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ORIGINAL ARTICLE

Characterization of IL-17-producing Treg cells in type 2 diabetes patients

Lei Zhu[1] · Haihan Song[2] · Li Zhang[1] · Haiyan Meng[3]

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Abstract

The association between type 2 diabetes (T2D) pathogenesis and immune-mediated tissue damage and insulin resistance suggests that T2D patients might benefit from the suppression of pathogenic inflammation. Foxp3$^+$ Treg cells are crucial suppressors of inflammation, but the differentiation of Foxp3$^+$ Treg cells is not static and is subject to conversion into IL-17-producing Th17-like cells upon receiving external signals. In this study, we examined the production of IL-17 by Treg cells. Compared to non-T2D controls, T2D patients presented significantly higher levels of IL-17-expressing cells in both Foxp3$^-$ CD4 T cells and Foxp3$^+$ Treg cells. The frequencies of IL-17-nonexpressing Foxp3$^+$ Treg cells, on the other hand, were not changed. Interestingly, IL-17-expressing Foxp3$^+$ Treg cells were mutually exclusive from IL-10-expressing and TGF-β-expressing Foxp3$^+$ Treg cells, suggesting that multiple subpopulations exist within the Foxp3$^+$ Treg cells from T2D patients. In T2D patients, the frequencies of IL-17-expressing Foxp3$^+$ Treg cells were positively correlated with the body mass index (BMI) and the HbA1c levels of T2D patients. The frequencies of IL-10-expressing Treg cells, on the other hand, were inversely associated with the BMI of both non-T2D controls and T2D patients. In addition, the suppressive activity of Treg cells was significantly lower in T2D patients than in non-T2D controls. Together, our study uncovered a dysregulation in Foxp3$^+$ Treg cells from T2D patients, characterized by high IL-17 expression and low suppression activity.

Keywords Type 2 diabetes · Treg · IL-17

Introduction

Type 2 diabetes (T2D) is characterized by a relative deficiency in insulin, resulted from islet β cell destruction and insulin resistance. Currently, T2D is one of the most prevalent diseases in the world, with an estimated number of 415 million patients living with the disease and another 193 million people living with undiagnosed T2D [1].

In recent years, accumulating evidence indicates that chronic low-grade inflammation is playing an augmenting role in T2D initiation and development. Obesity, a common comorbidity in T2D, can directly activate the immune system via the production of reactive oxygen species (ROS) as a result of energy overload and organelle destruction [2, 3]. The expanding adipose tissue may also cause local hypoxia, which then leads to the production of angiogenic cytokines [4]. Furthermore, saturated fatty acids and directly activate Toll-like receptor (TLR)2 and TLR4, and activate proinflammatory cytokine production [5, 6]. T2D patients in general display elevated serum concentrations of TNF-α and IL-6 [7]. These cytokines may stimulate the production of C-reactive protein (CRP), which increases ICAM-1 and MCP-1 expression by endothelial cells [8, 9]. This potentially allows immune cell infiltration and inflammation-induced tissue destruction. IL-6 and TNF-α also promote Th17 differentiation [10]. Th17 and its namesake cytokine IL-17 could sustain inflammation in the adipose tissue by promoting the expression of IL-1β, IL-6, and TNF-α in the positive feedback loop [11]. Foxp3$^+$ regulatory T cells (Tregs), on the other hand, may have a role to

✉ Lei Zhu
julyleizhu@sina.com

✉ Haiyan Meng
hy_meng@sohu.com

[1] Department of Endocrinology, Shandong Provincial Third Hospital, 11 Wuyingshan Middle Road, Jinan, Shandong, China

[2] DICAT Biomedical Computation Centre, Vancouver, British Columbia, Canada

[3] Department of Cardiology, Shandong Provincial Third Hospital, 11 Wuyingshan Middle Road, Jinan, Shandong, China

reverse proinflammatory disorders in T2D. Foxp3$^+$ Treg-treated mice presented significantly better insulin sensitivity and lower diabetic nephropathy [12]. Depletion of Foxp3$^+$ Treg cells in the visceral adipose tissue was found in insulin-resistant obese mice compared to lean mice [13].

Interestingly, previous studies have shown that the differentiation of Foxp3$^+$ Treg cells is not static and is subject to conversion into IL-17-producing Th17-like cells upon receiving external signals. IL-6 in the absence of TGF-β has been shown to induce IL-17 production in Foxp3$^+$ T cells [14–16]. The IL-17-producing Treg cells have suppressive capacities in vitro, but these capacities are lost after IL-1β and IL-6 stimulation [15]. This Treg to Th17 conversion has been observed arthritic joints, where CD25^{lo}Foxp3$^+$ CD4 T cells downregulate Foxp3 and become Th17 cells in an IL-6-dependent manner, with roles to exacerbate inflammation in autoimmune arthritis [17].

The association between T2D pathogenesis and immune-mediated tissue damage and insulin resistance suggests that T2D patients might benefit from the suppression of pathogenic inflammation. To this end, we investigated the characteristics of Treg cells and Th17 in T2D patients.

Method

Participating volunteers

This investigation was approved by the Shandong Provincial Third Hospital Ethics Committee and was performed in accordance with the declaration of Helsinki. All T2D patients recruited in this study were recently diagnosed and untreated. Control subjects were recruited from age- and sex-matched individuals, who were neither diabetic nor pre-diabetic. The T2D patient group included 13 females and 9 males between 50 and 65 years of age (median 61 years). The control group included 12 females and 10 males between 52 and 65 years of age (median 60 years). HbA1c levels were measured during blood collection. Exclusion criteria were concurrent cardiovascular disease, autoimmune diseases, malignancies, and chronic virus infections. All participants provided written informed consent.

Treg frequency and cytokine expression

Peripheral blood mononuclear cells (PBMCs) were harvested from blood using the standard Ficoll gradient centrifugation technique. Following isolation, PBMCs were incubated in unstimulated media or with human T-activator beads (anti-CD3/28-coated, 1 bead per T cell; Thermo Fisher Scientific) in the presence of brefeldin A and monensin (BD Biosciences). After 5 h, PBMCs were incubated with fluorescent anti-human CD3 and CD4 antibodies (BioLegend) and LIVE/DEAD Violet dead cell stain (Invitrogen) for 30 min, permeabilized with CytoFix/CytoPerm (BD Biosciences), and incubated with fluorescent anti-human Foxp3, IL-17, IL-10, and/or TGF-β antibodies (BioLegend). After washing, cells were fixed in 2% formaldehyde and acquired in FACSCanto cytometer system (BD Biosciences). Analyses were performed in FlowJo (Tree Star).

Cell sorting and Treg suppression assay

CD4$^+$ T cells were pre-sorted using a magnetic bead-based negative isolation method with the EasySep Human CD4 T Cell Enrichment kit (Stemcell Technologies). Isolated CD4$^+$ T cells were stained with fluorescent anti-CD25 and CD127 antibodies (BioLegend), and CD4$^+$CD25$^-$ T_{conv} cells and CD4$^+$CD25^{hi}CD127$^-$ T cells were sorted in FACSAria cytometer system (BD Biosciences). CD4$^+$CD25$^-$ T_{conv} cells were resuspended at 5×10^4 cells per mL and plated in a 96-well round-bottom plate at 100 μL per well. Human T-activator beads were added at 1 bead per T_{conv} cell. CD4$^+$CD25^{hi}CD127$^-$ T cells were added at varying concentrations accordingly. After a 72-h incubation, the cells were pulsed with 0.1 μCi/mL radioactive thymine (Amersham) for 5 h. The cells were then harvested to measure radioactivity. Triplicate experiments were performed for each condition.

Statistical analysis

Serum IL-17 level between non-T2D controls and T2D patients was examined using Student's t test. Comparisons of Treg cells between non-T2D controls and T2D patients were performed using two-way ANOVA followed by Tukey's test. Correlation studies were performed using Pearson correlation test. The value of p needs to be lower than 0.05 to be considered statistically significant.

Results

Treg frequency and IL-17 expression in T2D patients

In this study, we recruited recently diagnosed, untreated T2D patients and age/sex-matched non-T2D controls. The non-T2D controls were from patients who had no prior or current diagnosis of metabolic diseases or other diseases. First, the level of IL-17 in serum was evaluated. Overall, the serum IL-17 was slightly, but significantly, higher in T2D patients than in non-T2D controls (Fig. 1a); however, no distinctive difference between T2D patients and non-T2D controls was observed, as the ranges of values overlapped highly between the two groups. To evaluate the Treg and Th17 frequency in non-T2D controls and T2D patients, we performed intracellular staining to

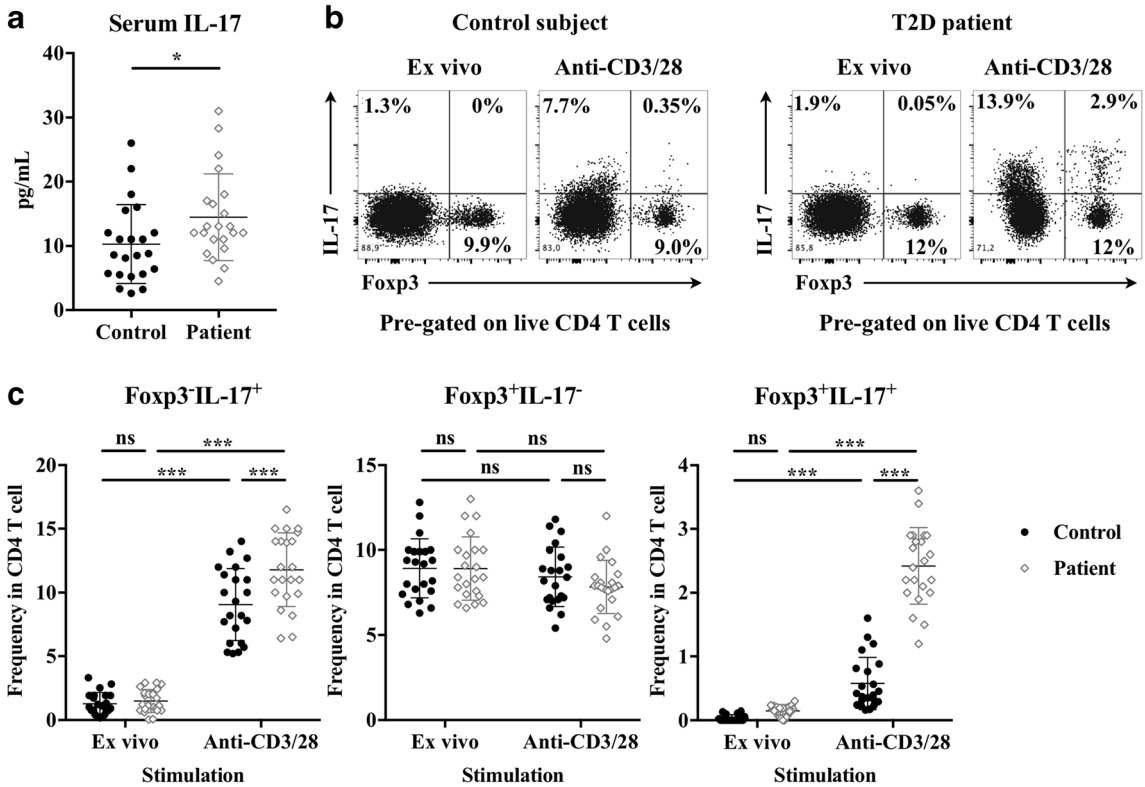

Fig. 1 Foxp3 and IL-17 expression in CD4 T cells from non-T2D controls and T2D patients. **a** Serum IL-17 level in non-T2D controls and T2D patients. Student's *t* test. $*p < 0.05$. **b** Foxp3 vs. IL-17 staining in pre-gated CD4 T cells, examined directly ex vivo or after anti-CD3/28 stimulation. One representative each from the controls and the patients is shown. **c** The frequencies of $Foxp3^{-}IL\text{-}17^{+}$ cells, $Foxp3^{+}IL\text{-}17^{-}$ cells, and $Foxp3^{+}IL\text{-}17^{+}$ cells in 22 controls and 22 patients, examined directly ex vivo or after anti-CD3/28 stimulation. Two-way ANOVA followed by Tukey's test. ns, not significant. $***p < 0.001$

examine the Foxp3-expressing cell frequency and IL-17-expressing cell frequency in circulating CD4 T cells, respectively. In addition, cells were examined under unstimulated condition directly ex vivo and after a 5-h stimulation using CD3 and CD28 ligation beads (anti-CD3/28). In unstimulated CD4 T cells, Foxp3-expressing Treg cells were readily detected, while IL-17-expressing cells were rarely found (Fig. 1b). After anti-CD3/28 stimulation, IL-17-expressing cells became readily detectable in both $Foxp3^{-}$ CD4 T cells and $Foxp3^{+}$ Treg cells (Fig. 1b). In the 22 controls and 22 patients recruited for this study, we found that the frequencies of $Foxp3^{-}IL\text{-}17^{+}$ CD4 T cells were significantly elevated by anti-CD3/28 stimulation, and were significantly higher in T2D patients than in non-T2D controls (Fig. 1c, left). The frequencies of $Foxp3^{+}IL\text{-}17^{-}$ Treg cells, on the other hand, were not changed by anti-CD3/28 stimulation and were comparable in T2D patients and in non-T2D controls (Fig. 1c, middle). The frequencies of $Foxp3^{+}IL\text{-}17^{+}$ Treg cells were significantly elevated by anti-CD3/28 stimulation and were significantly higher in T2D patients than in non-T2D controls (Fig. 1c, right). Overall, these data indicated that in T2D patients, both the Treg cells and non-Treg CD4 T cells were enriched with IL-17-expressing cells.

IL-17-expressing $Foxp3^{+}$ Treg cells did not produce IL-10 or TGF-β

Thymic-derived natural Treg cells express TGF-β while induced Treg cells express IL-10 in addition to TGF-β [18]. Both TGF-β and IL-10 are instrumental in Treg-mediated immune suppression. We wondered whether IL-17-expressing Treg cells could also express IL-10 and TGF-β; therefore, in anti-CD3/28-stimulated, pre-gated $Foxp3^{+}$ Treg cells, IL-10, and TGF-β expression were examined alongside IL-17. We found that the vast majority of IL-10-expressing Treg cells did not concurrently express IL-17 (Fig. 2a). In both non-T2D controls and T2D patients, the vast majority of IL-10-expressing Treg cells were found in the $IL\text{-}17^{-}$ proportion (Fig. 2b). In addition, the frequencies of $IL\text{-}10^{+}IL\text{-}17^{-}$ Treg cells were significantly lower in patients than in controls. In addition, the vast majority of TGF-β-expressing Treg cells also did not express IL-17 (Fig. 2c), and the frequencies of $TGF\text{-}\beta^{+}IL\text{-}17^{-}$ Treg cells were significantly lower in patients than in controls (Fig. 2d). Overall, these data indicated that IL-10-expressing and TGF-β-expressing Treg cells and IL-17-expressing Treg cells were mutually exclusive populations.

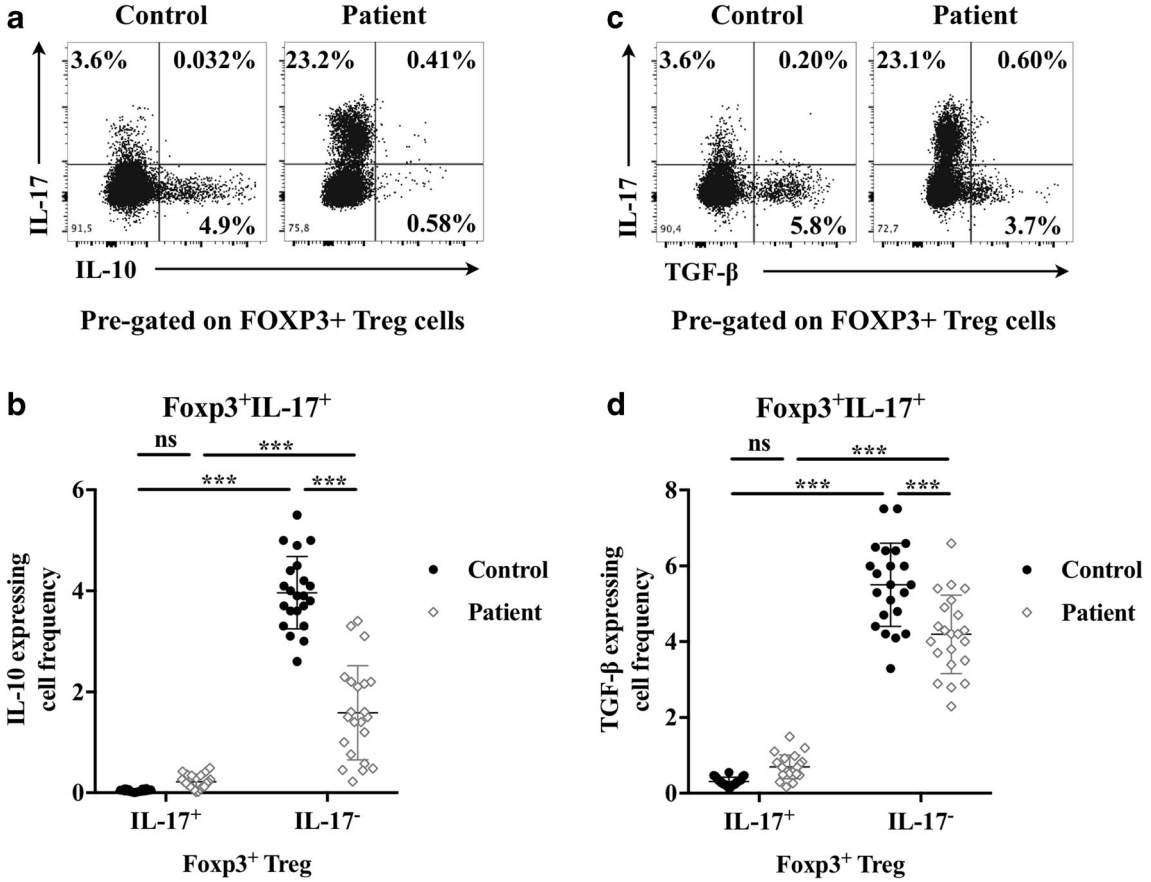

Fig. 2 IL-10 and TGF-β expression in IL-17$^-$ and IL-17$^+$ Foxp3$^+$ Treg cells from non-T2D controls and T2D patients. **a** IL-10 vs. IL-17 staining in pre-gated Foxp3$^+$ Treg cells, examined after anti-CD3/28 stimulation. One representative each from the controls and the patients is shown. **b** The frequencies of IL-10-expressing cells in IL-17$^+$ and IL-17$^-$ Foxp3$^+$ Treg cells in 22 controls and 22 patients. **c** TGF-β vs. IL-17 staining in pre-gated Foxp3$^+$ Treg cells, examined after anti-CD3/28 stimulation. One representative each from the controls and the patients is shown. **d** The frequencies of TGF-β-expressing cells in IL-17$^+$ and IL-17$^-$ Foxp3$^+$ Treg cells in 22 controls and 22 patients. Two-way ANOVA followed by Tukey's test. ns, not significant. ***$p < 0.001$

The frequencies of IL-17-expressing and IL-10-expressing Treg cells were associated with obesity and disease severity

Subsequently, we examined whether the cytokine expression characteristics of the Treg cells were associated with the clinical characteristics of the patients and the controls. First, the associations between the body mass index (BMI) of the patients and the controls and the frequency of IL-17, IL-10, and TGF-β-expressing Treg cells were examined. In T2D patients, the frequencies of IL-17-expressing Treg cells were positively correlated with the BMI (Fig. 3a). In non-T2D controls, a similar trend was observed, but the association was not statistically significant. The frequencies of IL-10-expressing Treg cells, on the other hand, inversely associated with the subjects' BMI in both non-T2D controls and T2D patients (Fig. 3b). No association between the frequencies of TGF-β-expressing cells and the subjects' BMI was found in either non-T2D controls or T2D patients (Fig. 3c).

In T2D patients, we also examined the association between Treg-mediated cytokine expression and glycated hemoglobin (HbA1c) level. The frequencies of IL-17-expressing Treg cells were positively correlated with HbA1c levels (Fig. 4a). The frequencies of IL-10-expressing Treg cells and TGF-β-expressing Treg cells, on the other hand, were not correlated with HbA1c levels (Fig. 4b, c).

Treg cells from T2D patients demonstrated lower suppression activity

Aside from cytokine expression, we also investigated the suppression activity of Treg cells in T2D patients and in non-T2D controls. Previous studies indicated that the CD4$^+$CD25^{hi}CD127$^-$ phenotype can be used to enrich for Foxp3$^+$ Treg cells [19]. For confirmation, CD4$^+$CD25^{hi}CD127$^-$ T cells and CD4$^+$CD25$^-$ T conventional (T_{conv}) cells were sorted from non-T2D controls and T2D patients, and the expression of Foxp3 was examined in each cell type. CD4$^+$CD25^{hi}CD127$^-$ T cells presented significantly

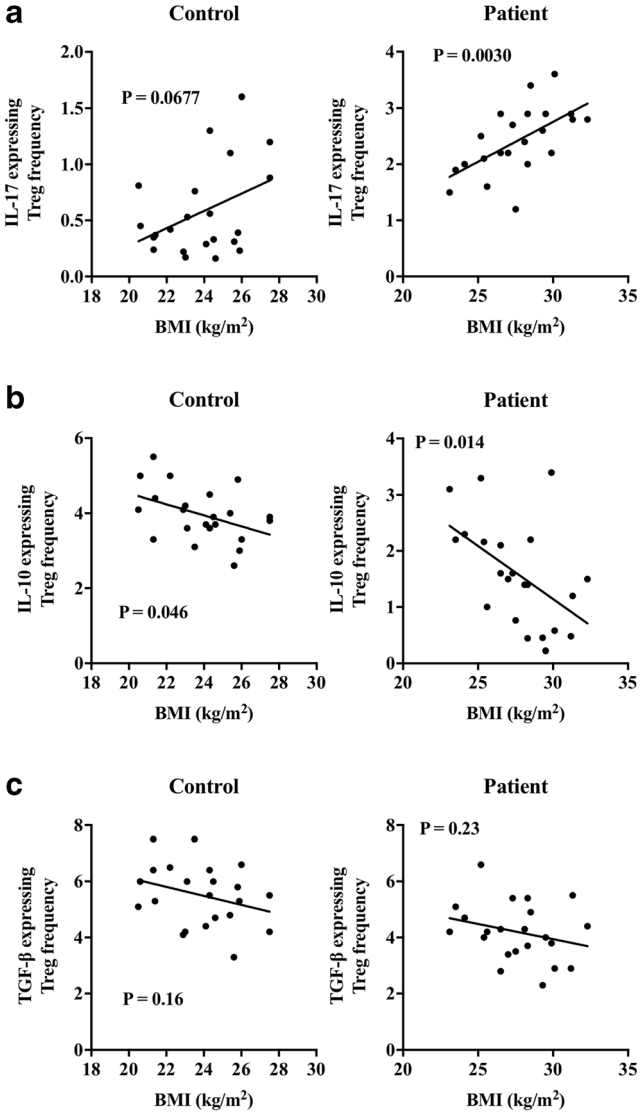

Fig. 3 Association between BMI and cytokine expression in Treg cells. **a** The association between BMI and IL-17-expressing Treg frequency. **b** The association between BMI and IL-10-expressing Treg frequency. **c** The association between BMI and TGF-β-expressing Treg frequency. Pearson correlation test

higher Foxp3 expression than T_{conv} cells (Fig. 5a). Subsequently, $CD4^+CD25^-$ T_{conv} cells from non-T2D controls and T2D patients were treated with anti-CD3/28 stimulation in the absence of $CD4^+CD25^{hi}CD127^-$ T cells or in the presence of increasing levels of $CD4^+CD25^{hi}CD127^-$ T cells. After a 72-h stimulation, a thymidine incorporation assay was performed to determine T_{conv} cell proliferation. The level of proliferation was decreased with increasing levels of $CD4^+CD25^{hi}CD127^-$ T cells (Fig. 5b). Interestingly, at 0 or low $CD4^+CD25^{hi}CD127^-$ T cell levels, the level of proliferation was comparable between non-T2D controls and T2D patients, but at high $CD4^+CD25^{hi}CD127^-$ T cell levels, the level of proliferation was significantly lower in non-T2D controls than in T2D patients. Overall, these data indicated that the suppressive activity of Treg cells was significantly lower in T2D patients than in non-T2D controls.

Discussion

Association between adiposity, obesity, and inflammation is increasingly recognized as a crucial player in T2D pathogenesis. $Foxp3^+$ Treg cells, as the master regulators of inflammation, are thought to suppress systemic inflammation to prevent exacerbation of the disease. However, in this study, we found that the $Foxp3^+$ Treg cells in T2D patients present several functional dysregulations. First, compared to $Foxp3^+$ Treg cells in control individuals, $Foxp3^+$ Treg cells in T2D patients expressed significantly higher levels of IL-17 and significantly lower levels of IL-10. Second, $CD4^+CD25^{hi}CD127^-$ T cells from T2D patients presented significantly lower suppression activity than $CD4^+CD25^{hi}CD127^-$ T cells from non-T2D controls. We further identified that the frequencies of IL-17-expressing $Foxp3^+$ Treg cells were positively correlated with the T2D patients' BMI and HbA1c level, while the frequencies of IL-10-expressing $Foxp3^+$ Treg cells were negatively correlated with BMI. In the past, many studies have shown that IL-17 overproduction can be a direct consequence of adipogenesis [20]. Adipocytes and macrophages in the adipose tissue may directly express IL-6, TNF, and IL-1β, which could promote IL-17 expression by CD4 T cells [21]. Hence, the upregulation of $IL\text{-}17^+$ Treg cells might have been a corollary of obesity. On the other hand, HbA1c level also positively correlated with $IL\text{-}17^+$ Treg frequency, which demonstrated that the bias toward IL-17 was directly associated with disease severity. It remains unclear whether $IL\text{-}17^+$ Treg cells directly participate in mediating insulin resistance and β cell damages. In the future, inhibition and removal of $IL\text{-}17^+$ Treg cells should be attempted to investigate the role of $IL\text{-}17^+$ Treg cells in T2D pathogenesis. At the same time, we observed that the level of $IL\text{-}10^+$ Treg cells was downregulated in T2D patients, which might also contribute to Treg impairment. To fully distinguish the effect of IL-10 downregulation and IL-17 upregulation in Treg cells, further animal studies would be required. Also, it should be investigated whether IL-17 upregulation could affect IL-10 expression by Treg cells.

IL-17 and Th17 cells have been implicated in the pathogenesis of various autoimmune diseases and proinflammatory disorders. In autoimmune type 1 diabetes (T1D), the peripheral blood CD4 T cell compartment is characterized by overwhelmingly strong IL-17 and Th17 responses [22]. In T2D patients, it is also found that the CD4 T cell compartment is enriched with IL-17-producing and IFN-γ-producing cells [23]. Similarly, IL-17-expressing Treg cells have been implicated in multiple inflammatory diseases. In the skin of human psoriasis

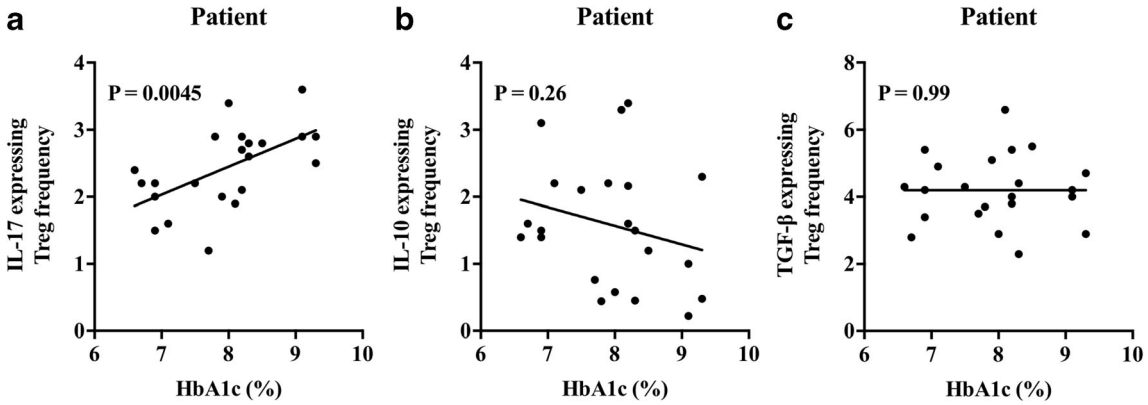

Fig. 4 Association between HbA1c and cytokine expression in Treg cells. **a** The association between HbA1c and IL-17-expressing Treg frequency. **b** The association between HbA1c and IL-10-expressing Treg frequency. **c** The association between HbA1c and TGF-β-expressing Treg frequency. Pearson correlation test

patients, $CD4^+Foxp3^+$ Treg cells presented high IL-17-expressing capacity [16, 24]. In rheumatoid arthritis, the level of IL-17-expressing Treg cells was significantly up-regulated [25]. IL-17- and IFN-γ-expressing Treg cells have also been found in the tumor microenvironment [26, 27]. The induction of these Treg cells is thought to be mediated by a Th17-inducing cytokine milieu. IL-1β, IL-6, and IL-23 are implicated in the induction of IL-17A from $Foxp3^+$ Treg cells [28]. IL-6-mediated STAT3 activation, together with RORγt and RORα, is required by Treg cells to express IL-17A [29]. Interestingly, IL-6 promotes PIM1 expression, a kinase that phosphorylates Ser422 in Foxp3 and negatively regulates Foxp3 activity [30]. In our study, we found that the IL-17-producing Treg cells and IL-10- or TGF-β-producing Treg cells were mutually exclusive populations. Hence, it appears that multiple subpopulations exist within the $Foxp3^+$ Treg cells. Further research is needed to examine the transcription and translation control factors that are dedicated to the cytokine expression in these subpopulations.

Interestingly, despite the production of proinflammatory cytokines, the IL-17-expressing Treg cells described in autoimmune diseases and cancer retained strong suppression activity [25–27], indicated by their capacity to suppress T_{conv} cell proliferation. In our study, we found that the Treg cells in T2D patients could suppress T_{conv} cell proliferation, but the efficacy was significantly reduced compared to the Treg cells in non-T2D controls. To what extent does Treg dysregulation impact T2D pathogenesis should be examined in future studies?

Overall, our data showed that in T2D patients, the Treg cells were dysregulated with higher IL-17-expressing cells,

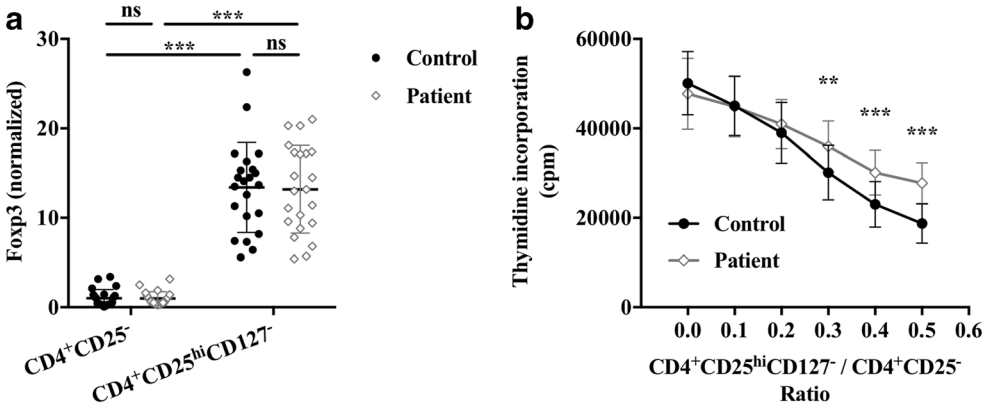

Fig. 5 The suppression activity of Treg cells in T2D patients and non-T2D controls. **a** The Foxp3 mRNA expression level in $CD4^+CD25^{hi}CD127^-$ T cells and $CD4^+CD25^-$ T_{conv} cells. The values were normalized to the average Foxp3 expression level by T_{conv} cells in non-T2D controls and T2D patients. **b** The thymidine incorporation level by $CD4^+CD25^-$ T_{conv} cells following coculture with $CD4^+CD25^{hi}CD127^-$ T cells at various ratios. $N = 22$ for both the non-T2D and the patient groups. Two-way ANOVA followed by Tukey's test. ns, not significant. $^{**}p < 0.01$. $^{***}p < 0.001$

lower IL-10-expressing cells, and lower suppression capacity. Furthermore, the extent of these dysregulations was associated with the BMI and the HbA1c levels, two parameters that were indicative of T2D disease severity. In the future, inhibition of IL-17 or removal of IL-17-producing Treg cells should be investigated to determine how Treg dysregulation could impact T2D pathogenesis.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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