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ORIGINAL RESEARCH PAPER

The increased T helper cells proliferation and inflammatory responses in patients with type 2 diabetes mellitus is suppressed by sitagliptin and vitamin D3 in vitro

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Abstract

Objective The probably effects of sitagliptin and vitamin D3 (VitD3) on proliferation capacity and cytokines production were investigated in type 2 diabetes mellitus (T2DM) in vitro.

Materials and methods Peripheral blood mononuclear cells (PBMCs) were isolated from 35 patients with T2DM and 26 healthy controls (HCs). CFSE-labeled PBMCs stimulated with phytohamagglutinin (PHA, 5 µg/mL) in the presence/absence of sitagliptin (200 mg/mL) with/without VitD3 (10^{-8} M) for 4 days. The proliferation of $CD4^+$ T helper cells and non-$CD4^+$ cells was analyzed using flow cytometry. The supernatant levels of IFN-γ, IL-17, IL-4, TGB-β and IL-37 were detected using ELISA.

Results The proliferation of $CD4^+$ T cells in response to PHA was higher in T2DM patients compared with HCs. The production of IFN-γ and IL-17 in PHA-stimulated cultures was higher, and the levels of IL-4 and IL-37 were lower in T2DM patients compared to HCs. The addition of sitagliptin or VitD3 to the cultures decreased the $CD4^+$ T cells and non-$CD4^+$ cells proliferation in patients and HCs. Sitagliptin with VitD3 was more effective in suppression of proliferation, decreasing of IL-17 and enhancing of IL-37 production.

Conclusion Sitagliptin plus VitD3 effectively reduces the proliferative T cells response and modulates pro-inflammatory/anti-inflammatory cytokines production.

Keywords T cell · Proliferation · Cytokine · Sitagliptin · Vitamin D3 · T2DM

Introduction

Type 2 diabetes mellitus (T2DM) is a chronic inflammatory disorder identified by insulin dysfunction and hyperglycemia [1]. Pathogenesis of T2DM is associated with alterations in immune cell subsets including $CD4^+$ T helper (Th) cells [2]. Th cells differentiate into pro-inflammatory (such as Th1 and Th17) and anti-inflammatory (such as Th2 and regulatory T cells) subtypes [3]. Th1 cells produce interferon (IFN)-γ, and Th17 cells produce interleukin (IL)-17, that mainly promote inflammatory condition and cell-mediated immunity. Th2 cells produce IL-4 and regulatory T (Treg) cells secrete transforming growth factor (TGF)-β, IL-10 and IL-37 cytokines, which are important to prevent inflammation and immune responses [4, 5]. IL-37 is recognized as a novel anti-inflammatory cytokine that contributed to reduce the inflammation and insulin resistance [6]. The serum concentration of IL-37 was changed in autoimmune or inflammatory diseases [7, 8].

Balance of pro-inflammatory and anti-inflammatory Th subsets is important for the control of inflammatory conditions and hemostasis of immune system in T2DM [2]. It has been reported that the percentage of circulating pro-inflammatory Th1 cells (by phenotype: $CD8^-IFN\text{-}\gamma^+$) and Th17 cells (by phenotype: $CD8^-IL\text{-}17A^+$) was increased in patients with T2DM [1]. The ratios of Th1/Th2 cells and Th17/Treg cells were enhanced in T2DM patients, while

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the ratio of Treg/Th1 was reduced [1, 9]. The serum levels of pro-inflammatory IL-1β and TNF-α cytokines were increased in T2DM patients [10].

Sitagliptin is being used for the treatment of T2DM. Sitagliptin enhances insulin secretion, and it also decreases the level of HbA1c and fasting blood glucose [11]. Several studies indicated that sitagliptin exerts anti-inflammatory effects. In vivo treatment with sitagliptin can decrease the number of $CD4^+$ T cells and the percentage of inflammatory T cell subsets in T2DM [12]. Sitagliptin treatment reduces serum levels of pro-inflammatory cytokines such as TNF-α, and increases the anti-inflammatory cytokines such as IL-10 in vivo [13, 14].

Vitamin D3 (VitD3) plays an essential role in the regulation of immune system. The effects of VitD3 on T cells function and cytokines production have shown in previous studies.VitD3 down-regulates pro-inflammatory cytokines such as IFN-γ and IL-6, while enhances anti-inflammatory cytokines such as IL-10 in autoimmune diseases and healthy individuals [15–17]. Moreover, VitD3 decreases the proliferation of auto-reactive naive T cells in patients with autoimmune disorders [18]. There are several evidence suggesting that VitD3 plays a role in glycemic control and insulin secretion [19, 20].

Our previous studies showed that the in vivo treatment with both sitagliptin and VitD3 diminish pro-inflammatory serum cytokines, increase anti-inflammatory cytokines and up-regulate gene expression of Treg cells transcription factor, forkhead box protein 3 (FOXP3), in T2DM [3, 21]. We hypothesized that sitagliptin and VitD3 may exert another anti-inflammatory effects on immune cells function. To our knowledge the simultaneous in vitro effects of sitagliptin and VitD3 on immune cells proliferation and cytokines production have not been investigated in T2DM. In the current study, we investigated the effects of sitagliptin and VitD3 on $CD4^+$ T cells and non-$CD4^+$ cells proliferation in response to PHA using CFSE-based assay in the patients compared to healthy individuals. The effects of sitagliptin and VitD3 on Th1, Th2, Th17 and Treg cells cytokines were also evaluated in PHA-activated cell culture supernatants.

Materials and methods

Patients and healthy controls

Blood samples (10 mL) from 35 patients with T2DM and 26 healthy controls (HCs) were collected in tubes with EDTA (Becton–Dickinson, BD, USA, Cat no: 368589). The patients were recruited from the Internal Medicine Department of Shahid Beheshti Hospital, Hamadan University of Medical Science (UMSHA). Basically, diagnoses of diabetic patients were according to the 2016 criteria of American Diabetes Association. The patients were identified with random blood glucose ≥ 200 mg/dL and HbA1C ≥ 6.5%. All patients were treated with metformin (1000 mg/day) and gliclazide (160 mg/day) in the last 1 year. The details of the participants are shown in Table 1.

The exclusion criteria were autoimmune/allergy disorders, neoplasia/chronic inflammatory diseases, HbA1c > 9% according to National Glycohemoglobin Standardization Program, smoking and alcohol consumption. None of the patients had taken insulin, immunosuppressive drugs, glucagon such as peptide-1, dipeptityl peptidase-4 and sodium/glucose cotransporter 2 inhibitors. Healthy control subjects were controlled by physicians and they had normal blood glucose with no family history of diabetes/chronic inflammatory diseases. The mean serum level of 25-hydroxy VitD was in the normal range (> 30 ng/mL) in both T2DM patients (31.68 ± 12.18 ng/mL) and HC subjects (34 ± 12.99 ng/mL), without any significant difference between the patients and HC subjects. The study was approved by the local UMSHA ethics committee (no: IR.UMSHA.REC.1396.510). The informed consent was received from the participants.

Cell isolation

Peripheral Blood Mononuclear Cells (PBMCs) were isolated using ficoll (Histopaque-1077, Sigma-Aldrich, St Louis, MO, USA, Cat no: 10771) density method, as previously described [22]. Briefly, whole blood was diluted 1:1 with phosphate buffered saline (PBS) and centrifuged in 2200 rpm for 25 min at room temperature. PBMCs were washed with PBS and stored in complete cell culture medium until use in further experiments. Viability of the cells was determined by trypan blue. The complete cell culture medium was contain RPMI 1640 (Biosera, France, Cat no: LM-R1638), 10% heat-inactivated Fetal Bovine Serum (Biosera, France, Cat no: FB-1001), 10,000 U/mL penicillin and 100 mg/mL streptomycin (Biosera, France, Cat no: XC-A4122), and L-glutamine (2 mM, Biosera, France, Cat no: XC-T1715).

Cell culture and proliferation assay

The proliferation of PBMCs was assessed by carboxyfluorescin succinimidyl ester (CFSE, Sigma-Aldrich, St Louis, MO, USA, Cat no: 21888) dilution assay as described previously [23]. Briefly, PBMCs (2×10^6 cells) were labelled with 2 µM CFSE in PBS. After 12 min incubation in 37 °C, cells were washed twice. CFSE-labelled PBMCs (2.5×10^5 cells/well) stimulated with PHA (concentration: 5 µg/mL, Sigma, St Louis, MO, USA, Cat no: L1688) in absence or presence 200 mg/mL of sitagliptin (Santa Cruz, Bergheimer, Heidelberg, Germany, Cat no: sc482298) with or without VitD3 (concentration 10^{-8} M, Sigma, St Louis, MO, USA, Cat no:

Table 1 Demographic and clinical characteristics of the participants

Characteristic	T2DM ($n=35$)	HC ($n=26$)
Gender (female/male)	24/11	14/12
Age (year)	52.71 ± 7.28	47.38 ± 10.86
Weight (kg)	74.36 ± 6.85	70 ± 9.7
Height (m)	1.7 ± 0.07	1.69 ± 0.07
Body mass index (kg/m^2)	25.86 ± 2.1	24.3 ± 2.39
Fasting plasma glucose (mg/dL)	164.69 ± 44.71*	87.05 ± 8.43
Random blood glucose (mg/dL)	257.85 ± 90.71*	111.64 ± 10.55
Hemoglobin A1C (%)	7.4 ± 0.77*	4.7 ± 0.58
Albuminuria (mg/day)	11.54 ± 7.42	10.36 ± 5.09
Serum creatinine (mg/dL)	1.02 ± 0.15	0.98 ± 0.1
Glomerular filtration rate (mL/min/1.73 m^2)	72.67 ± 8.01	75.62 ± 11
Disease duration (year)	4.50 ± 2.16	–
Vitamin D3 (ng/mL)	30.75 ± 12.28	34 ± 12.99
Red blood cell (cells/mm^3)	4.87 ± 0.41	4.46 ± 0.62
White blood cell (cells/mm^3)	6384.62 ± 1096.85	5672.73 ± 1686.13
Lymphocyte (%)	48.17 ± 6.36	42.68 ± 8.12
Neutrophil (%)	47.09 ± 5.95	52.5 ± 6.78
Monocyte (%)	3.2 ± 1.87	2.37 ± 1.86
Eosinophil (%)	2.86 ± 1.83	2.17 ± 1.62
Platelet × 10^3 (cells/mm^3)	233.77 ± 48.89	223.55 ± 58.56
Serum triglyceride (mg/dL)	162 ± 30.87	146.91 ± 29.78
Total cholesterol (mg/dL)	159.73 ± 37.88	165.32 ± 28.18
Low-density lipoprotein (mg/dL)	86.5 ± 42.11	112.36 ± 29.03
High-density lipoprotein (mg/dL)	51.5 ± 14.86	52.27 ± 11.09
Hemoglobin (mg/dL)	14.5 ± 1.34	13.75 ± 1.57
Hematocrit (%)	42.57 ± 3.86	41.21 ± 4.87

Values expressed as means (± SD)

*$p < 0.001$ compared to values of HCs

D1530) in 96-well flat bottom plate (Jet biofil, China, Cat no: TCP001096). The cells were incubated in the complete cell culture media at 37 °C in 5% CO_2 for 4 days. After incubation period, the PBMCs were stained with PE-conjugated anti-CD4 monoclonal antibody (clone: SK3, BD, USA, Cat no: 345768) for 30 min [24]. The cells were washed in PBS and the proliferation assay was performed using flow cytometry instrument (Invitrogen, ATTUNE NxT, USA) and Attune™ NxT Software V2.5. The culture supernatants were collected and stored in − 80 °C until cytokine analysis.

Measurement of cytokines

The supernatant levels of IFN-γ (Biolegend, San Diego, CA, USA, Cat no: 430104), IL-17 (Biolegend, San Diego, CA, USA, Cat no: 433914), IL-4 (Invitrogen, San Diego, CA, USA, Cat no: 887046), TGF-β (Invitrogen, San Diego, CA, USA, Cat no: 8850390) and IL-37 (Invitrogen, San Diego, CA, USA, Cat no: 885210322) were detected by ELISA kit according to the manufacturer's instructions.

Statistical analysis

Statistical analysis was performed using SPSS version 21. Graphs were drawn with Graph Pad prism version 6.07. ANOVA with post hoc Bonferroni applied to assess differences between the groups. Data were expressed as mean ± SD. P value < 0.05 was considered statistically significant.

Results

Sitagliptin and VitD3 reduced the proliferation of CD4$^+$ T cells

To investigate the effects of sitagliptin and VitD3 on T cells proliferation ability, PBMCs labelled with CFSE and stimulated with PHA in the presence or absence of sitagliptin with or without VitD3 for 4 days, and the immune cells proliferation were detected by flow cytometry. Because experiments were initiated with PBMCs, the baseline percentage

of immune cells was compared between the patient and HC groups. There were no significant differences in percentage of $CD4^+$ T cells and non-$CD4^+$ cells between the patients and HCs (Fig. 1a). In the preliminary culture experiments, PBMCs were cultured with different concentrations of PHA (0, 2.5, 5 and 10 µg/mL) and the proliferation of $CD4^+$ T cells was analyzed. PHA was able to stimulate the proliferation of $CD4^+$ Th cells in a dose-dependent manner without any significant differences between 5 and 10 µg/mL concentrations. Based on present and previous observation [25], the dose of 5 µg/mL was selected for all experiments (Fig. 1b). In another preliminary experiment, PBMCs were cultured with PHA (5 µg/mL) with different doses of sitagliptin (100, 200 and 400 µg/mL) or VitD3 (10^{-7}, 10^{-8} and 10^{-9} M), and the cells proliferation was evaluated. We observed that the both sitagliptin and VitD3 were able to suppress the $CD4^+$ T cells proliferation in a dose-dependent manner, but the 400 µg/mL of sitagliptin and 10^{-7} M of VitD3 showed a toxic effect on the cells (Fig. 1c, d). Based on these observations, the concentration of 200 µg/mL for sitagliptin and 10^{-8} M for VitD3 was selected for all experiments [24].

Figure 2 shows representative plots for gating strategy of proliferation assay from a healthy individual, demonstrating sitagliptin and/or VitD3 diminished the proliferation of $CD4^+$ T cells ($CD4^+$ gate) and non-$CD4^+$ cells ($CD4^-$ gate). In general, the proliferation of $CD4^+$ T cells in response to PHA was significantly increased in T2DM patients compared with HC subjects ($p=0.03$, Fig. 3a). Similarly, the proliferation of $CD4^+$ T cells in cultures with sitagliptin, VitD3 and sitagliptin plus VitD3 in T2DM was significantly higher than HCs ($p=0.03$, $p=0.02$ and $p=0.02$). We found that the addition of sitagliptin or VitD3 to the cultures significantly decreased the proliferation of $CD4^+$ T cells, compared to cultures with PHA in both HC subjects ($p<0.001$ and $p<0.001$) and T2DM ($p<0.001$ and $p<0.001$) patients (Fig. 3a). Similarly, the addition of sitagliptin combined with VitD3 to the cultures diminished the proliferation of $CD4^+$ T cells, compared to cultures with PHA in both HCs ($p<0.001$) and T2DM ($p<0.001$) patients. The proliferation of $CD4^+$ T cells in cultures with sitagliptin plus VitD3 was significantly lower than in cultures with sitagliptin or VitD3 alone in both HCs ($p=0.004$ and $p=0.002$) and T2DM patients ($p<0.001$ and $p<0.001$).

Sitagliptin and VitD3 reduced the proliferation of non-$CD4^+$ cells

The results showed that the addition of sitagliptin,VitD3 or sitagliptin plus VitD3 to PBMC cultures diminished the proliferation of non-$CD4^+$ cells compared to cultures with PHA in both HCs ($p<0.001$, $p<0.001$ and $p<0.001$) and T2DM group ($p<0.001$, $p<0.001$ and $p<0.001$) (Fig. 3b). The proliferation of non-$CD4^+$ cells was reduced in cultures with sitagliptin plus VitD3 compared to the cultures with sitagliptin or VitD3 in HCs ($p=0.004$ and $p<0.001$) and T2DM patients ($p<0.001$ and $p<0.001$). There were no significant changes in the proliferation of these cells between the HCs and T2DM group.

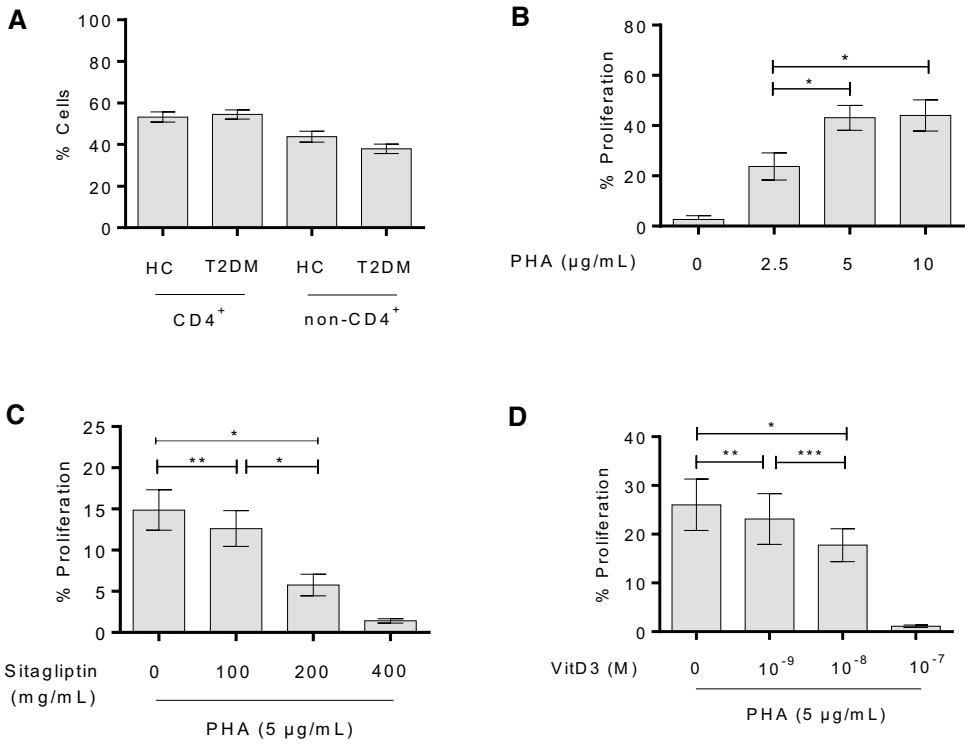

Fig. 1 The cells proliferation assay performed at different doses of PHA, sitagliptin and VitD3. **a** The percentage of $CD4^+$ T cells and non-$CD4^+$ cells in PBMCs gate are shown in HCs ($n=26$) and T2DM ($n=35$) patients. CFSE-labeled PBMCs from healthy controls ($n=6$) were stimulated with different doses of **b** PHA (0, 2.5, 5 and 10 µg/mL), **c** sitagliptin (100, 200 and 400 mg/mL) and **d** VitD3 (10^{-7}, 10^{-8} and 10^{-9} M) for 4 days and the proliferation of $CD4^+$ T cells was analyzed using flow cytometry. *$p<0.001$, **$p=0.01$, ***$p=0.04$. *PHA* phytohamagglutinin, *VitD3* vitamin D3

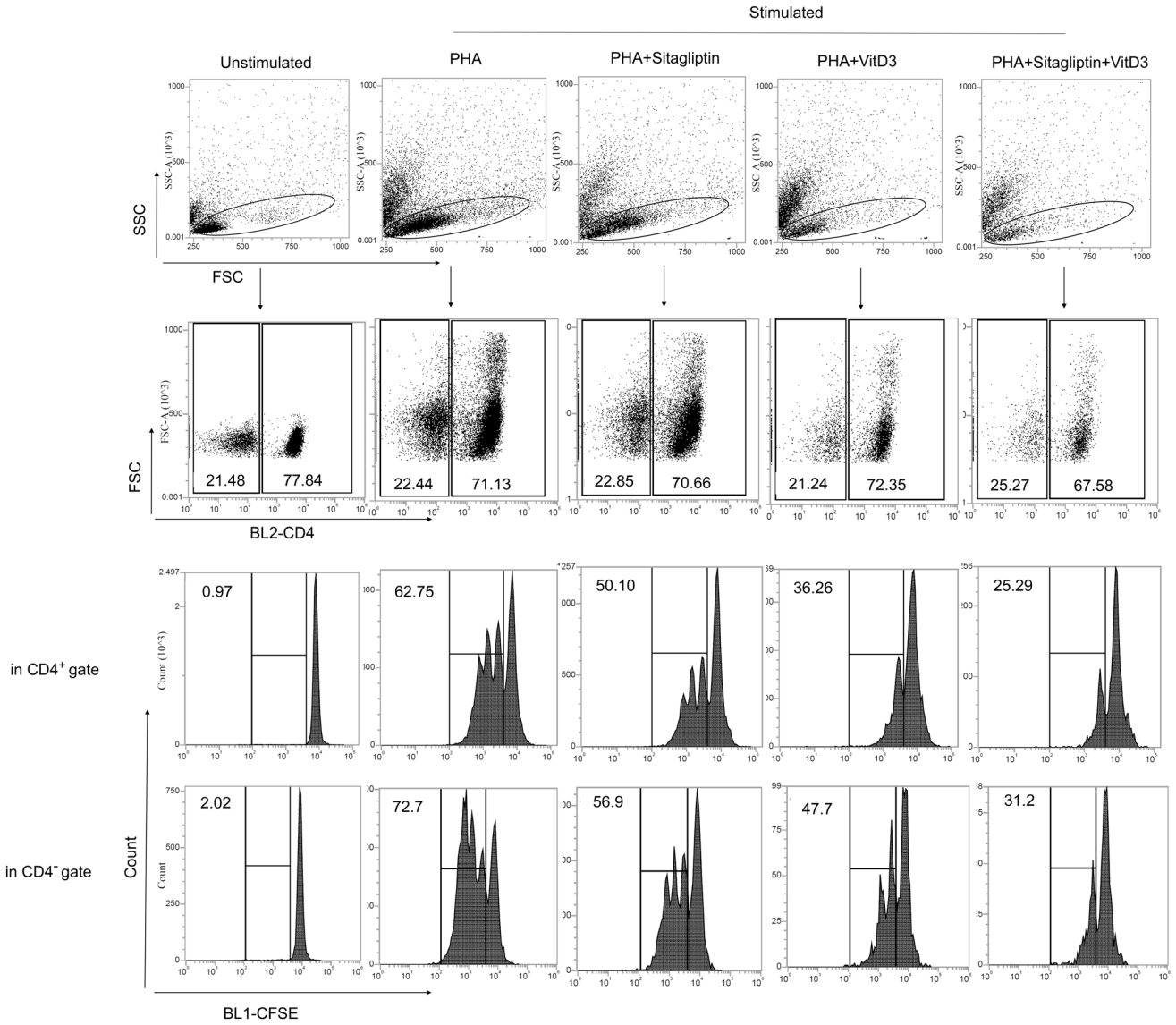

Fig. 2 Flow cytometry gating strategy for proliferation assay. PBMCs were labelled with CFSE and stimulated with PHA (5 µg/mL) in the absence or presence of sitagliptin (200 mg/mL) with ot without VitD3 (10^{-8} M) for 4 days. Representative plots from a HC subject that stained with anti CD4-PE and analyzed according to the gating strategy. Debris (SSC vs. FSC) were excluded and lymphocyte population were selected. $CD4^+$ and $CD4^-$ population were separated (FSC vs. BL2) based on the CD4 expression. The proliferation of $CD4^+$ T cells and non-$CD4^+$ cells were analyzed using CFSE-based assay in different culture condition (histograms)

Effect of sitagliptin and VitD3 on cytokine production

To investigate the effect of sitagliptin and VitD3 on cytokine secretion, the PBMCs were activated with PHA in the absence or presence of sitagliptin with or without VitD3, and the levels of cytokines were determined in the culture supernatants. The production of IFN-γ in cultures with PHA or sitagliptin was significantly higher in T2DM patients compared with HC subjects ($p=0.04$ and $p=0.04$) (Fig. 4a). The secretion of IFN-γ was reduced in cultures with VitD3 or sitagliptin plus VitD3 compared to the cultures with PHA in both HCs ($p<0.001$ and $p=0.04$) and T2DM patients ($p<0.001$and $p<0.001$). The level of IFN-γ was reduced in cultures with VitD3 compared to the cultures with sitagliptin in both HCs ($p<0.001$) and T2DM patients ($p<0.001$).

The production of IL-17 in the cultures that stimulated with PHA was significantly higher in T2DM patients compared with HCs ($p<0.001$) (Fig. 4b). The production of IL-17 was significantly decreased in cultures with sitagliptin, VitD3 and sitagliptin plus VitD3 compared to the cultures with PHA in both HCs ($p=0.02$, $p<0.001$ and $p<0.001$) and T2DM patients ($p<0.001$, $p<0.001$ and $p<0.001$). Addition of sitagliptin together with VitD3 to the cultures

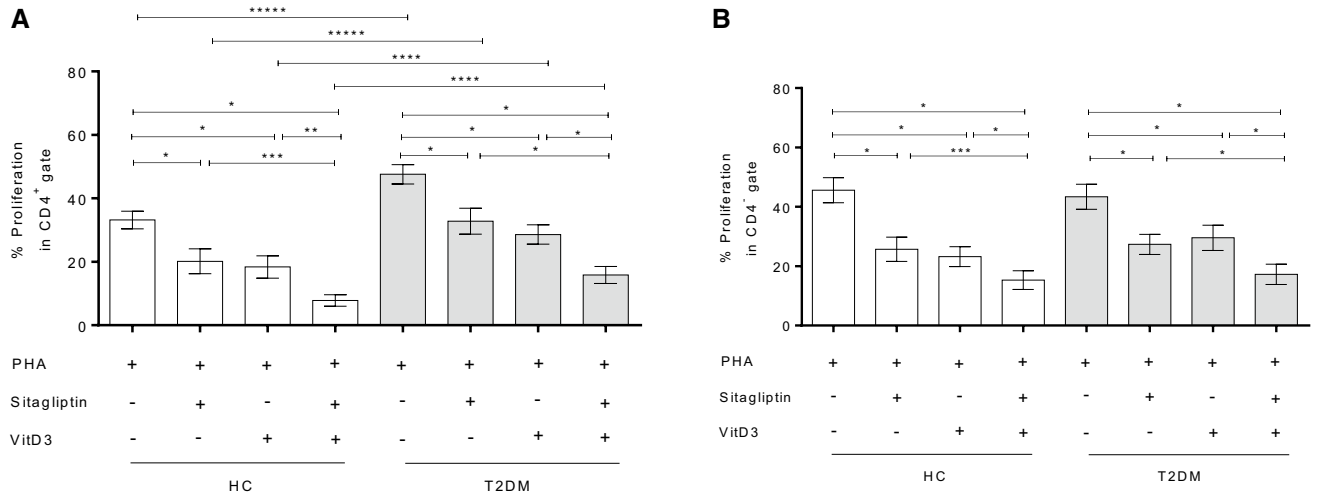

Fig. 3 Effect of sitagliptin and VitD3 on proliferation of $CD4^+$ T cells and non-$CD4^+$ cells. PBMCs were labelled with CFSE and stimulated with PHA (5 µg/mL) in absence or presence of sitagliptin (200 mg/mL) with or without VitD3 (10^{-8} M) for 4 days. Proliferation of the cells were analyzed using flow cytometry. **a**, **b** Scatter plots shows the proliferation of $CD4^+$ T cells and non-$CD4^+$ cells in HCs ($n=26$) and T2DM ($n=35$) patients. $p<0.05$ was considered significant. Data expressed as mean ± SEM. *$p<0.001$, **$p=0.002$, ***$p=0.004$, ****$p=0.02$, *****$p=0.03$. *PHA* phytohamagglutinin, *VitD3* vitamin D3, *HC* healthy control, *T2DM* type 2 diabetes mellitus

diminished the level of IL-17 compared to the cultures with sitagliptin or VitD3 in both HCs ($p=0.02$ and $p<0.001$) and T2DM ($p<0.001$ and $p<0.001$). IL-17 level was lower in cultures with VitD3 compared to cultures with sitagliptin in both HCs ($p=0.04$) and T2DM patients ($p=0.01$).

We observed that the production of IL-4 was significantly diminished in T2DM patients compared to HCs in cultures stimulated with PHA ($p<0.001$), sitagliptin ($p<0.001$), VitD3 ($p=0.01$), and sitagliptin plus VitD3 ($p=0.04$) (Fig. 4c). We found no significant changes in IL-4 cytokine levels in different activated cultures between the patient and HC groups. The level of TGF-β did not change between the cultures with PHA, VitD3, sitagliptin and sitagliptin plus VitD3 in patient and HC groups. Similarly, its level did not change between the patient and HC groups (Fig. 4d). The production of IL-37 in cultures with PHA was lower in T2DM patients compared with HCs ($p=0.04$) (Fig. 4e). Its level was significantly elevated in the culture with sitagliptin plus VitD3 compared to the cultures with PHA, sitagliptin or VitD3 alone in both HCs ($p<0.001$, $p<0.001$ and $p=0.04$) and T2DM patients ($p<0.001$, $p=0.04$ and $p=0.01$).

Discussion

It has been shown that the pro-inflammatory subsets of T cells and their cytokines play an essential role in pathogenesis of T2DM [10]. Combined treatment with sitagliptin and VitD have been shown to improve β-cells function and attenuate disease severity in patient with latent autoimmune diabetes in vivo [26]. In the current study, we assessed the mono- or combined effects of sitagliptin and VitD3 on proliferation of $CD4^+$ T cells and non-$CD4^+$ cells and cytokines production in vitro. Our main findings were that the basal proliferation level of $CD4^+$ T cells was higher in T2DM patients compared to HCs. The addition of sitagliptin or VitD3 to the cultures decreased proliferation of immune cells in both T2DM patients and HC subjects. Sitagliptin in combination with VitD3 was more effective in the suppression of proliferation compared to sitagliptin or VitD3 alone. The basal production levels of IFN-γ and IL-17 were enhanced in response to PHA, whereas the level of IL-4 and IL-37 was reduced in patients compared to HCs. The addition of sitagliptin inhibited IL-17, and VitD3 diminished both IFN-γ and IL-17 in vitro. Sitagliptin plus VitD3 potently enhanced IL-37 and decreased IL-17 production.

Previous studies indicated that the proliferation of T cells increased in autoimmune diseases including rheumatoid arthritis and type 1 diabetes mellitus [27]. Our previous study revealed that the proliferation of isolated naïve T cells increased in T2DM patients following stimulation with polyclonal anti-CD3/CD28 in vitro, and the T cells were auto-reactive in the patients [28]. In this study, we confirmed that the proliferation of $CD4^+$ T enhanced in T2DM patients following stimulation with PHA in PBMCs culture environment. It has been demonstrated that Tregs in visceral adipose tissue play a key role in the modulation of insulin resistance, local pro-inflammatory state and progression of T2DM [29]. The complementary studies showed that defect in Tregs function promotes inflammatory condition

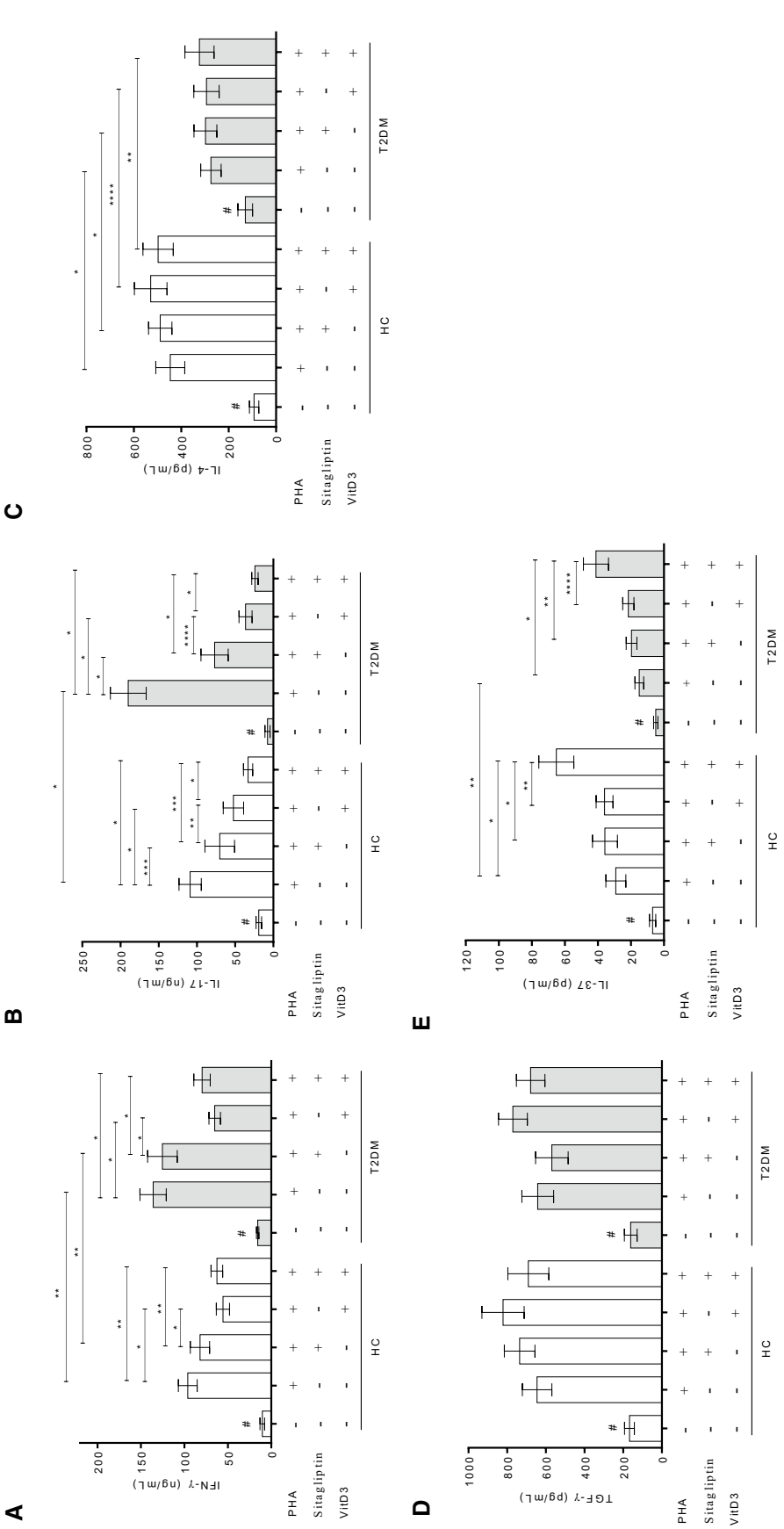

Fig. 4 Effect of sitagliptin and VitD3 on cytokine production. PBMCs were stimulated with PHA (5 µg/mL) in absence or presence of sitagliptin (200 mg/mL) with or without VitD3 (10^{-8} M) for 4 days, and the cytokine production in HCs ($n=20$) and T2DM ($n=21$) were determined. Scatter plots shows the level of **a** IFN-γ, **b** IL-17, **c** IL-4, **d** TGF-β and **e** IL-37 in the patients and HCs. $p<0.05$ was considered significant. Data expressed as mean ± SEM. $^{\#}p<0.001$ compared to cultures with PHA, $^{*}p<0.001$, $^{**}p=0.04$, $^{***}p=0.02$, $^{****}p=0.01$. *PHA* phytohamagglutinin, *VitD3* vitamin D3, *HC* healthy controls, *T2DM* type 2 diabetes mellitus

[9]. We previously reported that the function of Treg cells were defective in T2DM [28]. Based on pervious and current studies, it seems that functional impairment of Tregs may be responsible for the enhanced proliferation ability of T cells in T2DM.

Dipeptidyl peptidase IV or CD26, as a receptor for sitagliptin, is a cell surface antigen expressed on $CD4^+$ and $CD8^+$ T cells which plays a role in T cell activation and proliferation [25]. Sitagliptin can alter T cells function and differentiation by binding to CD26 [12]. The effects of sitagliptin on immune cells have been shown in several studies. Aso and colleagues showed that the total number of $CD4^+$ T cells was reduced after sitagliptin treatment in vivo [12]. Our result showed that the addition of sitagliptin to the cultures diminished the proliferation of $CD4^+$ T cells and non-$CD4^+$ cells (mainly composed of $CD8^+$ T cells) in the patients [30]. It seems that sitagliptin may reduce the proliferation of the cells by inhibition of CD26 signaling pathway, but further investigations are needed to confirm these possibilities.

Vitamin D3 exerts its effects by interaction with vitamin D receptor (VDR) [19]. Expression of VDR was induced on T cells by TCR mitogen-activation [31]. Some studies explained that VitD3 inhibits the proliferation of auto-reactive $CD4^+$ T cells in autoimmune and inflammatory diseases [24, 32]. We confirmed that the addition of VitD3 to the cultures diminished the proliferation of $CD4^+$ T cells and non-$CD4^+$ cells in T2DM patients. Sitagliptin plus VitD3 can suppress the immune cells proliferation more effective than sitagliptin or VitD3 alone. These finding suggested that increased proliferation of T cells in T2DM can be effectively suppressed by double combination of sitagliptin and VitD3 in the patients.

The possible effects of sitagliptin and VitD3 on cytokine production were also investigated in this study. IFN-γ is a pro-inflammatory cytokine which is mainly produced by Th1 subset. Previous studies proposed that increased levels of IFN-γ are associated with inflammation and insulin resistance in diabetic patients [10]. In this study, we found that the production level of IFN-γ was enhanced in PHA-stimulated cell culture environment in T2DM patients compared with HC subjects. Recently, we also reported that the serum levels of IFN-γ and IL-17 and the production of these cytokines in anti-CD3 activated cell culture experiments were increased in untreated (without sitagliptin and VitD3) nephropathic and non-nephropathic T2DM patients compared with healthy controls [21]. We did not observe any significant changes in secretion of IFN-γ following sitagliptin treatment. Previously, we published that the addition of VitD3 to the anti CD3/CD28 stimulated $CD4^+$ T cell cultures, decreased IFN-γ production in HC subjects [16]. In this study, we confirmed that VitD3 alone or in combination with sitagliptin can inhibit IFN-γ production in PHA-activated PBMCs cultures of T2DM patients, in agreement with our previous in vivo studies [3, 21]. Moreover, we did not found any significant changes in IFN-γ comparison between the VitD3 cultures and VitD3 plus sitagliptin cultures. It seems that only VitD3 (not sitagliptin) has immunosuppressive effects on IFN-γ.

IL-17, as a main cytokine for Th17, plays a key role in the pathogenesis of inflammatory disorders including T2DM [2]. Studies indicated that the proportion of $CD3^+CD8^-IL\text{-}17^+$ T cells increased in T2DM [10]. We confirmed that the production of IL-17 was increased in PHA-stimulated cell culture environment in T2DM patients compared with HCs. It has been reported that sitagliptin reduced the frequency of peripheral Th17 cells in T2DM patients in vivo [12]. We observed that sitagliptin diminished the level of IL-17 in the culture experiments in T2DM patients, in agreement with in vivo studies [12]. Previous studies showed that VitD3 can decrease production of IL-17 both in vitro and in vivo [17, 33]. Our results reveled that combined sitagliptin and VitD3 treatment was more effective for reducing IL-17 in the patients, in agreement with our previous studies in vivo [3, 21]. Reduction of IL-17 can be one of the underlying mechanisms for anti-inflammatory action of sitagliptin and VitD3 in the patients. These observations suggest that the combined use of sitagliptin and VitD3 may have a more anti-inflammatory effect on Th17 cells in the patients.

IL-4 is an inhibitory cytokine for Th1 cells that mainly produced by activated Th2 cells. This study showed that the production of IL-4 in the PBMCs supernatants of T2DM patients was lower than in HCs following stimulation with PHA. Probably the diminished level of IL-4 may contribute to the induction of diabetes mellitus [34]. It has been shown that diminished gene expression of IL-4 is associated with development of diabetes in animal model [35]. The reduction of IL-4 may contribute to the onset of diabetes in animal models and humans [34, 36]. In addition, because of IL-4 and IFN-γ are opposing each other, it is possible that the increased IFN-γ in the patients reduced the IL-4 production or vice versa. Sromova and colleagues reported that in vivo administration of sitagliptin for 4 weeks did not change the serum level of IL-4 in the T2DM patients [14]. In vitro study has been demonstrated that the intracellular frequency of $CD4^+IL\text{-}4^+$ lymphocytes reduced after stimulation with PHA and sitagliptin after 48 h in vitro [25]. On the other hand, in vivo administration of VitD3 increases IL-4 gene transcripts and production [37]. We found no significant changes in the level of IL-4 cytokine after the addition of sitagliptin or VitD3 to PHA stimulated PBMC cultures in disagreement with our previous study in vivo [3], which could be as a result of inadequate sample size, dose of sitagliptin and type or duration of stimulation. Future studies should be investigated these observations with isolated $CD4^+$ T cells and with more sample size or different dose of sitagliptin and VitD3.

IL-37, TGF-β, IL-10 are anti-inflammatory cytokines which mainly produced by Treg cells and associated with inflammatory and autoimmune diseases [4, 5]. Previous studies have been reported that the serum level of IL-10 was diminished in T2DM patients, and the sitagliptin can increase IL-10 levels [13, 38]. In this study, we investigated the level of IL-37 as a novel cytokine, and TGF-β as a routine cytokine for Tregs. We found decreased level of IL-37 in T2DM patients compared to HC subjects in PHA-activated cultures. The addition of sitagliptin plus VitD3 potently enhanced IL-37 production in both patients and HCs. Recently, we also published that the in vivo production of IL-37 and FOXP3 expression were increased in the patients treated with sitagliptin (8 months) plus VitD3 (2 months) [21]. Enhancement of IL-37 can be proposed to another underlying mechanisms for anti-inflammatory action of sitagliptin and VitD3 in the patients. We found no significant changes in TGF-β levels in cultures with sitagliptin and VitD3 in agreement with previous studies [3, 21]. In vivo study revealed that there were no significant changes in its level after 4 weeks and 12 months of sitagliptin administration [14]. It should be noted that, cytokines are produced by a various cells, and the source of the TGF-β and IL-37 may be different from each other. This probability should be confirmed on isolated Tregs in the future studies.

The limitation of this study was the evaluation of cytokines production at PBMCs culture environment. Future studies should confirm these observations on isolated T cell subsets. This study investigated the cytokines in PBMCs, yet flow cytometric cytokines analysis should be included in future studies. In this study, we only focused on the effect of sitagliptin and VitD3 on proliferation of $CD4^+$ T cells and non-$CD4^+$ cells. An evaluation of the circulating $CD4^+$ T cell subsets, $CD8^+$ T cells, B cells and NK cells and the effect of sitagliptin and vitamin D3 on purified subsets would have investigate in future studies. Further studies are required to describe the more detail about intracellular molecular mechanisms of sitagliptin and VitD3 on T cell proliferation in the patients.

Conclusion

The results confirmed the increased proliferation of T helper cells in the T2DM patients. The production of IFN-γ and IL-17 in PHA-stimulated cultures was higher in the patients, whereas the levels of IL-4 and IL-37 were lower compared with healthy individuals. In this study, the in vitro immunomodulatory effect of sitagliptin was demonstrated by suppressing of $CD4^+$ T cell and non-$CD4^+$ cells proliferation and decreasing the production of IL-17 in T2DM patients. Sitagliptin plus VitD3 was more effective in the suppression of cells proliferation, decreasing of IL-17 and enhancing of IL-37. The results indicate that sitagliptin plus VitD3 effectively reduces the auto-reactive T cells function and reduces inflammatory response in the patients. Combined sitagliptin and VitD3 can be considered as a potential combination for the treatment of T2DM patients.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflicts of interest.

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