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CLINICAL TRIAL

Altered T-cell subsets and transcription factors in latent autoimmune diabetes in adults taking sitagliptin, a dipeptidyl peptidase-4 inhibitor: A 1-year open-label randomized controlled trial

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Keywords
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ABSTRACT

Aims/Introduction: Dipeptidyl peptidase-4 inhibitor has been proven to improve glycemic control and β-cell function in latent autoimmune diabetes in adults (LADA). The potential immune modulation mechanism is still unknown. Thus, we tested T-lymphocyte subsets and expression of relevant transcription factors in LADA patients with sitagliptin intervention for up to 1-year.

Materials and Methods: A total of 40 LADA patients were randomly assigned to sitagliptin and/or insulin treatment (SITA group; $n = 20$) or insulin alone treatment (CONT group; $n = 20$). Peripheral blood mononuclear cells were isolated at baseline, 6 months and 12 months. The percentage of T-lymphocyte subsets (T helper 1, T helper 2, T helper 17 and regulatory T cells) tested by flow cytometry, and the messenger ribonucleic acid expression (T box expressed in T cells [T-BET], GATA binding protein 3 [GATA3], forkhead box protein 3 [FOXP3] and related orphan receptor C [RORC]) tested by real-time polymerase chain reaction were determined at baseline, 6 months and 12 months.

Results: The percentage of regulatory T cells in the SITA group was significantly lower than that of the CONT group at baseline. The percentage of T helper 2 cells was higher than that of the CONT group at 6 months and 12 months. At 12 months, the percentage of T helper 17 cells was lower in the SITA group than that of the CONT group. After a 1-year visit, the messenger ribonucleic acid expression levels of T-BET expressed in T cells and RORC in the SITA group were significantly lower than at baseline. Whereas that of RORC in the CONT group were significantly lower than that at baseline.

Conclusions: The data confirmed that sitagliptin altered the phenotype of T cells and downregulated the expression of T-BET and RORC in LADA patients, and ameliorated glycemic control in LADA patients.

INTRODUCTION

Latent autoimmune diabetes in adults (LADA) is a subset of autoimmune diabetes, with clinical manifestation overlapping both type 1 diabetes and type 2 diabetes[1,2]. A variety of oral agents are applicable to LADA, such as sulfonylureas, insulin sensitizers (metformin and thiazolidinediones), incretins and sodium–glucose cotransporter inhibitors[3–5]. Dipeptidyl peptidase-4 (DPP-4) inhibitors, a novel hypoglycemic medication, have been widely applied in type 2 diabetes patients because of their cardiovascular safety, excellent glycemic controlling ability and rare relevant hypoglycemic events in large-scale multiple-centered randomized controlled trials, as reported[6–8].

Autoimmune diabetes is considered to be dominantly modulated by T cells. A potential pathogenic mechanism is the imbalance between pathogenic and regulatory T lymphocytes, which results in the actual destruction of the insulin-producing β-cells in the pancreatic islets. DPP-4, known as lymphocyte cell surface protein CD26, has multiple functions in diabetes[9], the cardiovascular system, solid tumor and so on because of the diversity of substrates cleaved by DPP-4. DPP-4 inhibitors

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could increase the incretin levels by blocking their degradation. It has also been proven to play a crucial role in immune regulation by several signaling pathways. In a non-obese diabetic mouse model[10–14], DPP-4 inhibitors were reported to delay the onset of diabetes, preserve β-cell mass and attenuate autoimmunity.

Several clinical trials[15–19] of DPP-4 inhibitors in autoimmune diabetes also confirmed that DPP-4 inhibitors might improve glycemic control and preserve β-cell function. However, DPP-4-mediated cell signaling and possible immune modulation is still not clearly identified in autoimmune diabetes.

In the present prospective randomized control trial, we investigated whether there are any differences in the profiles of the T-cell subpopulation and its associated transcription factors in LADA patients with sitagliptin intervention for up to 1 year of follow up.

METHODS

Patient selection

The criteria of LADA were: (i) diabetes diagnosed according to the report of the World Health Organization in 1999; (ii) age range 30–70 years; (iii) glutamic acid decarboxylase antibody (GADA)-positive; (iv) insulin independent within the first 6 months after the diagnosis of diabetes; (v) fasting C-peptide level >200 pmol/L or a 2-h postprandial C-peptide >400 pmol/L; and (vi) duration of diabetes of ≤3 years. The exclusion criteria were: (i) total insulin doses >0.8 U/kg/day; (ii) evidence of any other autoimmune diseases; (iii) evidence of chronic or acute infection; (iv) a history of any malignancy, congestive heart failure or secondary diabetes; (v) renal diseases or renal dysfunction with serum creatinine of ≥1.5 mg/dL for men and ≥1.4 mg/dL for women; (vi) women who were pregnant, had several miscarriages or were breast-feeding; and (vii) patients unable to abide by the treatment protocol.

A total of 40 LADA patients were recruited from December 2014 to December 2016. All of them were given written information and consent was obtained. The protocols were approved by the ethics committee of the Second Xiangya Hospital, Central South University. This study has been registered online (www.clinicaltrials.gov/identifier NCT01159847).

Treatment protocol

After a 3-month washing-in period, the 40 patients enrolled in this study were randomized at a 1:1 ratio to insulin with sitagliptin 100 mg/day (SITA group; $n = 20$) or insulin without sitagliptin (CONT group; $n = 20$). Patients included in this study had similar diet and lifestyle modifications, self-monitoring of blood glucose, and insulin dose adjustment. The regimen of insulin is administration of premixed insulin (30% insulin aspart and 70% insulin aspart protamine) twice or three times daily according to the patient's glycemic profile. Sulfonylureas, insulin sensitizers, other types of DPP-4 inhibitors and sodium–glucose cotransporter inhibitors were not applied to the patients in the present study. The intervention lasted for 12 months, and the follow-up visits took place at baseline, 6 months and 12 months. All 40 participants completed the treatment protocol and attended the visits at all three time-points.

Fasting blood samples were tested for hemoglobin A1c (HbA1c), GADA, fasting blood glucose and C-peptide; 2-h postprandial blood samples were tested for postprandial blood glucose and C-peptide.

GADA assay

GADA was tested by radioligand assay confirmed by the Diabetes Antibody Standardization Program (2012) and sponsored by the Immunology of Diabetes Society. The cut-off indices of positivity for GADA was ≥18 U/mL (World Health Organization units), and the sensitivity was 78.0% and specificity was 96.7% (Diabetes Autoantibody Standardization Program 2010)[20].

Assessment of HbA1c and β-cell function

A standard 543.6-kcal, mixed-meal tolerance test (44.4% of calories as carbohydrate, 47.7% calories as fat and 7.9% calories as protein) was given. The HbA1c levels were measured by automated liquid chromatography (HLC-723G8; Tosoh, Yamaguchi, Japan). Plasma glucose and C-peptide levels were measured at 0 and 2 h after the standard meal. C-peptide was detected by a chemiluminescence method (Advia Centaur System, Munich, Germany).

T helper 1/T helper 2/T helper 17/regulatory T Cell phenotype testing

Fresh blood samples, collected at baseline, 6 and 12 months, were gathered in sodium heparin tubes from fasting participants and processed in 2 h. Peripheral blood mononuclear cells were separated by Ficoll-Paque density-gradient. Cells were stimulated with 1 μg/mL ionomycin and 50 ng/mL phorbol-12-myristate-13-acetate for 6 h in the presence of Brefeldin A and GolgiStop. Cells were harvested and stained with viability dye (Invitrogen, Carlsbad, CA, USA) and flurophore conjugated anti-interferon-γ, anti-interleukin-4, anti-interleukin-17A and anti-forkhead box protein 3 (FOXP3) by fluorescence-activated cell sorting. Data were gathered in a Canto flow cytometer (BD, Franklin Lakes, NJ, USA) and analyzed with Flowjo 7.6 software (Tree Star, Inc., Ashland, OR, USA). The gating strategy is shown in Figure 1.

Quantification of T-cell transcription factors

Total ribonucleic acid (RNA) was obtained by the TRIZOL (Invitrogen) method and frozen at −70°C according to the manufacturer's protocol. The RNA was first isolated with a RNA extraction kit (Zymo Research, Orange County, CA, USA), and was then converted to complementary deoxyribonucleic acid (cDNA) with a cDNA Reverse Transcription Kit (GoScript™; Promega, Madison, WI, USA). The cDNAs

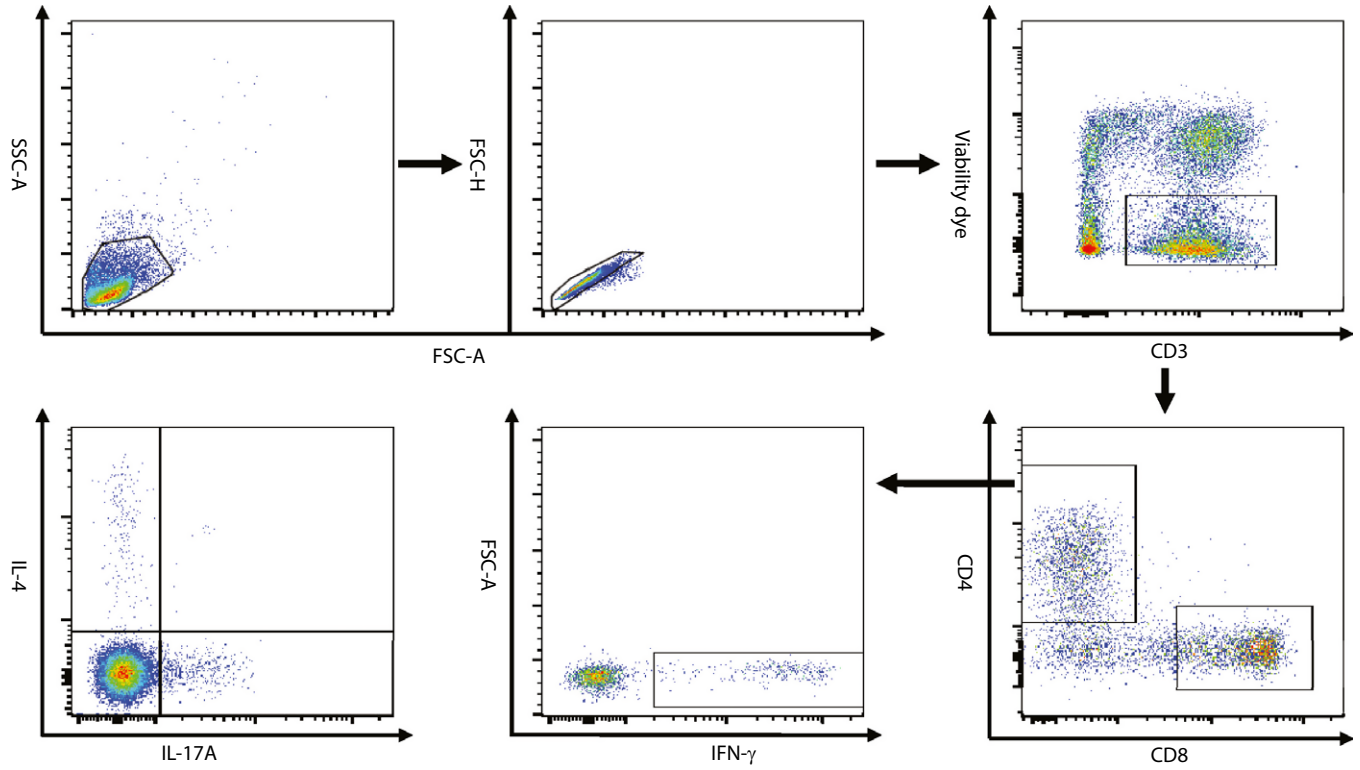

Figure 1 | Representative plots and the gating strategy for the fluorescence-activated cell sorting analysis of CD4 helper T cell subsets. $CD4^+$ T cells were sequentially gated on lymphocytes, singlets, live T cells and $CD4^+$ T cells, and the expression of interferon (IFN)-γ, interleukin (IL)-4 and IL-17A was further analyzed. FSC-A, forward scatter-area; FSC-H, forward scatter-height; SSC-A, side scatter-area.

collected were amplified with specific oligonucleotides for T box expressed in T cells (T-BET), GATA binding protein 3 (GATA3), forkhead box protein 3 (FOXP3) and related orphan receptor C (RORC) with SYBR Green I and quantified by real-time polymerase chain reaction in an ABI PRISM Step One Sequence Detection System (PE Applied Biosystems™ machine; Carlsbad, CA, USA). Primers are listed in Table 1. The relative messenger RNA (mRNA) gene expression of T-cell transcription factors (T helper 1 [Th1] = T-BET, T helper 2 [Th2] = GATA3, T helper 17 [Th17] = RORC, regulatory T cells [Treg] = FOXP3) were quantified as a fold change against the actin control sample by using the $2^{-\Delta\Delta CT}$ method. These associated transcription factors were tested at baseline, 6 and 12 months.

Statistical analysis

Data are presented as mean ± standard error of the mean or median (25th–75th percentile). A paired *t*-test was used for a comparison from the baseline within the same group. Categorical variables were compared by one-way ANOVA. We used SPSS version 17.0 (IBM Corporation, Chicago, IL, USA) and GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). A two-tailed $P < 0.05$ was considered a significant difference.

RESULTS

Anthropometric and metabolic data

We randomly assigned these 40 LADA patients into the SITA group (n = 20, women/men 7/13) and CONT group (n = 20,

Table 1 | Forward and reverse primers for T-cell transcription factors

Target gene	Primer forward	Primer reverse
β-Actin	5′-CGGGAAATCGTGCGTGAC-3′	5′-GGAAGGAAGGCTGGAAGAG-3′
T-BET	5′-CAACGCTTCCAACACGCAT-3′	5′-GACTCAAAGTTCTCCCGGAA-3′
GATA3	5′-TCATTAAGCCCAAGCGAAGG-3′	5′-GTCCCCATTGGCATTCCTC-3′
RORC	5′-GCAGCGCTCCAACATCTTCT-3′	5′-ACGTACTGAATGGCCTCGGT-3′
FOXP3	5′-CACCTGGCTGGGAAAATGG-3′	5′-GGAGCCCTTGTCGGATGA-3′

FOXP3, forkhead box protein 3; GATA3, GATA binding protein 3; RORC, related orphan receptor C; T-BET, T box expressed in T cells.

women/men 9/11). The mean age in the SITA group and CONT group was 47.8 ± 13.1 vs 51.9 ± 10.2 years, respectively. The GADA titer in the SITA group was 483.6 U/mL (30.9–1,134.7 U/mL), and 285.1 U/mL (18.5–1,190.5 U/mL) in the CONT group. No significant differences were present among duration, sex, age and GADA titer. No patient progressed to an insulin-dependent state during the 1-year follow up.

Table 2 summarizes the characteristics of the participants in the SITA group and CONT group at baseline, 6 and 12 months. After the 12-month visit, postprandial blood glucose and fasting blood glucose in the CONT group were significantly higher than baseline. No significant difference could be found in body mass index, insulin dose, HbA1c, fasting blood glucose, fasting C-peptide, postprandial C-peptide.

Th1/Th2/Th17/Treg phenotype

We investigated the percentage of T-lymphocyte subsets (Th1, Th2, Treg and Th17 cells) in the SITA group and CONT group by flow cytometry at baseline, 6 and 12 months.

The percentage of Treg to $CD4^+$ T cells in the SITA group was significantly lower than that in the CONT group (2.36 ± 0.35% vs 3.92 ± 0.50%, $P = 0.016$) at baseline. The percentage of Th2 to $CD4^+$ T cells was higher than that of the CONT group at 6 months (4.08 ± 0.58% vs 2.18 ± 0.42%, $P = 0.015$) and 12 months(5.17 ± 0.92% vs 2.51 ± 0.33%, $P = 0.012$). At 12 months, the percentage of Th17 to $CD4^+$ T cells was lower in the SITA group than that of the CONT group(0.74 ± 0.57% vs 1.20 ± 0.52%, $P = 0.020$; Figure 2).

Expression of T-cell transcription factors

We next extracted RNA from peripheral blood mononuclear cell samples in all 40 LADA patients at baseline, 6 months and 12 months, reversed them into cDNA, and tested the relative mRNA expression of T-cell transcription (T-BET, GATA3, RORC and FOXP3) by real-time polymerase chain reaction.

In Figure 3, no significant difference could be found in the mRNA expression in the SITA group and CONT group at baseline and 6 months, neither difference can be found compared with their baseline, respectively. After the 1-year visit, no significant difference could be found in these four transcription factors between the SITA group and CONT group. However, the mRNA expression levels of T-BET (0.29 ± 0.16% vs 0.85 ± 0.24%, $P = 0.012$) and RORC (0.11 ± 0.07% vs 0.85 ± 0.51%, $P = 0.009$) in the SITA group were significantly lower when compared with baseline in the SITA group. Whereas the mRNA expression levels of RORC (1.14 ± 0.84% vs 0.19 ± 0.17%, $P = 0.004$) were also significantly lower than that of its baseline in the CONT group. Although there was a descending tendency in the FOXP3 expression and ascending tendency in the GATA3 expression, the differences were not significant.

DISCUSSION

Type 1 diabetes is mainly mediated by T cells, and is associated with loss of immunological tolerance to self-antigens. LADA is a subclass of autoimmune diabetes. DPP-4 inhibitors, a novel class of oral hypoglycemic agent, have gained wide acceptance for the treatment of type 2 diabetes. They have also been documented to be protective and effective in autoimmune diabetes, not only in type 1 diabetic animal models[10–14], but also in LADA patients based on small-scale population studies[15–19]. However, it has not been fully understood whether immunological modulation is involved in the disease progression mediated by DPP-4 inhibition. Therefore, we attempted to elucidate the immunological profile of T-cell subsets and the main transcription factors with sitagliptin intervention in LADA patients.

Table 2 | Demographic and clinical characteristics at baseline, 6 months and 12 months

	Baseline		6 months		12 months	
	SITA group ($n = 20$)	CONT group ($n = 20$)	SITA group ($n = 20$)	CONT group ($n = 20$)	SITA group ($n = 20$)	CONT group ($n = 20$)
BMI (kg/m^2)	23.19 ± 0.64	24.46 ± 0.63	23.11 ± 0.65	24.41 ± 0.62	23.05 ± 0.67	24.27 ± 0.58
Insulin dose (U/day)	12.0 ± 2.6	14.0 ± 2.4	11.5 ± 2.5	15.2 ± 2.8	12.3 ± 2.6	18.9 ± 3.4
HbA1c (%)	6.33 ± 0.20	6.94 ± 0.41	6.35 ± 0.17	7.09 ± 0.43	6.48 ± 0.21	7.12 ± 0.36
FBG (mmol/L)	6.68 ± 0.40	7.54 ± 0.61	6.82 ± 0.42	7.62 ± 0.67	6.29 ± 0.29	7.50 ± 0.63
PBG (mmol/L)	12.72 ± 1.07	14.02 ± 1.36	12.45 ± 0.90	14.79 ± 1.30	11.68 ± 1.19	14.90 ± 1.28*
△BG (mmol/L)	6.05 ± 1.12	5.95 ± 0.92	5.63 ± 1.01	6.08 ± 1.13	5.37 ± 1.26	7.22 ± 0.92*
FCP (pmol/L)	479.5 ± 71.2	477.1 ± 55.5	420.9 ± 59.7	522.2 ± 59.7	441.3 ± 51.8	458.3 ± 43.4
2hCP (pmol/L)	1,458.9 ± 200.7	1,487.9 ± 175.5	1,557.7 ± 191.1	1,658.4 ± 184.8	1,691.3 ± 220.8	1,516.7 ± 150.9
△CP (pmol/L)	979.4 ± 166.2	1,010.8 ± 137.2	1,136.9 ± 148.5	1,136.15 ± 157.3	1,250.0 ± 179.7	1,058.4 ± 125.8

Data presented as mean ± standard error of the mean. *$P < 0.05$, compared with the insulin alone treatment (CONT) group at baseline. △CP, 2-h postprandial C-peptide – fasting C-peptide; 2hCP, 2-h postprandial C-peptide; BG, blood glucose; BMI, body mass index, FBG, fasting blood glucose; FCP, fasting C-peptide; CP, postprandial C-peptide; HbA1c, hemoglobin A1c; PBG, postprandial blood glucose; SITA, sitagliptin and insulin treatment.

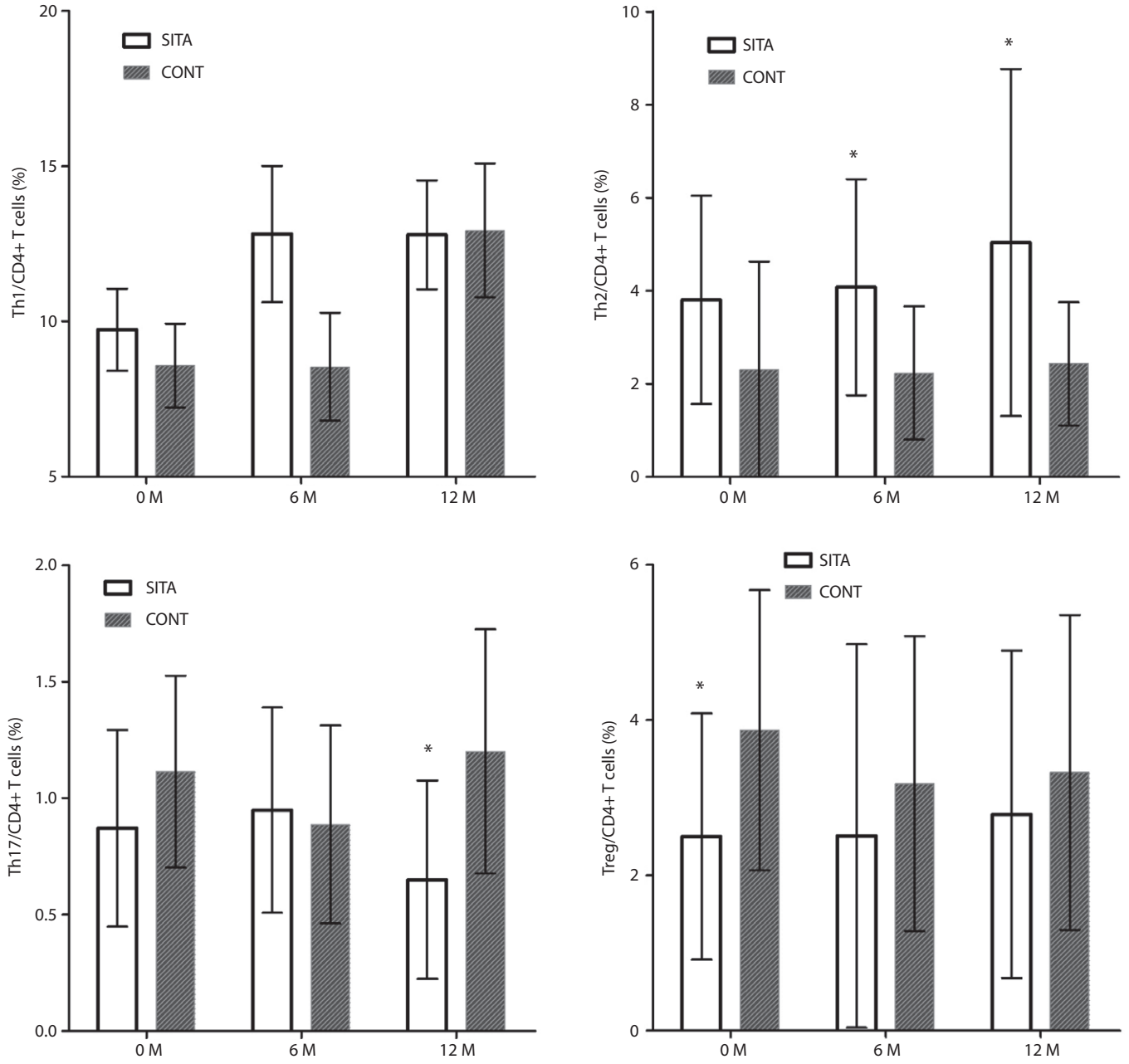

Figure 2 | Percentage of T-cell subsets between the sitagliptin and insulin treatment (SITA) group and insulin alone treatment (CONT) group at baseline, 6 and 12 months. *$P < 0.05$, compared with the CONT group. Th1, T helper 1 cells; Th2, T helper 2 cells; Th17, T helper 17 cells; Treg, regulatory T cells.

We investigated the frequency of T-cell subsets in LADA patients taking sitaliptin and LADA patients not taking sitaliptin. We found that the percentage of Th2 in the SITA group was statistically higher at 6 months and 12 months, whereas Th17 was dramatically lower than the CONT group after 1-year follow up. These results confirmed that sitagliptin might alter the subsets of T cells by increasing the protective Th2 and decreasing the pathogenic Th17. This finding shares similarities with another *in vitro* study[21] in which peripheral blood mononuclear cells were extracted from healthy volunteers, confirming sitagliptin inhibition had a suppression effect on Th1, Th2 and Th17 lymphocytes differentiation. The complicated local milieu in the pancreatic islets of Langerhans and the interplay between non-immune cells and immune cells *in vivo* might explain this discrepancy. Previous studies[22–25] have shown a decline in Th1 and Th2, lower cytokine secretion, and

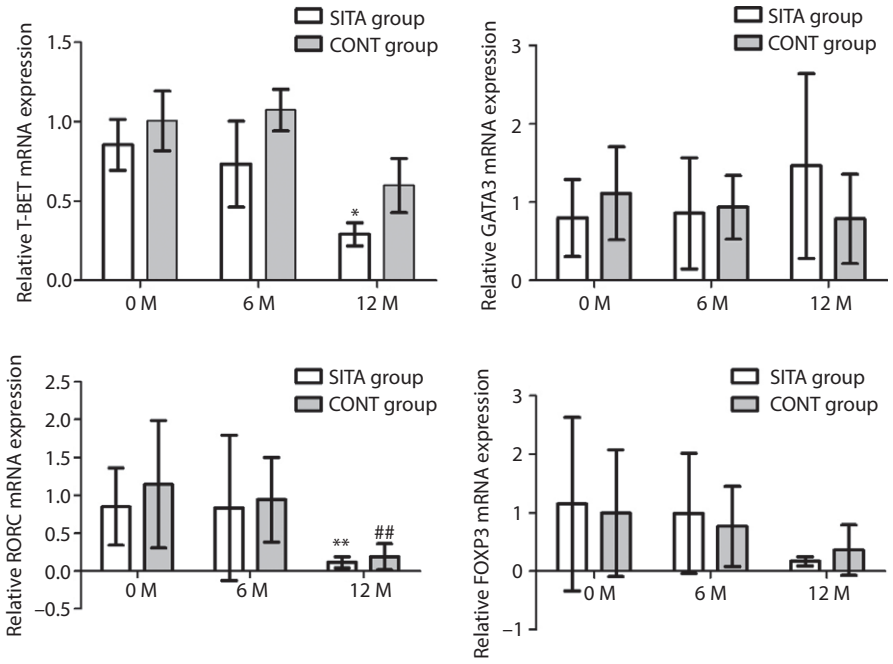

Figure 3 | Expression of T-cell transcription factors at baseline, 6 and 12 months between the sitagliptin and insulin treatment (SITA) group and insulin alone treatment (CONT) group. Real-time polymerase chain reaction was carried out to test the messenger ribonucleic acid (mRNA) expression of transcription factors (T helper 1 cells = T box expressed in T cells [T-BET], T helper 2 cells = GATA binding protein 3 [GATA3], regulatory T cells = forkhead box protein 3 [FOXP3] and T helper 17 cells = related orphan receptor C [RORC]) in the SITA group and CONT group at baseline, 6 and 12 months. $^{*}P < 0.05$, compared with baseline in the SITA group; $^{**}P < 0.01$, compared with baseline in the SITA group; and $^{\#\#}P < 0.01$, compared with baseline in the CONT group.

a low expression of T-cell-specific transcription factors in the progression of autoimmune diabetes, suggesting an immune dampening and exhaustion at the onset of autoimmunity. Seldom do clinical trial studies focus on the alteration of the T-cell phenotype with sitagliptin intervention. Interestingly, the present prospective study observed that although the quantities of Th1 did not significantly decrease, its expression of T-BET was still obviously decreased in the SITA group. In addition, the percentage of protective Th2 was elevated, and that of the pathological Th17 was decreased. Furthermore, blood glucose was improved, especially postprandial blood glucose, inferring that sitagliptin intervention might alter the quantities of T-cell subsets in LADA patients and further contribute to better glucose control, resulting in a protective effect on pancreatic β-cells.

In type 1 diabetes, there was a decreased frequency of Treg[24]. Defects in Treg number and/or function are a major factor in the progression of type 1 diabetes[25]. In our previous published study[26], we found a lower expression of FOXP3 in LADA patients; a further study[27] confirmed that the FOXP3 promoter region was hypermethylated in $CD4^{+}$ T cells from LADA patients. In the present study, after the 1-year visit, there was neither a significant alteration in the percentage of Treg nor its corresponding mRNA expression (FOXP3) compared with those in the control group with sitagliptin intervention, though a significant difference of Treg at baseline between these two groups unexpectedly occurred. Sitagliptin did not alter the frequency of Treg and the level of its associated gene expression at 1-year follow up, and a further long-term study is required to further confirm this.

Nevertheless, the expression levels of T-BET and RORC were significantly lower with sitagliptin, suggesting that sitagliptin might downregulate the expression of pathogenic transcription factors. It seems that the percentage of T cells analyzed by fluorescence-activated cell sorting was unexpectedly not in accordance with the expression of their associated transcription factors. Conflicting results were also found in another type 1 diabetes study[28]. This unexpected inconsistency might arise from an abnormal translational or post-translational modulation of protein.

Case reports[29,30] and clinical trials[14–18] in autoimmune diabetes treated with DPP-4 inhibitor alone or in combination with other drugs showed that sitagliptin significantly improved glycemic control and reduced insulin requirements, accompanied by a good tolerance profile. Although a recent meta-analysis showed that DPP-4 inhibitor gives a neutral result for HbA1c[31] in type 1 diabetes. The present observed results found that sitagliptin, as an add-on therapy, seemed to be a better choice to improve the postprandial blood glucose in LADA patients. Although, in regard to C-peptide and HbA1c, the

result from our 1-year study was not in line with previous studies[15–17], possibly because of the small sample size, different population and relatively short follow-up period.

In conclusion, the current data first implied that sitagliptin, a DPP-4 inhibitor, could alter the frequency of $CD4^+$ T-cell subsets on both a cellular and mRNA level in LADA patients. The downregulation of the expression of pathological mRNAs, including RORC and T-BET, as well as the decreased Th17 and elevated Th2 cells, might contribute to immune suppression and glycemic control in LADA. A further larger cohort study is warranted to confirm these outcomes from clinic to bench.

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DISCLOSURE

The authors declare no conflict of interest.

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