods

Ethics statement. All questionnaires and medical procedures were approved by the Institutional Review Board of Fudan University (Shanghai, China). Written informed consent was prospectively obtained from all study participants.

Patients. A total of 59 patients (age range, 18-60 years), diagnosed with type I HT at the Fifth People's Hospital of Shanghai, Fudan University between March and November 2013, were enrolled in the present study and assigned to the HT experimental group. An additional 38 healthy age- and gender-matched volunteers were recruited to the control group. The diagnosis criteria for type I HT were as follows: Normal thyroid function and characteristic clinical manifestations associated with elevated serum anti-thyroid peroxidase antibodies (anti-TPO) or anti-thyroglobulin antibodies (anti-Tg) at ≥60 U/ml (23). The clinical manifestations were detected twice for each patient, with a 1-week interval. The exclusion criteria were as follows: Patients with a previous and/or new diagnosis of DM, determined by oral glucose tolerance test (OGTT), patients with a confirmed diagnosis of hyperthyroidism, and those who received anti-thyroid drugs or thyroid hormone replacement therapy. In addition, patients with cardiovascular and cerebrovascular diseases, severe liver or kidney dysfunction, malignant tumors, severe mental disorders, were currently taking glucocorticoids, or were pregnant or breastfeeding were excluded. The diagnoses of DM, impaired glucose regulation and hypertension were made according to the guidelines approved by the World Health Organization (24).

Sample collection. Questionnaires containing demographic information, medical history of hypertension, weight, height, body mass index (BMI), and waist and hip circumferences were administered to all study participants. An OGTT was performed using fasting blood samples and blood samples collected 30 and 120 min following drinking 75 g glucose dissolved in 250-300 ml water. The levels of plasma glucose, serum lipid, C-peptide and insulin were measured, and thyroid function was assessed. The insulin level in the blood at the time points of 0, 30, and 120 min were designated as Ins0, Ins30 and Ins120, respectively. In addition, the plasma glucose levels at those time points were designated as Glu0, Glu30 and Glu120, with the average glucose and insulin levels in the OGTT designated as GluAve and InsAve, respectively. The area under the curve (AUC) for insulin and glucose (InsAuc and GluAuc, respectively) were determined by the insulin secretion curve adjusted for glucose.

Biochemical analyses. The plasma glucose levels were measured using a glucose oxidation assay kit (Shanghai Institute of Biological Products Co., Ltd. Shanghai, China), using a Beckman Glucose Lab Analyzer 2 (Model 6517; Beckman Coulter, Inc., Danvers, MA, USA). The serum lipid contents, including total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C), were measured using an enzymatic colorimetric analyzer (Hitachi 7600 Clinical Analyzer; Hitachi, Ltd., Tokyo, Japan). The serum levels of C-peptide and insulin, and the thyroid function indicators total triiodothyronine (TT_3), total thyroxine (TT_4), free triiodothyronine (FT_3), free thyroxine (FT_4), thyroid stimulating hormone (TSH), anti-Tg and anti-TPO were measured using specific radioimmunoassay kits purchased from Linco Research, Inc. (St. Charles, MO, USA) in a Beckman immunoassay analyzer (Beckman Coulter, Inc.).

To assess β-cell function and insulin sensitivity, the following parameters were measured: i) homeostasis model assessment of β-cell function (HOMA-β) [20 x Ins0 (μU/ml)]/[Glu0 (mmol/l)-3.5]; ii) change in insulin and glucose ratio at 30 min OGTT (ΔI30/ΔG30) [Ins30-Ins0 (μU/ml)]/[Glu30-Glu0 (mmol/l)]; iii) early-phase insulin secretion(InsAuc30/GluAuc30)[Ins0+Ins30(pmol/L)]/[Glu0+Glu30 (mmol/l)]; iv) ratio of insulin to glucose AUC values at 120 min (InsAuc120/GluAuc120) [Ins0+4 x Ins30+3 x Ins120 (pmol/l)]/[Glu0+4'Glu30+3 x Glu120 (mmol/l)]; v) HOMA of insulin resistance (HOMA-IR) calculated as [Ins0 (μU/ml) x Glu0 (mmol/l)/22.5]; vi) disposition index (DI) calculated as [HOMA-β/HOMA-IR]; vi) Matsuda index (ISI_M) of insulin sensitivity [10,000/((Glu0 (mg/dL) x Ins0 (μU/ml) x GluAve (mg/dl) x InsAve (μU/ml))$^{1/2}$]; vii) DI at 30 min OGTT (DI30) [InsAuc30/GluAuc30 x ISI_M]; and viii) DI at 120 min OGTT (DI120) [InsAuc120/GluAuc120 x ISI_M].

Flow cytometry. Venous blood was obtained from the study participants by EDTA-anticoagulation. For examination of different lymphocyte populations, two 100-μL blood aliquots were incubated with CD4-FITC/CD8-PE-Cy5/CD28-PE and CD19-PE-Cy5/CD24-PE/CD27-APC/CD38-FITC (BD Biosciences, San Jose, CA, USA) for 20 min at room temperature in the absence of light. The stained samples were treated with Simultest™ (Beckman Coulter, Inc.) to lyse erythrocytes.

Table I. Comparison of demographic characteristics and laboratory measurements between HT and control groups.

Characteristic	HT (n=59)	Control (n=38)	P-value
Age (years)	47.2±14.7	40.6±16.9	NS
Gender (female/male)	53/6	34/4	NS
Waist circumference (cm)	83.4±9.4	77.1±8.7	0.043
Hip circumference (cm)	94.6±7.3	93.5±4.1	NS
SBP (mmHg)	120.5±8.8	130.7±22.9	NS
DBP (mmHg)	75.5±5.3	71.2±5.4	NS
BMI (kg/m^2)	23.3±3.7	23.7±3.2	NS
FT_3 (pg/ml)	2.9±0.6	2.9±0.7	NS
FT_4 (ng/dl)	1.1±0.3	1.1±0.1	NS
TT_3 (ng/ml)	1.1±0.4	1.4±1.5	NS
TT_4 (μg/dl)	8.5±3.0	9.3±1.7	NS
TSH (μU/ml)	2.1±1.2	2.2±1.3	NS
Anti-TPO (U/ml)	790.1±572.3	30.3±16.6	<0.001
Anti-TG (U/ml)	216.5±29.3	16.1±9.7	<0.001
TC (mmol/l)	4.9±1.3	4.5±0.9	NS
TG (mmol/l)	1.4±0.8	1.1±0.8	NS
HDL-C (mmol/l)	1.5±0.4	1.3±0.6	NS
LDL-C (mmol/l)	3.3±1.3	3.0±0.7	NS
GADA (%)	0	0	NS
IAA (%)	0	0	NS
ICA (%)	0	0	NS
IGR (%)	39.1	30.8	NS

HT, Hashimoto's thyroiditis; SBP, systolic blood pressure; DBP, diastolic blood pressure; BMI, body mass index; FT_3, free triiodothyronine; FT_4, free thyroxine; TT_3, total triiodothyronine; TT_4, total thyroxine; TSH, thyroid stimulating hormone; TPO, thyroid peroxidase; TG, triglyceride; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; GADA, glutamic acid decarboxylase antibodies; IAA, insulin autoantibodies; ICA, islet cell antibodies; IGR, impaired glucose regulation; NS, not significant.

The samples were washed with PBS twice prior to flow cytometric analysis. The following isotype controls were used: IgG1-FITC, IgG1-PE-Cy5, IgG2a-PE and IgG1-APC. For examination of intracellular IL-10, 100 μl whole blood was cultured in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 25 μg/l phorbol 12-myristate 13-acetate, 1 mg/l ionomycin and 20 μg/l monensin (Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) at a density of 2x10^6 cells/ml. Following incubating for 4-6 h at 37˚C and in 5% CO_2, CD19-Cy5 (BD Biosciences) was added to the cells for 30 min at room temperature. Subsequently, the cells were permeabilized according to the manufacturer's protocol (Beckman Coulter, Inc.), followed by incubation with the monoclonal antibodies IL-10-PE (20 μl, cat. no. JES3-9D7; BD Biosciences) or isotype control IgG2a-PE (BD Biosciences) for 30 min at room temperature. The stained cells were then analyzed on a FACSCalibur flow cytometer (BD Biosciences). Lymphocytes were collected to measure the percentages of $CD8^+$, $CD8^+CD28^-$, $CD19^+CD24^+CD27^+$, $CD19^+CD24^{hi}CD38^{hi}$ and $CD19^+IL\text{-}10^+$ (B10) cell subpopulations.

Statistical analysis. Data are expressed as the mean ± standard deviation or percentage. Comparison of continuous data between groups was performed using either a parametric test, such as Student's t-test and one-way analysis of variance), or a nonparametric test (Mann-Whitney U or Kruskal-Wallis H tests). The comparison of categorical data between groups was performed using a χ^2 test. Pearson's correlation analysis was used to investigate the association between different parameters. P<0.05 (two-tailed) was considered to indicate a statistically significant difference. SPSS version 17.0 (SPSS Inc., Chicago, IL, USA) was used to analyze data.

Results and Discussion

Comparison of biochemical parameters between HT and control groups. Laboratory measurements were recorded in patients with type I HT (HT group; n=59) and healthy volunteers (normal group; n=38). The results are summarized in Table I. No significant differences were found in the mean age (47.2±14.7, vs. 40.6±16.9 years) or female-to-male ratio between the HT and control groups. Average hip circumference was similar between the two groups, however, a marginal but significant increase in waist circumference was observed in the HT group (P<0.05). Abdominal circumference has previously been associated with diabetes and cardiovascular disease, suggesting that those in the HT group may be

Table II. Comparison of β-cell function and insulin sensitivity between HT and control groups.

Parameter	HT (n=59)	Control (n=38)	P-value
Secretory capacity of pancreatic β-cells			
HOMA-β	132.4±95.7	187.1±153.6	NS
ΔI30/ΔG30	20.3±18.5	17.3±13.2	NS
InsAuc30/GluAuc30	45.3±31.2	32.2±14.1	0.015
InsAuc120/GluAuc120	61.9±38.5	45.1±22.2	0.047
β-cell compensation for IR			
DI	63.0±30.4	108.6±78.6	0.002
DI30	217.5±161.4	196.3±89.1	NS
DI120	294.5±197.6	266.0±107.8	NS
Insulin sensitivity			
HOMA-IR	2.5±1.9	1.8±1.0	NS
ISI_M	6.1±4.8	6.9±3.2	NS

HT, Hashimoto's thyroiditis; OGTT, oral glucose tolerance test; HOMA-β, homeostasis model assessment of β-cell function; ΔI30/ΔG30, change in insulin and glucose ratio at 30 min OGTT; InsAuc30/GluAuc30, early-phase insulin secretion at 30 min; InsAuc120/GluAuc120, ratio of insulin to glucose area under curve values at 120 min; DI, disposition index; DI30, DI at 30 min OGTT; DI120, DI at 120 min OGTT; HOMA-IR, homeostasis model assessment of insulin resistance; ISI_M Matsuda index; NS, not significant.

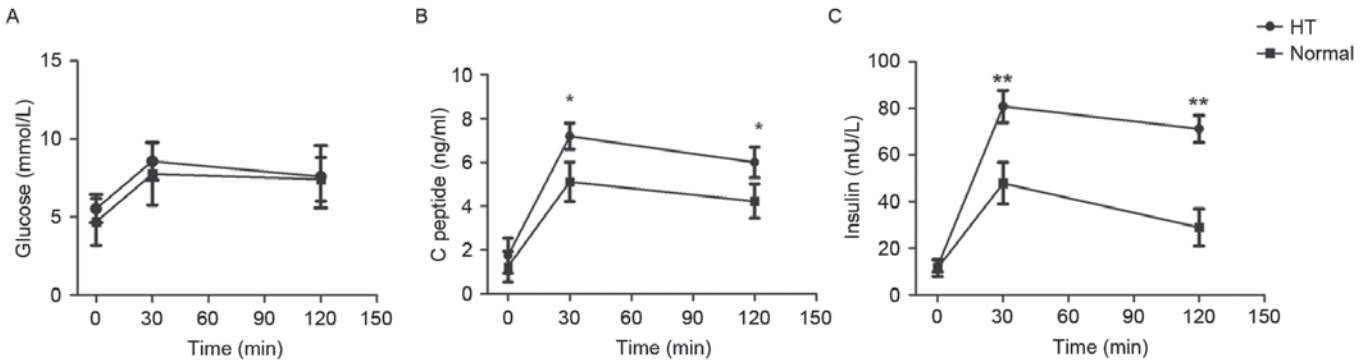

Figure 1. Measurements of plasma glucose, C-peptide and insulin in patients with HT and healthy controls. Oral glucose tolerance tests were administered. Blood samples were collected at 0, 30 and 120 min following oral glucose intake, and the concentrations of (A) glucose, (B) C-peptide and (C) insulin in the blood were measured. Data are presented as the mean ± standard deviation. Differences in concentrations were compared between the HT and healthy control (normal) groups; **P<0.01; *P<0.05. HT, Hashimoto's thyroiditis.

predisposed to such risk factors. Biochemical parameters in the blood, including those for pancreatic and thyroid functions, were also measured. No significant differences were observed in blood pressure, BMI, lipid profiles (TC, TG, HLD-C and LDL-C), thyroid indicators (TT_3, TT_4, FT_3, FT_4 and TSH) or diabetes-associated autoantibodies (GADA, IAA and ICA) between the two groups. The incidence of impaired glucose regulation was also similar between the two groups (Table I). Consistent with the diagnosis, the levels of anti-Tg and anti-TPO, two specific indicators for HT, were significantly higher in the HT group, compared with those in the control group (P<0.05). The level of C-peptide at 30 min was significantly different between the groups (Fig. 1B). In addition, OGTT was performed to determine insulin resistance, and the results showed markedly elevated insulin levels at 30 and 120 min following glucose intake in the HT group (Fig. 1C).

Comparison of pancreatic β-cell function and insulin sensitivity between HT and control groups. As shown in Table II, the parameters for HOMA-β, insulin response (ΔI30/ΔG30), and estimated insulin resistance in the fasting state (HOMA-IR) and postprandial state (ISI_M) showed no significant differences between the HT and control groups. Furthermore, the early-phase DI (DI30) and the total DI (DI120) were similar between the two groups. However, the early-phase and total insulin secretions (InsAuc30/GluAuc30 and InsAuc120/GluAuc120, respectively) were markedly higher in the HT group, compared with those in the control group. In addition, the basal insulin DI, which reflected the capacity of β-cell compensation function for IR in the fasting state, was significantly lower in the HT group (P<0.05). These results indicated that the patients with type I HT had increased insulin secretion in the postprandial state. No significant differences were found in the fasting glucose or insulin secretion, indicated by plasma C-peptide and insulin

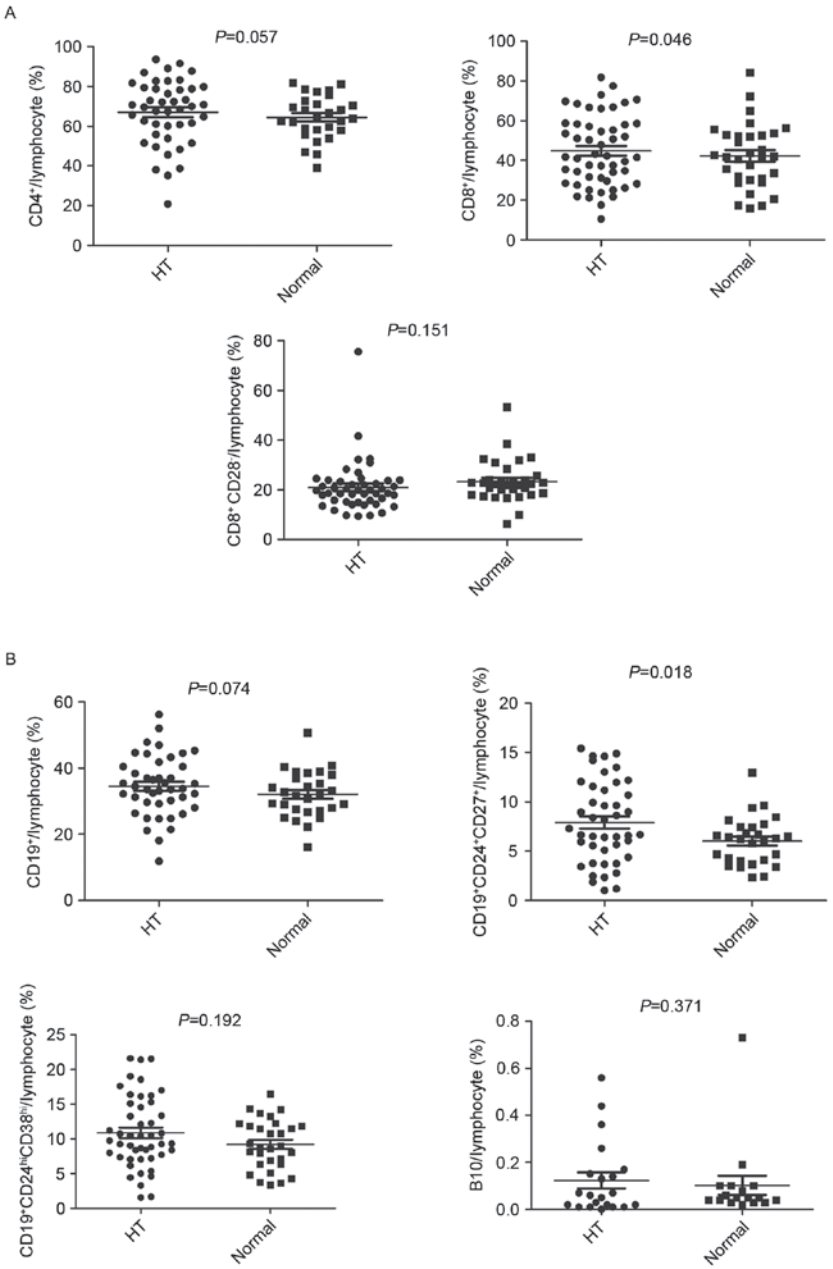

Figure 2. Percentages of lymphocyte populations in patients with HT and healthy controls. Peripheral blood was collected from the HT and control (normal) groups and lineage analysis was performed using specific markers and flow cytometry. Percentages of (A) T cell subsets and (B) B cell subsets in the total lymphocytes were measured. HT, Hashimoto's thyroiditis.

differences between the HT and control groups (Fig. 1), however, the fasting β-cell compensation function (indicated by DI) was markedly reduced in the HT group (Table I).

Comparison of lymphocyte populations between HT and control groups. To analyze lymphocyte populations, peripheral blood was obtained from patients in the HT group and the healthy controls. The percentages of different lymphocyte populations were measured using flow cytometry. No significant differences in the overall percentage of $CD4^+$ T cells or the $CD8^+CD28^-$ TReg cell subset were observed between the HT and control groups (Fig. 2A, upper left and lower panels). However, patients in the HT group had an increased percentage of $CD8^+$ T cells, compared to the control group ($P<0.05$; Fig. 2A, upper right panel).

To assess the percentages of different B cell subsets, cells were stained with specific lineage markers. As shown in Fig. 2B, the percentage of the specific Breg cell subset, $CD19^+CD24^+CD27^+$, was significantly higher in the HT group ($P<0.05$) (upper right panel). However, no differences in the other regulatory Breg cell subset ($CD19^+CD24^{hi}CD38^{hi}$) or the B10 cells were found between the HT and control groups (lower panels).

Correlation of $CD19^+CD24^+CD27^+$ Breg cells with β-cell function and insulin sensitivity. To determine the correlation between the enhancement of Bregs with insulin sensitivity, the study subjects were divided into $CD19^+CD24^+CD27^+$ Breg enhancer and Breg control groups. A reference range for different subsets of peripheral lymphocytes was set as the

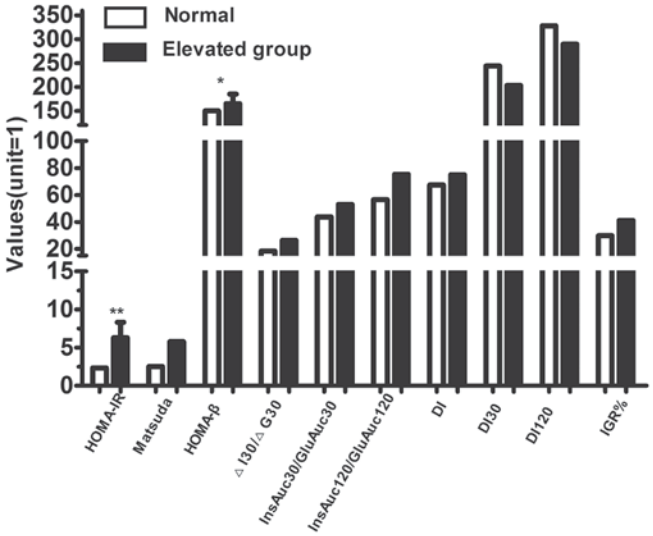

Figure 3. Comparison of pancreatic parameters for β-cell function and insulin sensitivity between individuals with normal and elevated $CD19^+CD24^+CD27^+$ Breg cells. All study subjects were divided according to their percentage of $CD19^+CD24^+CD27^+$ Breg cells. The parameters for β-cell function and insulin sensitivity were compared between the normal and elevated groups, and the values are shown on the vertical axis. The incidence of subclinical diabetes, in which the plasma glucose level reached the diagnostic criteria for diabetes without other clinical symptoms, and the incidence of IGR were calculated separately for each group. $^*P<0.05$; $^{**}P<0.01$. Breg, regulatory B cell; OGTT, oral glucose tolerance test; HOMA-IR, homeostasis model assessment of insulin resistance; HOMA-β, homeostasis model assessment of β-cell function; ΔI30/ΔG30, change in insulin and glucose ratio at 30 min OGTT; InsAuc30/GluAuc30; early-phase insulin secretion at 30 min; InsAuc120/GluAuc120, ratio of insulin to glucose area under the curve values at 120 min; DI, disposition index; DI30: disposition index at 30 min OGTT; DI120: disposition index at 120 min OGTT; IGR, impaired glucose regulation.

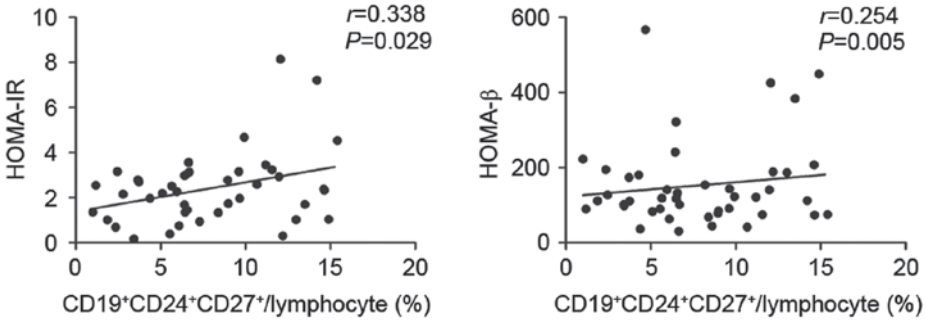

Figure 4. Correlation analysis between $CD19^+CD24^+CD27^+$ Bregs and indicators of β-cell function. The correlation coefficient (r) was calculated between the percentage of $CD19^+CD24^+CD27^+$ Bregs and HOMA-IR (left panel) or HOMA-β (right panel). Bregs, regulatory B cells; HOMA-IR, homeostasis model assessment of insulin.

interquartile range (25-75th percentile) of the percentages of lymphocyte subsets in the control group. According to the reference range, all study subjects were divided into two groups: Those with a higher percentage of $CD19^+CD24^+CD27^+$ Breg cells, compared with the reference range (>7.4325%; elevated group), and those with a percentage of $CD19^+CD24^+CD27^+$ Breg cells within the reference range (≤7.4325%; normal group). Pancreatic β-cell function and insulin sensitivity were compared between the two groups. As shown in Fig. 3, HOMA-β and HOMA-IR were significantly increased in the elevated group, indicating that the insulin secretory capacity of β-cells and the insulin resistance in the fasting state were enhanced in individuals with an elevated level of $CD19^+CD24^+CD27^+$ Breg cells. However, no differences were observed in the insulin secretion index in the postprandial state (InsAuc30/GluAuc30 and InsAuc120/GluAuc120) between groups.

To investigate whether the elevated level of $CD19^+CD24^+CD27^+$ Breg cells was associated with alterations in insulin secretion and insulin sensitivity, correlation analysis was performed. The results demonstrated that the $CD19^+CD24^+CD27^+$ Bregs were positively correlated with HOMA-IR (r=0.338; P=0.029) and HOMA-β (r=0.254; P=0.005; Fig. 4).

Insulin resistance is commonly observed in patients with HT accompanied with clinical or subclinical hypothyroidism (1). Previous investigations to elucidate the mechanism of insulin resistance in patients with HT have yielded inconclusive results. The present study demonstrated for the first time, to the best of our knowledge, the action of insulin in patients with type I HT with preserved normal thyroid function. The results indicated that these patients had insufficient insulin secretion following adjusting insulin resistance (DI) and increased insulin secretion in the postprandial state. An elevated level of $CD19^+CD24^+CD27^+$ Bregs was observed in these patients, which was positively correlated with insulin secretion and insulin resistance in the fasting state. These results revealed a

close association between immune dysregulation and insulin resistance in type I HT.

The observation of a normal blood glucose level and elevated insulin level, described as hyperinsulinemia, indicates the presence of insulin resistance. Insulin resistance contributes to the development of various medical conditions, including type 2 DM, metabolic syndromes, obesity, hypertension, dyslipidemia, elevated free fatty acid and a deregulated stress responses (25). In the present study, the levels of plasma glucose remained normal during OGTT in the HT group. However, the early-phase and total insulin secretion increased significantly, compared with the levels in the control group, indicating the possibility of postprandial insulin resistance. The fasting insulin secretion and insulin resistance levels in the HT group were normal, however, fasting β-cell compensation function was impaired, as indicated by a significant reduction in DI. DI reflects the ability of β-cells to compensate for insulin resistance; a reduced DI indicates impaired compensation. When insulin resistance increases, insulin secretion consequently increases. However, if the insulin secreted from β-cells is insufficient to compensate for insulin resistance, then the DI decreases, indicating impaired β-cell compensation function (26). In the present study, the fasting DI was significantly decreased in the HT group, compared with that in the control group, suggesting that the patients with HT had impaired β-cell compensation function and that insulin levels were not sufficient to compensate for the insulin resistance.

Previously, immune components, including $CD8^+$ cytotoxic T lymphocytes, proinflammatory T cells, and Tregs, have been shown to be important in the pathophysiology of autoimmune HT. Notably, the present study found no significant differences in $CD4^+$ or $CD8^+CD28^-$ T cells, but found a decrease in the overall percentage of $CD8^+$ T cells. However, previous studies have shown that the infiltration of proinflammatory T cells and overproduction of inflammatory mediators are largely present in the liver and muscle tissues of diabetic patients, which have been implicated in the development of insulin resistance (27,28). Under normal circumstances, the activation and proliferation of these proinflammatory T cells are strictly regulated by their interplay with Tregs, thus the immune balance is carefully maintained.

The present study is the first, to the best of our knowledge, to report on the involvement of $CD19^+CD24^+CD27^+$ Bregs in HT and associated insulin resistance. The patients with HT had reduced DI, and the elevated $CD19^+CD24^+CD27^+$ Breg population was positively correlated with fasting insulin secretion and insulin resistance. Although the regulatory mechanism of Bregs remains to be elucidated, studies in diabetic patients may provide insight to facilitate understanding of HT. Rather than producing the anti-inflammatory cytokine IL-10, Bregs in diabetic patients upregulate the expression of proinflammatory cytokines, including IL-6 and IL-8, and promote inflammation (29,30). In the present study of type I HT, the percentage of $CD19^+CD24^+CD27^+$ Bregs was elevated significantly, compared with that in the healthy controls. This increase in $CD19^+CD24^+CD27^+$ Bregs may also increase the expression of IL-6 and IL-8 in HT, promoting increased autoimmune inflammation, and thereby increasing risk of insulin resistance and decreased β-cell function. There may also be a feedback mechanism by which the Bregs are activated and proliferate in response to the tissue damage caused by infiltrating inflammatory cells. The causal association between Breg dysregulation and insulin resistance remains to be elucidated. Further investigation is required to define the exact role of Bregs in the development of HT.

In the patients with type I HT examined in the present study, insulin resistance, indicated by increased early-phase and total insulin secretion, but a normal glucose response was observed during OGTT. The increase in the percentage of $CD19^+CD24^+CD27^+$ Breg cells was correlated with insulin resistance in the fasting state and homeostatic β-cell function. However, no significant association was found between the postprandial secretion of insulin and alterations in the $CD19^+CD24^+CD27^+$ Breg population. These results indicated potential contributions from other cellular components or immune factors acting during the later phases of insulin sensitivity, which functioned with Bregs to regulate insulin signaling in the postprandial state.

The patients with HT examined in the present study showed increased postprandial insulin levels, but normal fasting insulin levels. A possible explanation for these differences may be due to the primary tissues involved in the action of insulin. Fasting insulin resistance is associated with decreased glucose uptake and utilization under the regulation of insulin in the liver, whereas postprandial insulin resistance is associated with reduced glucose uptake and utilization in muscle and adipose tissues. The required insulin level for glucose uptake in muscle or adipose tissues is substantially higher, compared with the level required in the liver. Therefore, fasting and postprandial insulin levels are increased in compensation in individuals with insulin resistance, although the postprandial insulin level is increased to a higher degree.

In conclusion, the present study demonstrated an increase in postprandial insulin secretion and impaired fasting β-cell compensation function, indicated by reduced DI, in patients with type I HT. An increased percentage of $CD19^+CD24^+CD27^+$ Breg cells was found in these patients, which was closely associated with β-cell function and insulin resistance in the fasting state. These results may assist in the development of novel therapeutic strategies, which target the fundamental immune components involved in the pathogenesis of HT.

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References

1. Maratou E, Hadjidakis DJ, Kollias A, Tsegka K, Peppa M, Alevizaki M, Mitrou P, Lambadiari V, Boutati E, Nikzas D, *et al*: Studies of insulin resistance in patients with clinical and subclinical hypothyroidism. Eur J Endocrinol 160: 785-790, 2009.
2. Fernandez-Real JM, Lopez-Bermejo A, Castro A, Casamitjana R and Ricart W: Thyroid function is intrinsically linked to insulin sensitivity and endothelium-dependent vasodilation in healthy euthyroid subjects. J Clin Endocrinol Metab 91: 3337-3343, 2006.
3. Roos A, Bakker SJ, Links TP, Gans RO and Wolffenbuttel BH: Thyroid function is associated with components of the metabolic syndrome in euthyroid subjects. J Clin Endocrinol Metab 92: 491-496, 2007.

4. Dimitriadis G, Mitrou P, Lambadiari V, Boutati E, Maratou E, Panagiotakos DB, Koukkou E, Tzanela M, Thalassinos N and Raptis SA: Insulin action in adipose tissue and muscle in hypothyroidism. J Clin Endocrinol Metab 91: 4930-4937, 2006.
5. Jornayvaz FR, Lee HY, Jurczak MJ, Alves TC, Guebre-Egziabher F, Guigni BA, Zhang D, Samuel VT, Silva JE and Shulman GI: Thyroid hormone receptor-α gene knockout mice are protected from diet-induced hepatic insulin resistance. Endocrinology 153: 583-591, 2012.
6. Stanická S, Vondra K, Pelikánová T, Vlcek P, Hill M and Zamrazil V: Insulin sensitivity and counter-regulatory hormones in hypothyroidism and during thyroid hormone replacement therapy. Clin Chem Lab Med 43: 715-720, 2005.
7. Handisurya A, Pacini G, Tura A, Gessl A and Kautzky-Willer A: Effects of T4 replacement therapy on glucose metabolism in subjects with subclinical (SH) and overt hypothyroidism (OH). Clin Endocrinol (Oxf) 69: 963-969, 2008.
8. Dessein PH, Joffe BI and Stanwix AE: Subclinical hypothyroidism is associated with insulin resistance in rheumatoid arthritis. Thyroid 14: 443-446, 2004.
9. Finucane JF: Carbohydrate tolerance in autoimmune thyroiditis. Diabetes 24: 829-832, 1975.
10. Kornete M, Mason ES and Piccirillo CA: Immune regulation in T1D and T2D: Prospective role of foxp3+ treg cells in disease pathogenesis and treatment. Front Endocrinol (Lausanne) 4: 76, 2013.
11. Herold KC, Hagopian W, Auger JA, Poumian-Ruiz E, Taylor L, Donaldson D, Gitelman SE, Harlan DM, Xu D, Zivin RA and Bluestone JA: Anti-CD3 monoclonal antibody in new-onset type 1 diabetes mellitus. N Engl J Med 346: 1692-1698, 2002.
12. Penaranda C, Tang Q and Bluestone JA: Anti-CD3 therapy promotes tolerance by selectively depleting pathogenic cells while preserving regulatory T cells. J Immunol 187: 2015-2022, 2011.
13. Herold KC, Gitelman S, Greenbaum C, Puck J, Hagopian W, Gottlieb P, Sayre P, Bianchine P, Wong E, Seyfert-Margolis V, *et al*: Treatment of patients with new onset type 1 diabetes with a single course of anti-CD3 mAb Teplizumab preserves insulin production for up to 5 years. Clin Immunol 132: 166-173, 2009.
14. Pescovitz MD, Greenbaum CJ, Krause-Steinrauf H, Becker DJ, Gitelman SE, Goland R, Gottlieb PA, Marks JB, McGee PF, Moran AM, *et al*: Rituximab, B-lymphocyte depletion, and preservation of beta-cell function. N Engl J Med 361: 2143-2152, 2009.
15. Winer DA, Winer S, Shen L, Wadia PP, Yantha J, Paltser G, Tsui H, Wu P, Davidson MG, Alonso MN, *et al*: B cells promote insulin resistance through modulation of T cells and production of pathogenic IgG antibodies. Nat Med 17: 610-617, 2011.
16. Filaci G, Fravega M, Negrini S, Procopio F, Fenoglio D, Rizzi M, Brenci S, Contini P, Olive D, Ghio M, *et al*: Nonantigen specific CD8+ T suppressor lymphocytes originate from CD8+CD28- T cells and inhibit both T-cell proliferation and CTL function. Hum Immunol 65: 142-156, 2004.
17. Yang M, Rui K, Wang S and Lu L: Regulatory B cells in autoimmune diseases. Cell Mol Immunol 10: 122-132, 2013.
18. Mizoguchi A, Mizoguchi E, Takedatsu H, Blumberg RS and Bhan AK: Chronic intestinal inflammatory condition generates IL-10-producing regulatory B cell subset characterized by CD1d upregulation. Immunity 16: 219-230, 2002.
19. Mizoguchi A and Bhan AK: A case for regulatory B cells. J Immunol 176: 705-710, 2006.
20. Kalampokis I, Yoshizaki A and Tedder TF: IL-10-producing regulatory B cells (B10 cells) in autoimmune disease. Arthritis Res Ther 15 (Suppl 1): S1, 2013.
21. Shi L, Bi M, Yang R, Zhou J, Zhao S, Fan C, Shan Z, Li Y and Teng W: Defective expression of regulatory B cells in iodine-induced autoimmune thyroiditis in non-obese diabetic H-2(h4) mice. J Endocrinol Invest 37: 43-50, 2014.
22. Lin Y and Sun Z: Thyroid hormone potentiates insulin signaling and attenuates hyperglycemia and insulin resistance in a mouse model of type 2 diabetes. Br J Pharmacol 162: 597-610, 2011.
23. Slatosky J, Shipton B and Wahba H: Thyroiditis: Differential diagnosis and management. Am Fam Physician 61: 1047-1052, 1054, 2000.
24. Alberti KG and Zimmet PZ: Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: Diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. Diabet Med 15: 539-553, 1998.
25. Samuel VT and Shulman GI: Mechanisms for insulin resistance: Common threads and missing links. Cell 148: 852-871, 2012.
26. Bergman RN, Ader M, Huecking K and Van Citters G: Accurate assessment of beta-cell function: The hyperbolic correction. Diabetes 51 (Suppl 1): S212-S220, 2002.
27. Kintscher U, Hartge M, Hess K, Foryst-Ludwig A, Clemenz M, Wabitsch M, Fischer-Posovszky P, Barth TF, Dragun D, Skurk T, *et al*: T-lymphocyte infiltration in visceral adipose tissue: A primary event in adipose tissue inflammation and the development of obesity-mediated insulin resistance. Arterioscler Thromb Vasc Biol 28: 1304-1310, 2008.
28. Pickup JC: Inflammation and activated innate immunity in the pathogenesis of type 2 diabetes. Diabetes Care 27: 813-823, 2004.
29. Jagannathan M, McDonnell M, Liang Y, Hasturk H, Hetzel J, Rubin D, Kantarci A, Van Dyke TE, Ganley-Leal LM and Nikolajczyk BS: Toll-like receptors regulate B cell cytokine production in patients with diabetes. Diabetologia 53: 1461-1471, 2010.
30. DeFuria J, Belkina AC, Jagannathan-Bogdan M, Snyder-Cappione J, Carr JD, Nersesova YR, Markham D, Strissel KJ, Watkins AA, Zhu M, *et al*: B cells promote inflammation in obesity and type 2 diabetes through regulation of T-cell function and an inflammatory cytokine profile. Proc Natl Acad Sci USA 110: 5133-5138, 2013.