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ORIGINAL ARTICLE

# The imbalance of Th17/Th1/Tregs in patients with type 2 diabetes: relationship with metabolic factors and complications

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**Abstract** Immune disorders are linked to the development of type 2 diabetes (T2D) and its complications. The relationship of $CD4^+CD25^{hi}$ T regulatory cells (Treg) and pro-inflammatory Th17 and Th1 subsets in T2D patients with metabolic disorders and complications need to be determined. The ratios of $CD4^+CD25^{hi}$ Treg/Th17 cells and $CD4^+CD25^{hi}$ Treg/Th1 cells, but not Th17/Th1 cells, were significantly decreased in T2D patients. The thymic output $CD4^+Foxp3^+Helios^+$ Tregs were normal but peripheral induced $CD4^+Foxp3^+Helios^-$ Tregs were decreased in T2D patients. The Bcl-2/Bax ratio decreased in $CD4^+CD25^{hi}$ Tregs in T2D patients, supporting the increased sensitivity to cell death of these cells in T2D. $CD4^+CD25^{hi}CD127^-$ Tregs in T2D patients with microvascular complications were significantly less than T2D patients with macrovascular complications. Importantly, $CD4^+CD25^{hi}CD127^-$ Tregs were positively correlated with plasma IL-6, whereas $IL\text{-}17^+CD4^+$cells were negatively related to high-density lipoprotein (HDL). Our data offered evidence for the skewed balance of anti- and pro-inflammatory T cell subsets in T2D patients and identified that HDL closely modulate T cell polarization. These results opened an alternative explanation for the substantial activation of immune cells as well as the development of T2D and complications, which may have significant impacts on the prevention and treatment of T2D patients.

**Keywords** Type 2 diabetes · $CD4^+CD25^+$ T regulatory cells · Th17 · Th1 · Immune tolerance · High-density lipoprotein

**Abbreviations**

| | |
|---|---|
| CPM | Counts per minute |
| FBS | Fetal bovine serum |
| FCM | Flow cytometry |
| FITC | Fluorescein isothiocyanate |
| Foxp3 | Forkhead box P3 |
| Th | T helper cells |
| mAbs | Monoclonal antibodies |
| MMC | Mitomycin C |
| PBMCs | Peripheral blood mononuclear cells |
| PE | Phycoerythrin |
| PI | Propidium iodide |
| Teff | T effector cells |
| PMA | Phorbol-12-myristate-13-acetate |
| Treg | T regulatory cells |
| T2D | Type 2 diabetes |
| CTL | Control |
| WBC | White blood cells |

Chun Zeng, Xiaoyun Shi, and Baojun Zhang contributed equally to this work and thus share the first authorship.

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| | |
|---|---|
| HbA1c | Hemoglobin A1c |
| HDL | High-density lipoprotein |
| LDL | Low-density lipoprotein |
| HLA | Human leucocyte antigen |

## Introduction

Type 2 diabetes (T2D) is characterized by insulin resistance and islet β-cell dysfunction with the progressive loss of insulin release being responsible for ever-increasing glucose concentrations without treatment. T2D was believed to be caused by insufficient insulin action due to obesity-induced insulin resistance and glucolipotoxicity in the liver and skeletal muscles [1]. However, today the pathogenesis of T2D was considered to be linked to both innate and adaptive immunity. It is reported patients with T2D displayed subclinical, low-grade systemic inflammation including increases in acute-phase proteins, cytokines, and mediators associated with endothelial activation, although the degree of immune activation is far below that in acute infections [2]. Polymorphism of some immune genes like HLA [3, 4], TNF-α [5], TNF-α receptor 80 [6], and TGF-β [7] have been reported to be associated with T2D. Monocytes from T2D patients display a pro-inflammatory profile and secrete elevated levels of IL-6, IL-8, TNF-α, and IL-1β [8–10]. Furthermore, low-grade systemic inflammation and immune disorders are linked to the development of diabetic complications as we recognized previously and also likely related to T2D pathogenesis itself [11, 12]. Recently, it was demonstrated that oligomers of islet amyloid polypeptide (IAPP), a protein that forms amyloid deposits in the pancreas during T2D, triggered the NLRP3 inflammasome and generated mature IL-1β, which links IAPP to immune disorders during T2D process [11, 13]. Obviously, the alteration of the immune system in T2D patients and its mechanisms were far not fully addressed.

$CD4^+$ T cells are generally subdivided into pro-inflammatory cells and anti-inflammatory sublineages. Recently, the critical role of immunosuppressive $CD4^+CD25^{hi}$ T regulatory cells (Treg) in controlling the activation of T effector cells (Teff) and innate immune cells was demonstrated [14]. On the other hand, the newly defined inflammatory Th17 subset has emerged as crucial players in infection, inflammation, autoimmune diseases, and cancer [15]. The involvement of adipose tissue T cells in T2D patients was reported [16–18]. Very importantly, It was demonstrated in elegantly designed experiments that $CD4^+$ T cells, especially Th1 and $CD4^+CD25^{hi}$ Tregs, in visceral adipose tissue play a fundamental role in the regulation of body weight, adipocyte hypertrophy, insulin resistance, glucose tolerance and T2D progression in mice and humans [19, 20]. These studies showed that an elevation in the Th17 and Th1 subsets with a decrease in the $CD4^+CD25^{hi}$ Tregs culminates in inflammation and insulin resistance [19, 20]. More recently, it was reported that T2D patients are skewed toward pro-inflammatory subsets [21]. However, the relationship of the alteration of peripheral $CD4^+$ T cell subsets including Th1, Th17, and $CD4^+CD25^{hi}$ Tregs in T2D patients with metabolic disorders of T2D such as hyperglycemia and dyslipidemia as well as the occurrence of diabetic complications urgently needs to be identified. In the present study, significantly reduced $CD4^+CD25^{hi}$ Treg/Th17 and $CD4^+CD25^{hi}$ Treg/Th1 ratios were detected in T2D patients, while the ratio of Th17/Th1 cells was normal. The percentage of $CD4^+CD25^{hi}$ Tregs in T2D patients with microvascular complications was significantly lower than T2D patients with macrovascular complications or none. Importantly, the levels of $CD4^+CD25^{hi}$ Tregs were positively correlated with IL-6 while Th17 negatively related to plasma high-density lipoprotein (HDL). Thus, this study offered, for the first time to our knowledge, the strong evidence for the relationship of hyperglycemia and dyslipidemia with the alteration of $CD4^+$ T cell subsets in T2D patients. The strikingly decreased $CD4^+CD25^{hi}$ Tregs to Th17 or Th1 ratios in T2D patients may contribute to the chronic low-degree activation of innate immunity and, subsequently, the further development and complications of T2D. The present study opened one potential approach to preventing the development of diabetic complications by regulating host $CD4^+CD25^{hi}$ Tregs and Th17 cells, which may have significant impacts on the prevention and treatment of T2D and diabetic complications in clinics.

## Materials and methods

### T2D patients and healthy control subjects

Fresh peripheral blood samples from 181 patients with T2D and 117 control subjects were collected at the General Hospital of Chinese People's Armed Police Forces. Age-matched normoglycemic control subjects were normal of fasting blood glucose, lacked family history of T2D as well as no clinical diagnosed diseases. Patients were diagnosed as T2D according to the standard criteria, and their baseline clinical parameters were summarized in Table 1. This study was cross-sectional and blinded. The study was approved by the ethics committee of the hospital and institution.

### Monoclonal antibodies and reagents

Phycoerythrin (PE)-CY5-anti-hCD4 mAb (PRA-T4), PE-anti-hFoxp3 (PCH101) mAb, PE-anti-hCD45RO mAb (UCHL1), PE-anti-CD127 mAb (RDR5), fluorescein isothiocyanate (FITC)-anti-hIL-17 mAb (DEC17), FITC-anti-CD45RA

**Table 1** Demographic features of controls and patients

| | Control | T2D patients |
|---|---|---|
| Patient number | 117 | 181 |
| Age (years) | 51.81±1.32 (24–81) | 59.59±1.01 (28–85) |
| Sex (female/male) | 0.58 (43/74) | 0.6(68/113) |
| TG | 1.42±0.19 (0.5–4.29) | 1.74±0.09 (0.38–4.72) |
| CH | 5.4±0.19 (3.7–7.87) | 4.66±0.1 (2.61–7.92) |
| HDL | 1.3±0.36 (0.99–1.7) | 1.03±0.21 (0.43–1.6) |
| LDL | 3.3±0.15 (1.94–5.41) | 2.85±0.09 (1.24–5.94) |
| FBG (mM) | 5.15±0.07 (4.34–6.04) | 8.57±0.34 (3.46–31.22) |
| 2 h PBG (mM) | n.a. | 16.56±0.83 (6.62–26.17) |
| HbA1c (%) | n.a. | 8.42±0.18 (4.6–13.3) |
| ICA | | Negative |
| GAD65/A[A] | | Negative |
| IA-2A[B] | | Negative |
| Duration(years) | | 9.13±0.63 (0.1–40) |

*TG* triglyceride, *CH* cholesterol, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein, *FBG* fasting blood glucose, *PBG* postprandial blood glucose, *HbA1c* hemoglobin A1c, *ICA* islet-cell antibodies, *GAD65/AA* anti-glutamic acid decarboxylase 65KD, *IA-2AB* tyrosine phosphatase-like protein IA-2, *n.a.* not available; data were shown as mean+SEM

mAb (Hi100), FITC-anti-hCD25 mAb (M-A251), and PE-anti-hIFN-γ mAb (4S.B3) were purchased from eBioscience (San Diego, CA). PE-anti-CD25 mAb (BC96), FITC-anti-helios mAb (22F6), and isotype control monoclonal antibodies (mAbs) were purchased from Biolegend. FITC-anti-Bcl-2 mAb and FITC-anti-Bax mAb were purchased from Santa Cruz. FITC-anti-Annexin V mAb were purchased from Baosai (Beijing, China).

Mitomycin C ($C_{15}H_{18}N_4O_5$ and MMC) was obtained from Kyowa Hakko Co, Ltd. (Tokyo, Japan). [$^3$H]thymidine was purchased from China institute of atomic energy (Beijing, China). The culture medium used in this study was RPMI 1640 (Hyclone, Logan, UT) supplemented with 10% heat-inactivated FCS, 100 U/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, 10 mM HEPES, and 50 μM 2-ME (Sigma, St. Louis, MO).

## Immunofluorescence staining and flow cytometry

Peripheral blood mononuclear cells (PBMCs) from controls and patients were isolated by ficoll-hypaque gradient centrifugation (Amersham). After washed twice with ice-cold flow cytometry (FCM) buffer (PBS with 0.1% BSA and 0.1% $NaN_3$), $2\times10^5$ PBMCs were re-suspended in 100 μL buffer and incubated with mAbs of cell surface molecules for 30 min at 4°C in the dark. Cells were washed twice and assayed using a FASCalibur flow cytometry (Becton Dickinson, CA). The data were analyzed with CellQuest software.

For intracellular staining, cells were treated with 1× permeabilization buffer (Biolegend) and stained with Bcl-2 or Bax mAb. For Foxp3 staining, cells were fixed and permeabilized with fix/Perm solution (eBioscience) and stained with Foxp3 mAb, according to the instruction provided by the manufacturer (eBioscience, San Diego, CA). Negative control fluorescence was assessed using the isotype-matched control mAb.

## Cytokine measurement

For intracellular cytokine staining, PBMCs were incubated with 100 ng/mL PMA, 1μg/mL Ionomycin and Golgiplug for 6 h. After harvest, cells were stained as described above. Assessments of plasma cytokine profiles were performed using human Th1/Th2/Th17 cytometric bead array kit, according to the instruction provided by the manufacturer (BD Biosciences PharMingen).

## Isolation of $CD4^+CD25^{+/hi}$ and $CD4^+CD25^-$ T cells

$CD4^+$ T cells were isolated from PBMCs by negative selection using magnetic cell sorting technology (MACS; Miltenyi Biotech, Germany). $CD4^+$ T cells were separated into $CD25^+$ and $CD25^-$ populations by MACS, using anti-CD25 microbeads (Miltenyi biotech). The isolation of $CD25^{+/hi}$ cells was optimized by varying the duration and bead concentration during purification. In some cases, the $CD4^+CD25^{hi}$ and $CD4^+CD25^-$ populations were sorted by FCM. The purity of enriched $CD4^+CD25^{+/hi}$ and $CD4^+CD25^-$ T cells was approximately 93% and 99%, respectively, as determined by FCM.

## $CD4^+CD25^{+/hi}$ Treg cell immunosuppressive assay

The immunosuppressive ability of $CD4^+CD25^+$ Tregs was determined using classic in vitro Treg and Teff co-culture system. Briefly, sorted $CD4^+CD25^-$ ($5\times10^3$/well) Teff cells were co-cultured with autogenic $CD4^+CD25^+$ Tregs at various ratios (1:0, 1:1, 1:1/2, 1:1/4, and 1:1/8) in the

presence of $5\times10^4$ MMC-pretreated autogenic accessory cells (25 mg/mL MMC at 37°C for 30 min). These cells were stimulated with plate-bound anti-CD3 (clone UCHT1; plates were pre-incubated with 100 μL/well PBS that contained 5 μg/mL anti-CD3 antibody overnight at 4°C and then washed twice in PBS) and soluble anti-CD28 (clone CD28.2; 1 μg/mL) antibodies (BD Pharmingen). On day 4 of culture, 0.5 μCi of [$^3$H]thymidine was added for the final 18 h of culture before harvesting. Cells were recovered with an automatic well recover (Tomtec, Toku, Finland). The radioactivity of each sample was assayed in a Liquid Scintillation Analyzer (Beckman Instruments, Fullerton, CA). Values are expressed as counts per minute (cpm) of triplicate wells. The percentage of suppression was determined as: (1-cpm in the Treg and Teff cell co-culture/cpm of Teff alone)×100%.

### Statistical analysis

Data analysis was performed by using SPSS version 13.0. Data are mainly presented as the mean±SEM. Student's unpaired *t* test was used for comparisons of group means as indicated in the figure legends. A Pearson test was used to identify correlations for all applicable panels. A *P* value of less than 0.05 was considered to be statistically significant.

## Results

### Significantly decreased functional $CD4^+CD25^{hi}$ Tregs in T2D patients

In order to get the basic data on the levels of immune cells in patients with T2D, we firstly assayed the counts of white blood cells (WBCs) in the peripheral blood. As shown in Fig. 1 in the Electronic supplementary material (ESM), significantly enhanced WBC counts were detected in patients with T2D compared with the age-matched healthy subjects, regardless of the duration of T2D ($P<0.001$; Fig. 1a, b in the ESM). Strikingly, the percentage and cell number of neutrophils were increased in the peripheral blood of patients with T2D, whereas the percentage of lymphocytes was significantly decreased ($P<0.001$; Fig. 1c, d in the ESM), suggesting significant alteration of the subpopulation of immune cells in the periphery of patients with T2D. The enhanced neutrophil levels in the blood of T2D patients is somewhat consistent with the increased numbers of polymorphonuclear leukocytes in the retina of diabetic monkeys [22].

$CD4^+CD25^+$ Tregs represent one of the important subsets of Tregs, which play a key role in preventing autoimmunity and immune diseases. In humans, $CD4^+CD25^{hi}$ T cells are more likely to be Tregs than $CD4^+CD25^{low}$ T cells. As shown in Fig. 1, though the percentage of $CD4^+$ T cells in the peripheral blood of T2D patients was unchanged ($P>0.05$; Fig. 1a), the percentage of $CD4^+CD25^{hi}$ T cells was significantly decreased ($P<0.001$; Fig. 1b, c). The level of $CD4^+CD25^{low}$ T cells, which likely are not Tregs, did not show detectable changes ($P>0.05$; Fig. 1e, f in the ESM). To further confirm whether these $CD4^{\pm}CD25^{hi}$ T cells are Tregs, we detected the expression of Foxp3 and CD127, which are critical markers for Tregs [23], by multi-color FCM. The majority of $CD4^{\pm}CD25^{hi}$ T cells did not express CD127 but expressed Foxp3, as previously reported (Fig. 1d; Fig. 2a in the ESM) [24]. Importantly, the percentage and cell number of $CD4^+CD25^{hi}CD127^-$ T cells were significantly decreased in patients with T2D compared with the age-matched healthy subjects ($P<0.01$; Fig. 1e, f). Identical results for $CD4^{\pm}CD25^{hi}Foxp3^{\pm}$ T cells in T2D patients were observed (Fig. 2b, c in the ESM).

The function of the sorted $CD4^+CD25^{hi}$ Tregs isolated from either T2D patients or healthy volunteers was determined using a classic immunosuppressive assay. As shown in Fig. 1g, $CD4^+CD25^{hi}$ Tregs of T2D patients or healthy control dose-dependently inhibited the proliferative response of Teff cells in a similar manner, indicating that $CD4^+CD25^{hi}$ Tregs in diabetes patients are functional without detectable alteration. Therefore, peripheral functional $CD4^+CD25^{hi}$ Tregs were significantly decreased in patients with T2D.

### The peripheral decreased $CD4^+CD25^{hi}$Tregs was due to the impaired survival ability but not the thymic output in T2D patients

Peripheral $CD4^+CD25^{hi}$ Tregs include $CD4^+Foxp3^+Helios^+$ Tregs which may be mainly developed in the thymus and $CD4^+Foxp3^+Helios^-$ Tregs mainly differentiated in the periphery of mice and humans [25], although the review that the transcription factor Helios is used as a marker for discriminating thymic-derived natural Tregs from induced Tregs has recently been challenged by others [26]. To find whether the peripheral decreased $CD4^+CD25^{hi}$ Tregs was due to the poor thymic output or/and impaired peripheral induction/survival in T2D patients, we assayed the $CD4^+Foxp3^+Helios^+$ Tregs in these patients. As shown in Fig. 2, the percentage of thymic-derived $CD4^+Foxp3^+Helios^+$ Tregs in the peripheral blood of T2D patients did not show detectable changes compared with the age-matched healthy subjects ($P>0.05$; Fig. 2a, b), but the percentage of inducing $CD4^+Foxp3^+Helios^-$ Tregs was significantly decreased in T2D patients ($P<0.01$; Fig. 2c), indicating that the decreased peripheral $CD4^+CD25^{hi}$ Tregs was caused by the peripheral components but not by the thymic output of $CD4^+CD25^{hi}$ Tregs in T2D patients.

The peripheral pool of $CD4^+CD25^{hi}$ Tregs was elegantly maintained by the induction, proliferation and survival of these cells except of its income from the thymus. As FOXP3

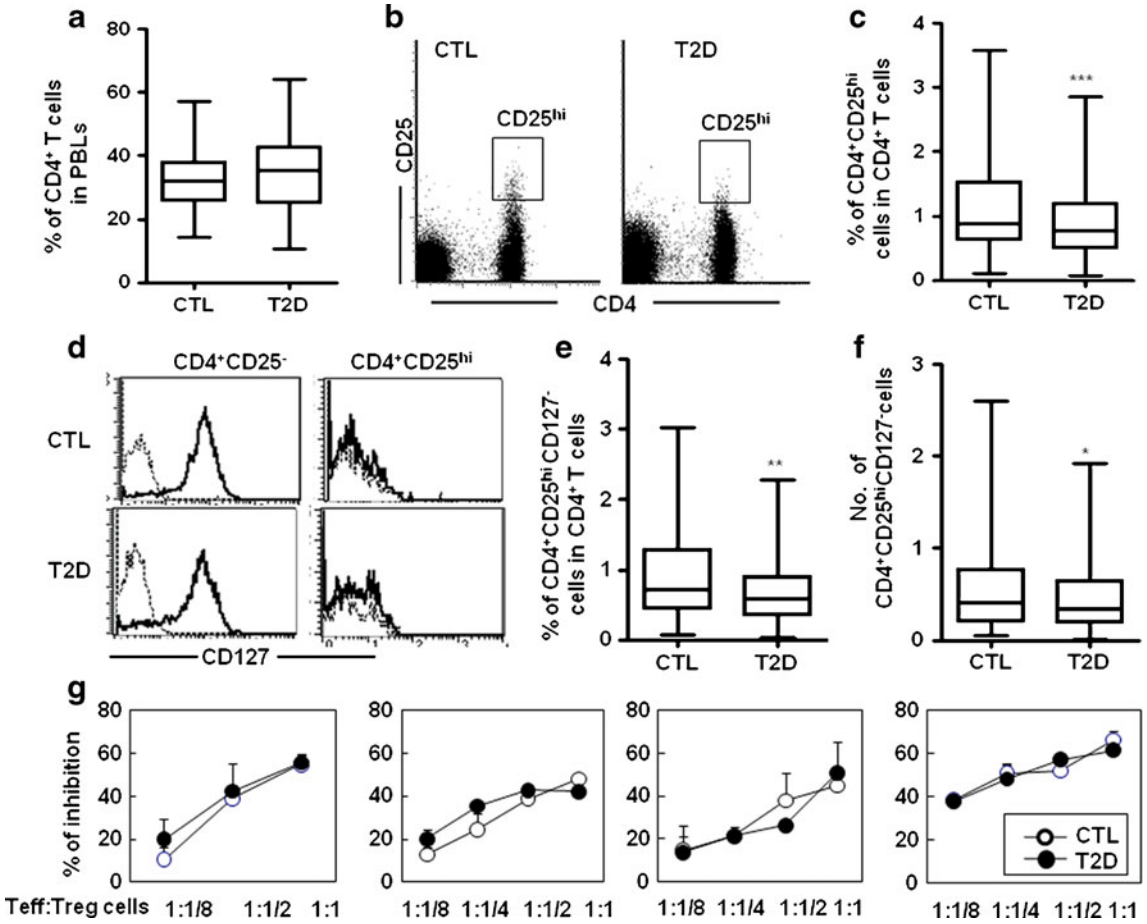


**Fig. 1** Significantly decreased functional $CD4^+CD25^{hi}$ T cells in peripheral blood of patients with T2D. 117 healthy subjects (*CTL*) and 181 patients with T2D were assayed. **a** Percentage of $CD4^+$ T cells in peripheral blood of patients with T2D. **b** Representative FACS staining for $CD25^{hi}$ in gated $CD4^+$ T cells. **c**. Percentage of $CD4^+CD25^{hi}$ T cells in peripheral blood. **d** One representative for staining of anti-CD127 in the gated $CD4^+CD25^{hi}$ cells. **e** Percentage of $CD4^+CD25^{hi}CD127^-$ T cells in peripheral blood. **f** Cell number of $CD4^+CD25^{hi}CD127^-$ T cells in peripheral blood. **g** The immunosuppressive ability of enriched $CD4^+CD25^{hi}$ T cells from four CTL and four patients were determined in vitro using the classic assays as described in the materials and methods. Data were shown as mean±SEM. $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$ compared with CTL

expression is not restricted to Tregs but can be transiently induced by a significant proportion of Teff cells after TCR activation [24], it is hard for us to directly investigate the possible alteration of $CD4^+CD25^{hi}$ Treg induction from naïve $CD4^+$ Teffs isolated from T2D patients in vitro. We thus compared the molecule expression of anti-apoptosis and

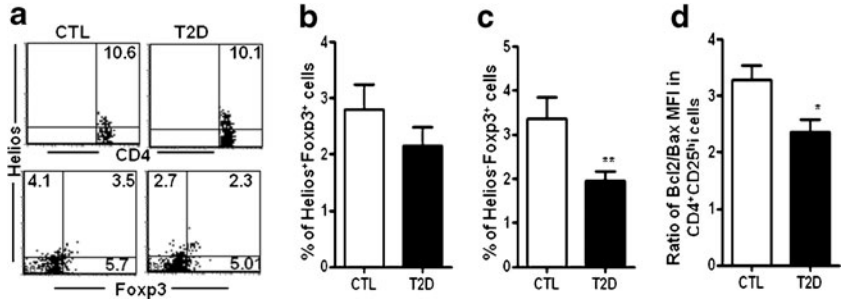


**Fig. 2** The peripheral decreased $CD4^+CD25^{hi}$ Tregs was due to the impaired survival ability but not the thymic output in T2D patients. **a** Representative FACS staining for Helios and Foxp3 in gated $CD4^+$ T cells. **b** The percentages of $Helios^+Foxp3^+$iTregs in $CD4^+$ T cells from CTL ($n=3$) and T2D patients ($n=4$). **c** The percentages of $Helios^-Foxp3^+$nTregs in $CD4^+$ T cells from CTL($n=3$) and T2D patients($n=4$). **d** Bcl-2 and Bax expression levels of $CD4^+CD25^{hi}$ cells obtained from CTL ($n=10$) and T2D patients ($n=10$). The ratios of Bcl-2/Bax mean fluorescence intensity (*MFI*) are above the histograms. Data were shown as mean±SEM. $^*P<0.05$ compared with CTL

apoptosis genes in $CD4^+CD25^{hi}$ Tregs of control and T2D patients in purpose to address whether peripheral $CD4^+CD25^{hi}$ Tregs in T2D patients are more sensitive to cell death than control. The balance of Bcl-2 and Bax controls the sensitivity to cell death. The ratio of Bcl2/Bax in $CD4^+CD25^{hi}$ Tregs of T2D patients was significantly lower than those in control subjects ($P<0.05$; Fig. 2d). Thus, the increased susceptibility to cell death of $CD4^+CD25^{hi}$ Tregs in T2D may be one of the reasons for the decreased $CD4^+CD25^{hi}$ Tregs in these patients.

## Strikingly decreased ratios of $CD4^+CD25^{hi}$ Tregs to Th17 or Th1 cells in T2D patients

The balance between $CD4^+CD25^{hi}$ Tregs and inflammatory T subsets such as Th1 and Th17 cells is critical for the immune homeostasis and response. We determined the levels of Th1 and Th17 cells in T2D patients in addition to $CD4^+CD25^{hi}$ Tregs. As shown in Fig. 3, significantly higher levels of IL-$17^+CD4^+$ and IFN-$\gamma^+CD4^+$ cells in the peripheral blood of T2D patients were detected compared with healthy subjects (Fig. 3a–c). The ratio of IL-$17^+CD4^+$ cell and IFN-$\gamma^+CD4^+$ cells in T2D patients was similar as control ($P>0.05$; Fig. 3d), but the ratios of $CD4^+CD25^{hi}CD127^-$ Tregs/IL-$17^+CD4^+$ cells and $CD4^+CD25^{hi}CD127^-$ Tregs/IFN-$\gamma^+CD4^+$ cells were strikingly decreased in T2D patients ($P<0.001$; Fig. 3e, f). Similar results were observed in regards to the ratios of $CD4^+CD25^{hi}Foxp3^+$ Tregs/IL-$17^+CD4^+$ cells and $CD4^+CD25^{hi}Foxp3^+$ Tregs/IFN-$\gamma^+CD4^+$ cells in T2D patients ($P<0.001$; Fig. 3a, b in the ESM). These data strongly indicate that the remarkable imbalance of $CD4^+$ T subsets presents in T2D patients.

## Correlations between $CD4^+CD25^{hi}CD127^-$ Tregs, Th17 or Th1 cells with the T2D complications and duration

Diabetic complications include microvascular complications, such as retinopathy, neuropathy and nephropathy, and macrovascular complications, including cardiovascular, peripheral vascular disease and cerebrovascular diseases [27]. To investigate the potential relationship between $CD4^+$ T cell subset alteration with the types of diabetic complications, we compared the levels of $CD4^+CD25^{hi}CD127^-$ Tregs, Th17 and Th1 cells in T2D patients with or without different types of complications. As shown in Fig. 4, the percentage of $CD4^+CD25^{hi}CD127^-$ Tregs in T2D patients with microvascular complications was significantly lower than healthy subjects and T2D patients with macrovascular complications ($P<0.05$ or 0.01; Fig. 4a). The percentage of $CD4^+CD25^{hi}$-$Foxp3^+$ Tregs in T2D patients were markedly reduced, too ($P<0.001$; Fig. 4a in the ESM). The percentages of IL-$17^+CD4^+$ T cells and IFN-$\gamma^+CD4^+$ T cells were significantly increased in T2D patients than the age-matched healthy subjects, regardless whether these patients with complications or not (Fig. 4b, c). Impressively, the ratios of $CD4^+CD25^{hi}CD127^-$ Tregs/IL-$17^+CD4^+$cells and $CD4^+CD25^{hi}CD127^-$ Tregs/

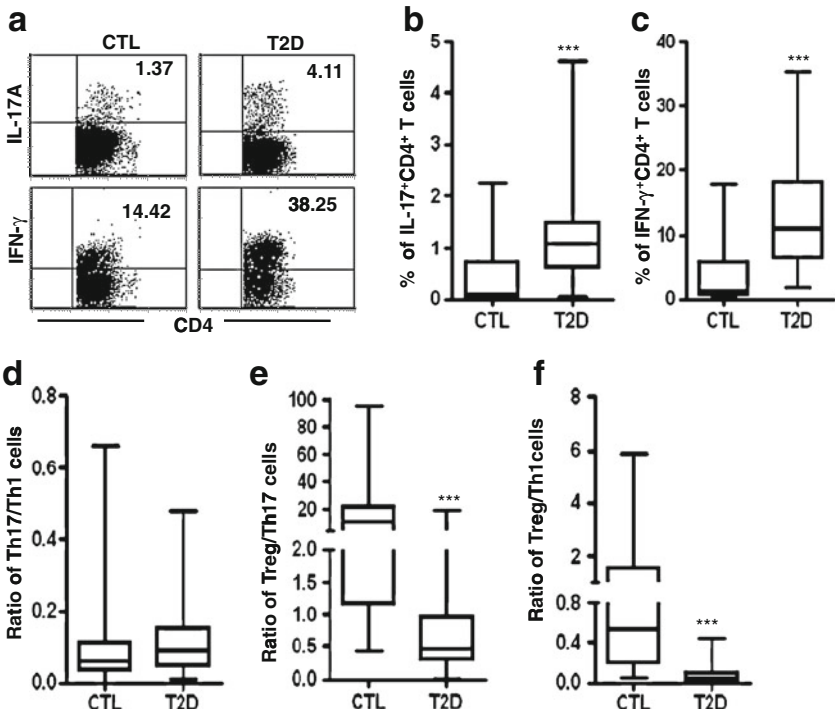


**Fig. 3** Elevated production of IL-17A and IFN-γ by PBMCs from T2D patients. PBMCs from control ($n=64$) or T2D ($n=58$) donors were stimulated by PMA and inomomycin for 6 h, and analyzed IL-17A and IFN-γ by FCM. **a** Representative FACS staining for IL-17A and IFN-γ in gated $CD4^+$ T cells. **b** The percentages of IL-17A in $CD4^+$ T cells. **c** The percentages of IFN-γ in $CD4^+$ T cells. The ratio of Th17/Th1 (**d**), Treg/Th17 (**e**), and Treg/Th1 (**f**) were shown as mean±SEM. ***$P<0.001$ compared with CTL

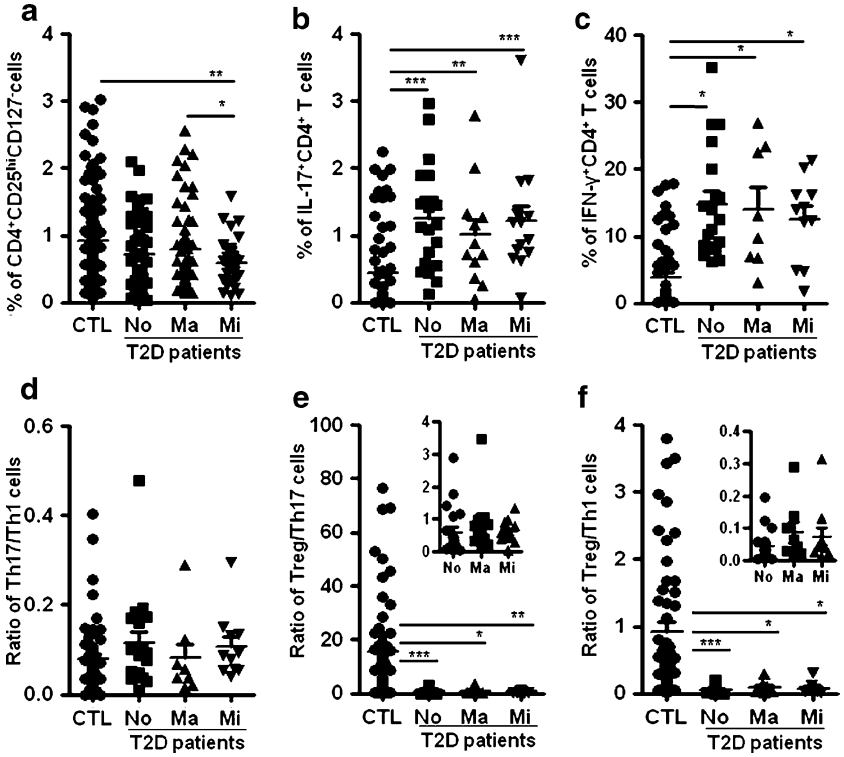


**Fig. 4** The correlations between $CD4^+CD25^{hi}$ $CD127^-$ Tregs, Th17, and Th1 cells with the T2D complications. Percentages of $CD4^+CD25^{hi}CD127^-$ T cells (**a**), IL-17A (**b**), and IFN-γ (**c**) in $CD4^+$ T cells and the ratio of Th17/Th1 (**d**), Treg/Th17 (**e**), and Treg/Th1 (**f**) in peripheral blood of diabetes patients with different complications. *$P<0.05$; **$P<0.01$; ***$P<0.001$ compared with indicated groups

$IFN\text{-}\gamma^+CD4^+$ cells, but not $IL\text{-}17^+CD4^+$ cells/$IFN\text{-}\gamma^+CD4^+$ cells, were strikingly decreased in T2D patients in all groups ($P<0.001$; Fig. 4d, f).

On the other hand, the decreased percentage of $CD4^+CD25^{hi}CD127^-$ Tregs and the enhanced percentages of $IL\text{-}17^+CD4^+$ cells and $IFN\text{-}\gamma^+CD4^+$ cells in T2D patients were detected in T2D patients, no matter they suffered from diabetes for <5 years or >10 years (Fig. 5a–c). The decreased ratios of $CD4^+CD25^{hi}CD127^-$ Tregs/$IL\text{-}17^+CD4^+$ cells and $CD4^+CD25^{hi}CD127^-$ Tregs/$IFN\text{-}\gamma^+CD4^+$ cells occurred no

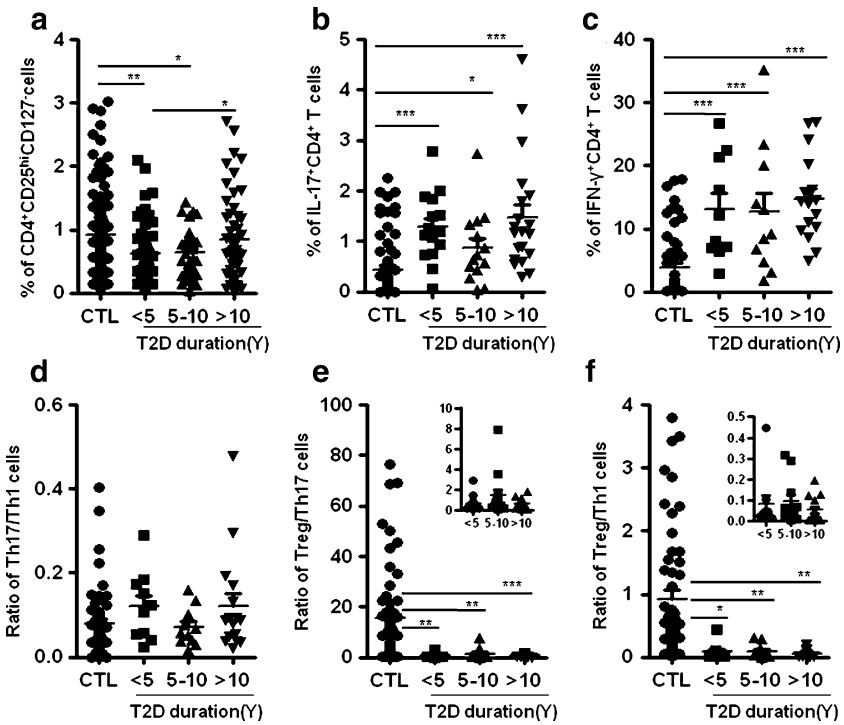


**Fig. 5** The correlations between $CD4^+CD25^{hi}$ $CD127^-$ Tregs, Th17, and Th1 cells with the T2D duration. Percentages of $CD4^+CD25^{hi}CD127^-$ T cells (**a**), IL-17A (**b**), and IFN-γ (**c**) in $CD4^+$ T cells and the ratio of Th17/Th1 (**d**), Treg/Th17 (**e**), and Treg/Th1 (**f**) in peripheral blood of diabetes patients with different duration. *$P<0.05$; **$P<0.01$; ***$P<0.001$ compared with indicated groups

matter how long the patient suffered from T2D, while the ratio of IL-17$^+$CD4$^+$cells/IFN-γ$^+$CD4$^+$ cells in T2D patients were kept as healthy control ($P$>0.05; Fig. 5d, f). Identical results were observed in regards to the percentage of CD4$^+$CD25$^{hi}$-Foxp3$^+$ Tregs in T2D patients ($P$<0.001; Fig. 4b in the ESM). These data suggest that the changes of CD4$^+$ T cell subsets in T2D patients may happen before the occurrence of complications and at the early stage of diabetes.

## Peripheral CD4$^+$CD25$^{hi}$ Treg, Th17, and Th1 cells are not correlated with blood HbA1c levels in T2D patients

T2D is a group of metabolic conditions characterized by abnormally increased levels of blood glucose due to impairment in insulin action and/or insulin secretion [28]. HbA1c is the most common marker used in the clinic to determine the control of hyperglycemia, one of the key pathophysiological changes for T2D. We evaluated the correlations of CD4$^+$ T cell subset imbalance with the levels of blood HbA1c. The percentages of CD4$^+$CD25$^{hi}$CD127$^-$ Tregs, IL-17$^+$CD4$^+$ cells, and IFN-γ$^+$CD4$^+$ cells as well as the ratios of CD4$^+$CD25$^{hi}$CD127$^-$ Tregs/IL-17$^+$CD4$^+$ cells, CD4$^+$CD25$^{hi}$CD127$^-$ Tregs/IFN-γ$^+$CD4$^+$ cells, and IL-17$^+$CD4$^+$ cells/IFN-γ$^+$CD4$^+$ cells were not correlated with the blood HbA1c in T2D patients ($p$>0.05; Fig. 5a–c in the ESM). In addition, the percentages of CD4$^+$CD25$^{hi}$Foxp3$^+$ Tregs were also not related to blood HbA1c ($P$>0.05; Fig. 5d, f in the ESM). In addition, the percentages of CD4$^+$CD25$^{hi}$Foxp3$^+$ Treg cells were also not related to blood HbA1c ($P$>0.05; Fig. 6a in the ESM). Thus, hyperglycemia seems not to modulate the balance of immunosuppressive CD4$^+$CD25$^{hi}$ Tregs and inflammatory IL-17$^+$CD4$^+$ cells and IFN-γ$^+$CD4$^+$ cells in humans.

## The relevance of CD4$^+$CD25$^{hi}$ Treg, Th17, and Th1 cells to dyslipidemia in T2D patients

T2D usually displayed accumulation of triacylglycerol and fatty acid metabolites, lowering HDL, with hyperglycemia due to the impaired response to insulin and β-cell dysfunction. The relevance of CD4$^+$ T cell subset imbalance to dyslipidemia (such as hypertriglyceridemia and low HDL cholesterol) was evaluated. In T2D patients, the levels of CD4$^+$CD25$^{hi}$CD127$^-$ Tregs, IFN-γ$^+$CD4$^+$ cells, and CD4$^+$CD25$^{hi}$Foxp3$^+$ Tregs were unrelated with blood HDL levels ($P$>0.05; Fig. 6a, b; Fig. 6b in the ESM), but the percentages of IL-17$^+$CD4$^+$ cells were significantly related to the blood HDL levels in a negative manner ($P$<0.05; Fig. 6c). The ratios of IL-17$^+$CD4$^+$ cells/IFN-γ$^+$CD4$^+$ cells, but not CD4$^+$CD25$^{hi}$CD127$^-$ Tregs/IL-17$^+$CD4$^+$ cells and CD4$^+$CD25$^{hi}$CD127$^-$ Tregs/IFN-γ$^+$CD4$^+$ cells, were also negatively related with blood HDL levels in T2D patients ($P$<0.05; Fig. 6d, f). However, All these CD4$^+$ T cell subsets were not meaningfully correlated with the blood triglyceride, cholesterol and low-density lipoprotein (LDL), respectively ($P$>0.05; Fig. 7 in the ESM). Therefore, these data support that among triacylglycerol and fatty acid metabolites, HDL is the key mediator which negatively regulates inflammatory Th17 subpopulation in T2D patients.

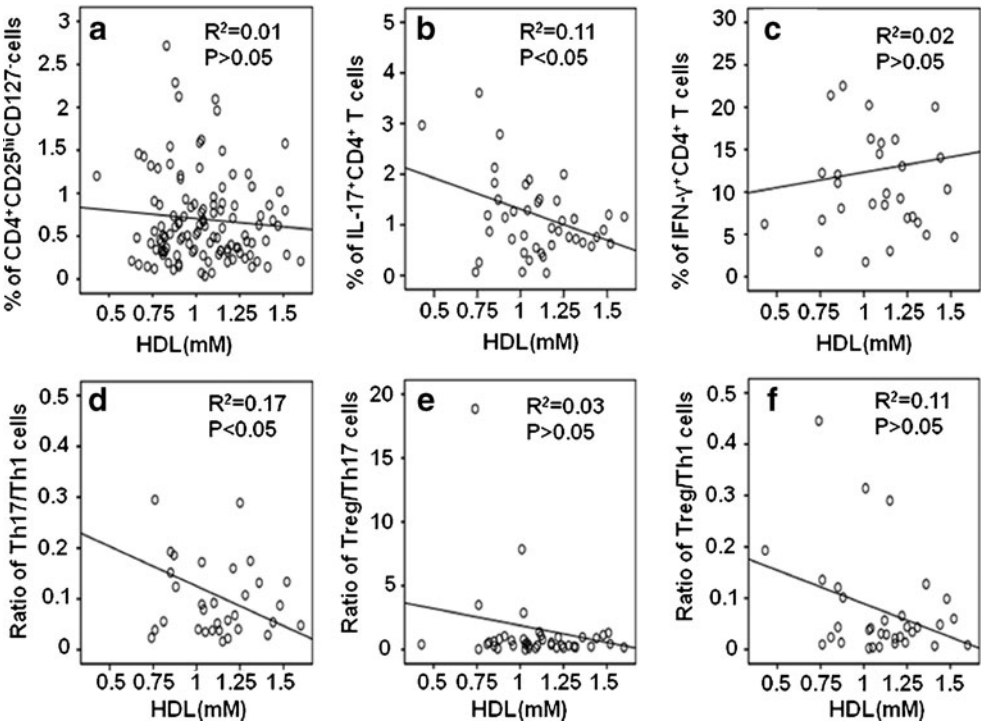


**Fig. 6** The correlations between CD4$^+$CD25$^{hi}$ CD127$^-$ Tregs, Th17, and Th1 cells with HDL in patients with T2D. Relationship between the percentage of CD4$^+$CD25$^{hi}$CD127$^-$ T cells (**a**), IL-17A (**b**), and IFN-γ (**c**) with the levels of HDL in T2D patients. The relationship between the ratio of Th17/Th1, Treg/Th17, and Treg/Th1 with the levels of HDL in T2D patients is shown in (**d**–**f**). The $P$ value and $R^2$ were indicated in the graphs

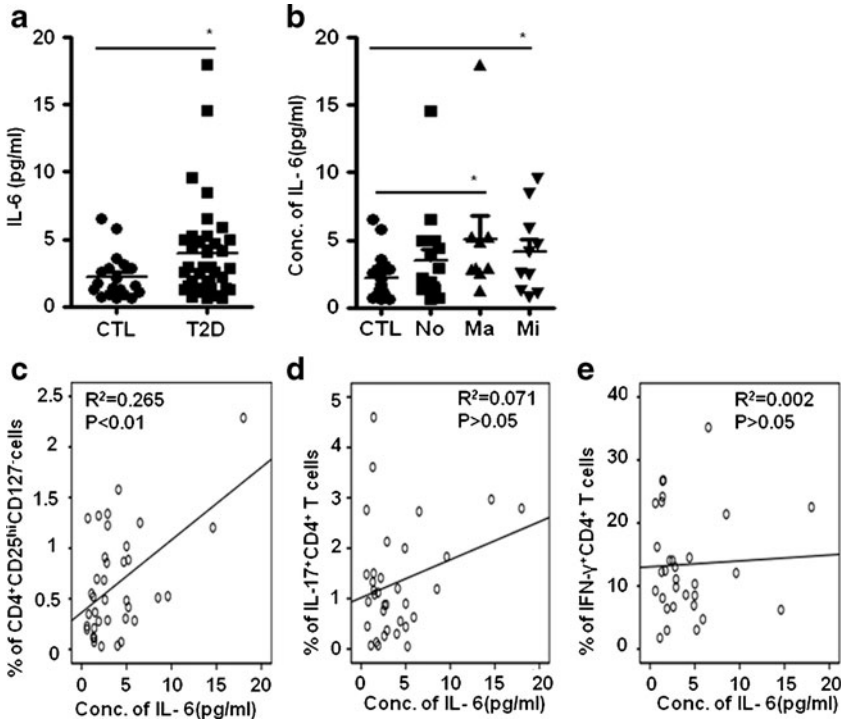


**Fig. 7** $CD4^+CD25^{hi}$ Tregs were positively related to the plasma IL-6 levels in T2D patients. **a** IL-6 concentration from CTL ($n$=19) and T2D ($n$=38) donors were detected by human Th1/Th2/Th17 cytometric bead array kit. **b** IL-6 concentration in peripheral blood of diabetes patients with different complications. **c** Relationship between the percentage of $CD4^+CD25^{hi}CD127^-$ T cells with the concentration of IL-6 in T2D patients. **d** Relationship between the percentage of IL-17A cells with the concentration of IL-6 in T2D patients. **e** Relationship between the percentage of IFN-γ cells with the concentration of IL-6 in T2D patients. The $P$ value and $R^2$ were indicated on the graphs. *$P$<0.05 compared with CTL

### $CD4^+CD25^{hi}$ Tregs were positively related to the plasma IL-6 levels in T2D patients

T2D is associated with increased monocyte superoxide and pro-inflammatory cytokine release (IL-1β, IL-6, and TNF-α) [29, 30]. The concentrations of plasma cytokines including IFN-γ, IL-2, TNF-α, IL-4, IL-6, and IL-10 in 38 T2D patients and 19 control subjects were detected using CBA assays. The levels of IFN-γ, IL-2, TNF-α, IL-4, and IL-10 in blood of T2D patients did not show significant difference with healthy control subjects (Fig. 8a in the ESM). However, the IL-6 levels were significantly enhanced in T2D patients, regardless whether patients with complications or not ($P$< 0.05; Fig. 7a, b). The percentages of $CD4^+CD25^{hi}CD127^-$ Tregs were in a positive correlation with plasma IL-6 concentrations in T2D patients ($P$<0.01; Fig. 7c). The levels of $IL\text{-}17^+CD4^+$cells and $IFN\text{-}\gamma^+CD4^+$cells as well as the ratios of $CD4^+CD25^{hi}CD127^-$ Tregs/$IL\text{-}17^+CD4^+$ cells, $CD4^+CD25^{hi}CD127^-$ Tregs/$IFN\text{-}\gamma^+CD4^+$ cells, and $IL\text{-}17^+CD4^+$ cells/$IFN\text{-}\gamma^+CD4^+$ cells were no correlation with blood IL-6 levels in T2D patients ($P$>0.05; Fig. 7d, e; Fig. 8b–d in the ESM). These results suggest that IL-6 may up-regulate $CD4^+CD25^{hi}$ T cells in T2D patients, as recently reported in a mouse model [31].

## Discussion

The involvement of immune disorders in the process of T2D itself in addition to T2D complications caused attenuation today [11, 18, 32, 33]. In the present study, the levels of $CD4^+CD25^{hi}Foxp3^+$ Tregs and $CD4^+CD25^{hi}CD127^-$ Tregs are decreased in the periphery of patients with T2D, whereas Th17 and Th1 cells are increased, indicating that distinct $CD4^+$ T cell subset polarization occurred in T2D patients. On the other hand, Th17 but not $CD4^+CD25^{hi}CD127^-$ Treg and Th1 cells are negatively related to plasma HDL while all these $CD4^+$ T cell subsets did not show significant relation to plasma triglyceride, cholesterol, and LDL, supporting the importance of HDL in the regulation of T cells in T2D patients. This observation opened an alternative explanation for the substantial activation of innate immune cells and high incidences of inflammation-coursed complications in patients with T2D, that is, the enhanced immune activation and inflammation may be, at least partially, caused by the decreased immunosuppressive potentiality of $CD4^+CD25^{hi}$ Tregs and enhanced inflammatory Th17 and Th1 cells. Furthermore, it may provide one possible approach to prevent the development of diabetic complications by modification of $CD4^+$ T cell subpopulations.

It has been well demonstrated that $CD4^+CD25^{hi}$ Tregs play a pivotal role in self-tolerance and resistance to autoimmune disease via its ability to down-regulate the function of innate and adaptive immune effector cells. Dysfunction of $CD4^+CD25^{hi}$ Tregs promotes the development of autoimmune diseases in animals and human beings. It is reported that human or mouse $CD4^+CD25^+$ Tregs induce M2 macrophage differentiation from monocytes in vitro and in vivo [34, 35]. Evidence supports that macrophage activation and accumulation is one of the major causes for

the development of diabetic complications [36–38]. Thus, it is speculated that the decreased $CD4^+CD25^+$ Tregs and its ratio to Th17 and Th1 cells may display less immunosuppressive effect on monocytes/macrophages and likely drive these cells to an inflammatory state, which may contribute to the occurrence of diabetic complications. This hypothesis is supported by the observation showing that enhanced IL-6 and TNF-α productions and decreased IL-10 levels occurred in blood of patients with T2D [30, 39]. Up-regulation of $IL\text{-}17^+$, $IL\text{-}15^+$, and $Foxp3^+$ cells were observed in chronic periodontitis sites in T2D subjects, suggesting that periodontitis development in these subjects may be influenced by the Th17/Treg axis. Further evidence also includes that significantly lower percentages of $CD4^+CD25^+$ Tregs in T2D patients with microvascular complications was observed compared with T2D patients with macrovascular complications or none. On the other hand, the frequency of complications is inversely correlated with the poor blood sugar control, indicating the role of hyperglycemia in the promotion of inflammatory injury in diabetes patients [27, 40]. However, our data showed that $CD4^+CD25^{hi}$ Tregs are not negatively correlated with the blood levels of HbA1c and Th17 and Th1 cells were not positively related to HbA1c. Therefore, it is likely that hyperglycemia itself may not directly affect $CD4^+CD25^{hi}$ Tregs in diabetic patients.

The etiology of human T2D is multi-factorial, with genetic background and physical inactivity as two critical components [28]. The pathogenesis of T2D is not fully understood. Whether the peripheral alteration of $CD4^+$ T cell subpopulation is just a consequence of T2D or also a contributor to T2D development needs to be determined. Based on the recent literatures [13, 18–20, 41], it is very likely that there is a bidirectional regulation loop between T2D and $CD4^+$ T cells even and $CD8^+$ T cells during the initiation and progress of T2D, possibly via inflammatory and anti-inflammatory cytokines. It will be interesting and important to determine the relationship between the T cell subsets in obese adipose tissue and those in the peripheral blood in the future, which may lead to identify the new diabetes diagnostic parameters.

Our study showed identical levels of $CD4^+Foxp3^+$ $Helios^+$ Tregs in T2D patients with the age-matched healthy subjects, indicating that T2D may not influence the naturally occurring $CD4^+CD25^{hi}$ Treg output from the thymus if recognizing Helios as a marker for nTregs. However, recent study showed that peripheral induced Tregs may also express Helios, suggesting that Helios may be a reliable marker to identify the developmental origin of Tregs [26]. Thus, to safely speaking, T2D mainly influenced the $CD4^+Foxp3^+Helios^-$ Treg subpopulation. Our data suggest that dyslipidemia might be one of the important impact factors for the peripheral $CD4^+CD25^{hi}$ Tregs in T2D patients. The detailed molecular mechanisms for the T2D-related decrease of $CD4^+CD25^{hi}$ Tregs are not fully addressed at present. Our preliminary data showed that $CD4^+CD25^{hi}$ Tregs from T2D patients displayed less ratio of anti-apoptosis gene Bcl-2 to pro-apoptosis gene Bax compared with healthy control subjects indicating that $CD4^+CD25^{hi}$ Tregs in T2D patients may be more sensitive to cell death than control. However, $CD4^+CD25^{hi}$ Tregs are unexpectedly in a positive correlation with the enhanced plasma IL-6 levels in T2D patients, which is consistent with the recent observation in mice that IL-6 enhanced the level of $CD4^+CD25^{hi}$ Tregs [31]. All these data together suggest that both the environmental factors and the intrinsic signal pathways may be collectively involved in the regulation of $CD4^+$ T cells and the final outcome is the significantly decreased $CD4^+CD25^{hi}$ Tregs and increased Th17 and Th1 cells in T2D patients. However, one possibility that certain T2D-relevant genes are involved in both promotion of hyperglycemia, dyslipidemia and the $CD4^+$ T cell subset development simultaneously was not excluded in our present study.

It should be emphasized that inflammatory Th17 cells were negatively correlated with blood HDL levels in T2D patients. It is reported that HDL, neither triglycerides nor LDL, was inversely related with subsequent coronary events and that plasma HDL levels bear a strong independent inverse relationship with inflammation and atherosclerotic cardiovascular disease [42–44]. Thus, the inhibiting role of HDL on Th17 offered more evidence supporting the benefit of raising HDL to decrease the risk of complications.

In conclusion, the present study showed that the dramatically decreased ratios of $CD4^+CD25^{hi}$ Tregs to Th17 or Th1 cells in T2D patients may contribute to the enhanced immune activation and inflammation, as well as the subsequent complications. The decreased $CD4^+CD25^{hi}$ Tregs in T2D patients may be caused by the increased cell death, possibly by imbalanced Bcl-2/Bax axis. The levels of Th17 cells in the peripheral blood of T2D patients were negatively related to plasma HDL. This study gains insight into the understanding the alteration of $CD4^+$ T cells in T2D patients and offered important clue for the involvement of T cells in the development of diabetes and its complications, which may have significant impacts in clinical practice.

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