Exenatide regulates Th17/Treg balance via PI3K/Akt/FoxO1 pathway in db/db mice

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Research Article

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Abstract

Background

The T helper 17 (Th17)/T regulatory (Treg) cell imbalance is involved in the course of obesity and type 2 diabetes mellitus (T2DM). In the current study, the exact role of glucagon-like peptide-1 receptor agonist (GLP-1RA) exenatide on regulating the Th17/Treg balance and the underlying molecular mechanisms are investigated in obese diabetic mice model.

Methods

Metabolic parameters were monitored in db/db mice treated with/without exenatide during 8-week study period. The frequencies of Th17 and Treg cells in vivo and in vitro were assessed. The phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/Forkhead box O1 (FoxO1) pathway was detected by western blotting.

Results

Exenatide treatment obviously improved β-cell function and insulitis. Increased Th17 and decreased Treg cells in peripheral blood were present as diabetes progressed while exenatide corrected this imbalance. Progressive IL-17 + T cell infiltration of pancreatic islets was alleviated by exenatide intervention. In vitro study showed that palmitate could promote Th17 but suppress Treg differentiation along with down-regulating the phosphorylation of PI3K/Akt/FoxO1, which could be reversed by exenatide intervention. FoxO1 inhibitor AS1842856 could abrogate all these effects of exenatide against lipid stress.

Conclusions

Exenatide could protect β-cell function in db/db mice partially by restoring Th17/Treg balance via PI3K/Akt/FoxO1 pathway.

Background

It is recognized that a chronic low-grade inflammation and activated immune system play essential role in the insulin sensitivity, β-cell preservation, and the resultant development of type 2 diabetes mellitus (T2DM) (Lee et al. 2018; Zeyda et al. 2009). Interleukin (IL)-17-producing CD4 + T helper (Th17) cells are crucial for the chronic inflammation and autoimmunity (Singh et al. 2014) while regulatory T (Treg) cells can suppress inflammatory responses and maintain peripheral tolerance (Sakaguchi et al. 2008). The imbalance of these two lymphocytes has been identified to be involved in the pathogenesis of type 1 diabetes mellitus (T1DM) (Fabbri et al. 2019). Furthermore, we recently described the potential role of Th17 and Treg cells in obesity and T2DM (Wang et al. 2018). The proportion of Th17 cells was higher in

obese or T2DM patients accompanied by elevated level of inflammatory cytokines (Dalmas et al. 2014; Ip et al. 2016). In contrast, Tregs were precipitously decreased in newly diagnosed T2DM patients (Yuan et al. 2017). In addition, adoptive transfer of Tregs was found to be associated with reversal of insulin resistance in db/db mice (Eller et al. 2011). Thus, the regulation of Th17/Treg balance might exert a beneficial effect on the treatment of T2DM.

Glucagon-like peptide-1 (GLP-1) is an incretin hormone secreted from the gastrointestinal tract and functions to lower glucose concentrations by augmenting insulin secretion and suppressing glucagon release (Drucker 2018). In addition, emerging evidences have indicated a critical role for GLP-1 in modulating innate immunity and inflammation (Alicic et al. 2021; Lee et al. 2012), which may be mediated by regulating CD4 + T cell proliferation and decreasing the production of pro-inflammatory cytokines (Charpentier et al. 2021; Moschovaki Filippidou et al. 2020). Such immuno-inflammatory regulation effect is identified to be implicated in the improvement of β-cell function in T2DM (Cechin et al. 2012; Lee et al. 2012). Howbeit, the exact effects of GLP-1 on the maintenance of β-cell function and the Th17/Treg balance in T2DM remains largely undefined and the underlying mechanisms are yet to be elucidated.

Exenatide was synthetically developed as a recombinant structure of exendin-4 and was the first GLP-1 receptor agonist (GLP-1RA) for the treatment of T2DM (Mikhail 2006). In this study, it is aimed to investigate the exact role of short-acting exenatide on Th17/Treg equilibrium in db/db mice. The possible molecular mechanisms of GLP-1 on the modulation of innate immunity and inflammation under the context of obese T2DM is investigated as well.

Materials And Methods

Animals and groups

Sixteen 4-week-old male db/db mice were randomly divided into exenatide (n=8) or control group (n=8) and maintained in a specific-pathogen free, temperature-controlled environment under 12 h light/dark cycles. Mice in exenatide group received subcutaneous short-acting exenatide (Byetta, Baxter Pharmaceutical Solutions LLC., Indiana, USA) administrations (200 μg/kg body weight). Control mice received an equal volume of saline solution. After 8-week treatment, animals were sacrificed and samples were collected. All animal care and experimental protocols were approved by the Institutional Animal Care and Use Committee of Tongji Medical College.

Glucose tolerance and insulin measurement

Body weight and fasting glucose level were regularly measured every 3 days. Oral glucose tolerance test (OGTT) and fasting insulin were detected at week 0 and week 8, respectively. For OGTT, body weight was determined after 12 h overnight fasting and then mice were orally gavaged with 2 g/kg body weight glucose solution. 100uL blood samples were collected before and 30, 60, 90, 120 and 180 min after the gavage via capillary pipette from caudal vein and placed into EDTA-treated tube for glucose

measurement. Serum fasting insulin was assayed using Mouse Insulin ELISA Kit (#CSB-E05071m, RRID: AB_2916335, Cusabio, China). Homoeostasis model assessment (HOMA) of insulin resistance (HOMA-IR) and HOMA of β-cell function (HOMA-β) were calculated as follows: HOMA-IR= Glucose (mmol/L) × Insulin (mU/L) / 22.5; HOMA-β=20×Insulin (mU/L) / (Glucose (mmol/L)−3.5)%.

Culture of Th17 and Treg cells

Naïve CD4+T cells were isolated from the spleens of male C57BL/6J mice using Naïve CD4+T cells isolation kit (Miltenyi, Germany) and then cultured in 1640 medium containing 10% fetal bovine serum with plate-bound anti-CD3 (2 μg/mL, #14-0031-86, RRID: AB_467051, eBioscience, USA) and anti-CD28 (1 μg/Ml, #14-0281-85, RRID: AB_467191, eBioscience, USA) antibodies for 4 days. IL-2 (200 U/mL, Cusabio, China) and transforming growth factor-β (TGF-β) (0.5 ng/mL, Cusabio, China) were added to the culture medium for Treg cell differentiation; while IL-6 (10 ng/mL, Cusabio, China), IL-23 (10 ng/mL, Sinobiological, China) and TGF-β (2.5 ng/mL, Cusabio, China) were added to the culture medium for Th17 cell differentiation. Palmitate, one of the most common saturated free fatty acid (FFA), is used to induce lipotoxic environment (Shao et al. 2014). After replacement of culture medium, cells were cultured with 200 μM palmitate (PA) and/or 100 nM exenatide in the presence or absence of 10 μM Foxrkhead box protein O1 (FoxO1) inhibitor (AS1842856, MedChemExpress, USA) for 24 h.

Flow cytometry analysis

The frequencies of Th17 and Treg cells were investigated using flow cytometry. For Th17 assay, cells were stimulated with Cell Stimulation Cocktail (eBioscience, USA) for 6 h at 37℃ and 5% CO_2 and then stained with anti-mouse CD4-phycoerythrin (PE) (#12-0041-82, RRID: AB_465506, eBioscience, USA). Fixation and permeabilization were performed with fix/perm buffer (Servicebio, China) and then cells were incubated with anti-mouse IL-17-allophycocyanin (APC) (#506915, RRID: AB_536017, eBioscience, USA). For Treg assay, cells were incubated with anti-mouse CD4-fluorescein isothiocyanate (FITC) (#11-0043-82, RRID: AB_464900, eBioscience, USA) and anti-mouse CD25-PE (#12-0251-81, RRID: AB_465606, eBioscience, USA) at 4℃ for 30 min in darkness. After fixation and permeabilization (Biolegend, USA), cells were stained with anti-mouse forkhead box P3 (Foxp3)-Alexa Fluor 647 (#126407, RRID: AB_794503, Biolegend, USA) at 4℃ for 30 min in darkness. Data collection and analysis were performed on a FACSCalibur (Beckman, USA).

Quantitative real-time PCR

mRNA expression of FoxO1, IL-17 and Foxp3 was determined by real-time PCR. Total RNA was extracted using Trizol Reagent adhering to the manufacturer's instructions (Scrvicebio, China). RNA concentration and purity were assessed using ultramicro spectrophotometer (NanoDrop2000, Thermo, USA). Afterwards, cDNA was generated from mRNA by using RevertAid First Strand cDNA Synthesis Kit (Thermo, USA). qRT-PCR was performed on Real-Time PCR System (ABI, USA), using FastStart Universal SYBR Green Master (Rox, Servicebio, China). The relative gene expression levels were calculated using

the threshold cycle (CT), according to the $2^{-\Delta\Delta CT}$ method. GAPDH was set as reference. All RT-PCR specific primer sequences applied for PCR reaction amplification were presented in Table S1.

Western blot analysis

Protein samples were separated by SDS-PAGE and transferred to polyvinylidene fluoride membranes. After blocking with 5% non-fat milk in Tris-buffered saline Tween-20 (TBST), the membranes were incubated with primary antibodies against IL-17 (#AO688, RRID: AB_2757339, Abclonal Technology, China), Foxp3 (#GB11093, RRID:AB_2861434, Servicebio, China), phosphoinositide 3-kinase (PI3K) (#bsm-33219m, RRID: AB_2916342, Bioss, China), phospho (p)-PI3K (#bs-5570R, RRID: AB_11050832 Bioss, China), protein kinase B (Akt) (#GB11689, RRID:AB_2916343, Servicebio, China), p-Akt (#AF0908, RRID:AB_2834079, Affinity, USA), FoxO1 (#GB11286, RRID: AB_2916344, Scrvicebio, China), or p-FoxO1 (#9464T, RRID:AB_329842, Cell Signaling Technology, USA) at 4℃ overnight. The membranes were then incubated with horseradish peroxidase-conjugated secondary antibodies. Proteins were detected using an enhanced chemiluminescence system (Clinx Science Instruments, China).

Immunofluorescence staining

5 cm-thick paraffin sections of pancreatic tissues were subjected to immunofluorescence staining with antibodies against IL-17 (#GB11110, RRID: AB_2892098, Servicebio, China) or Foxp3 (#GB11093, RRID: AB_2861434, Servicebio, China) in combination with anti-insulin antibody (#GB12334, RRID: AB_2916346, Servicebio, China) according to the manufacturer's instructions. Nuclei were counterstained with DAPI stain (Servicebio, China). Immunostained images were acquired using Ortho-Fluorescent microscope imaging system (Nikon, Japan).

HE staining

Pancreatic tissues were placed in 10% neutral formalin for fixation, embedded in paraffin blocks. After deparaffination and rehydration, 5-μm-thick paraffin sections were stained with hematoxylin and eosin (HE). Insulitis scoring was calculated according to the following criteria under light microscope: 0, no immune cell infiltration; 1, <25% 25% of islet being infiltrated with immune cell; 2, 25-50%; 3, 50-75%; 4, >75%. Data are presented as the percentage of islets with each grade of insulitis.

Statistical analysis

All data were presented as mean ± standard error of the mean (SEM). Comparisons were performed using t-test or ANOVA. All probability values were two-tailed, and $P<0.05$ was considered significant.

Results

Exenatide improved body weight, insulin sensitivity and β-cell function.

To determine the therapeutic effect of exenatide on T2DM, the time course changes in body weight and fasting blood glucose levels were monitored in db/db mice, the model of obese T2DM, treated with either saline solution or exenatide (Figure 1 A,B). Compared with the control group, exenatide-treated mice showed significantly less weight gain within 3-week intervention (Exenatide 3 Week, 39.39±0.73 g vs Control 3 Week, 42.81±1.28 g, P=0.048). However, such effect did not persist and the body weight in exenatide group gradually increased as compared with control group (Exenatide 8 Week, 52.74±1.14 g vs Control 8 Week, 46.74±1.54 g, P=0.014). In addition, exenatide treatment could effectively control the glucose level in db/db mice and the fasting glucose ranged from 8.48 mmol/L to 15.22 mmol/L over the 8-week duration of the experiment (Exenatide 8 Week, 15.22±1.35 mmol/L vs Control 8 Week, 27.82±1.99 mmol/L, P<0.001).

Moreover, OGTT study showed significant improvement of glucose tolerance after 8-week exenatide treatment as shown in Figure 1C. In addition, at the end of the study, exenatide group showed obviously lower levels of fasting insulin than control group (Exenatide 8 Week, 10.06±1.57 μU/mL vs Control 8 Week, 14.72±0.75 μU/mL, P=0.028, Figure 1D). Furthermore, HOMA-IR and HOMA-β were calculated and it was demonstrated that exenatide intervention could markedly improve either insulin sensitivity (Exenatide 8 Week, 6.80±1.18 vs Control 8 Week, 18.34±1.92, P<0.001, Figure 1E) or β-cell function (Exenatide 8 Week, 18.20±3.69 vs Control 8 Week, 12.33±0.86, P>0.05, Figure 1F).

Exenatide alleviated the severity of islet inflammation with the progression of T2DM

We next investigated the effect of exenatide on islet inflammation. According to the HE staining, the percentage of islets with immune cell infiltration was significantly increased in control diabetic mice at week 8 (Control 8 Week, 87.5% vs Control 0 Week, 0%, Figure 1 G,H), indicating low-grade inflammatory state in pancreatic islets as the development of T2DM. On the contrary, the inflammatory state was largely alleviated after 8-week treatment of exenatide (Exenatide 8 Week, 25% vs Control 8 Week, 87.5%, Figure 1 G,H), indicating the potential effect of GLP-1 on the regulation of inflammation state in obese T2DM.

Exenatide restored periphery Th17/Treg balance in db/db mice

To further investigate the immunoregulatory effects of exenatide, we detected the frequencies of Th17 and Treg cells in the peripheral blood of db/db mice by flow cytometry. Along with the progression of diabetes, we found that the proportion of CD4+IL-17+Th17 cells was increased from 0.99±0.07% to 2.46±0.30% after 8-week treatment (P=0.001, Figure 2 A,B) while CD4+CD25+Foxp3+Treg cells were markedly decreased (Control 8 Week, 0.08±0.01% vs Control 0 Week, 0.19±0.04%, P=0.037, Figure 2 C,D). These data further verified the imbalance of Th17/Treg as the development of T2DM. Of note, it was demonstrated that there was no significant difference of Th17 frequency in exenatide group before and after 8-week treatment (Figure 2 A,B), indicating that exenatide intervention could block the increase of peripheral Th17. Similar results were found for Treg cells (Figure 2 C,D). Taken together, these results disclosed that exenatide may exert its immuno-inflammatory regulation effect in obese diabetic mice through correcting the imbalance of Th17/Treg cells.

Exenatide prevented Th17 cells from infiltrating into islets

To examine whether Th17/Treg imbalance also occurred in pancreas in obese T2DM, we detected the expression of IL-17 and Foxp3 in pancreas by real-time PCR and western-blot. Surprisingly, both the mRNA and protein levels of IL-17 were decreased significantly as the progression of T2DM (Figure 3 A,B,C), which was inconsistent with the findings in peripheral blood. In addition, exenatide intervention had no impact on the mRNA expression of IL-17 (Exenatide 8 Week, 71.96±4.28% vs Control 8 Week, 73.17±6.68%, *P*>0.05, Figure 3 A). Similar results were shown in western blot detection (Exenatide 8 Week, 0.83±0.06 vs Control 8 Week, 0.74±0.07, *P*>0.05, Figure 3 B,C). We further detected the expression of IL-17 and Foxp3 via immunofluorescence analysis. The findings demonstrated that, as the development of T2DM, IL-17+T cells gradually migrated from the periphery of pancreas islet to the center of the islet; while Th17 cells maintained to infiltrate surrounding the islet in exenatide-treated mice (Figure 3D), disclosing that exenatide treatment could change the distribution rather than the proportion of IL-17+ T cells in pancreas. Of note, neither PCR nor western could detect the expression of Foxp3 in pancreas (Data not shown). Foxp3+Treg cells were undetectable in pancreas by immunofluorescence staining as well. Accordingly, it is speculated that GLP-1 may exert its protective effect on islet inflammation and β-cell function, partly, through preventing Th17 cells infiltrating into pancreatic islets rather than modulating their frequencies.

Exenatide regulated the proliferation of Th17 and Treg cells in vitro while FoxO1 inhibitor blocked this effect

To further verify the effect of GLP-1 in regulating the proliferation of Th17 and Treg cells, Naïve CD4+T cells from the spleens of C57BL/6J mice were collected. Since lipotoxicity is the most critical condition in obesity and induces a chronic low-level inflammation in metabolic tissues (Longo et al. 2019), the percentages of Th17 and Treg cells were detected with the administration of PA and exenatide. It was found that PA administration significantly increased the proportion of Th17 cells (PA, 6.67±0.22% vs Control, 4.86±0.12%, *P*=0.004, Figure 4 A,B) while decreased the percentage of Treg cells (PA, 0.13±0.02% vs Control, 0.26%±0.03%, *P*=0.039, Figure 4 C,D). Significantly, the treatment of exenatide corrected palmitate induced changes of both Th17 (Exenatide+PA, 4.75±0.20% vs PA, 6.67±0.22%, *P*=0.006, Figure 4 A,B) and Treg (Exenatide+PA, 0.24±0.01% vs PA, 0.13±0.02%, *P*=0.021, Figure 4 C,D).

FoxO1, a member of Forkhead transcription factor O family, has been reported to have crucial roles for the development of Th17 and Treg cells (Laine et al. 2015; Ouyang et al. 2010). The supplementation of FoxO1 inhibitor AS1842856 blocked the regulating effect of exenatide on both Th17 (Exenatide+PA+AS, 6.80±0.38% vs PA, 6.67±0.22%, *P*>0.05, Figure 4 A,B) and Treg (Exenatide+PA+AS, 0.12±0.02% vs PA, 0.13±0.02%, *P*>0.05, Figure 4 C,D).

Taken together, these findings verified that exenatide treatment could correct Th17/Treg imbalance under lipotoxic stress. In addition, such effect could be blocked when FoxO1 was inhibited, disclosing that FoxO1 signal pathway may be involved in.

Exenatide regulated Th17/Treg proliferation via the PI3K/Akt/FoxO1 pathway

To explore whether exenatide-modulated proliferation of Th17/Treg was mediated by FoxO1 signal pathway, the expression levels of FoxO1 and p-FoxO1 were measured in vivo and in vitro. Our findings demonstrated that mRNA level of FoxO1 in peripheral blood mononuclear cells (PBMCs) from exenatide-treated db/db mice increased significantly at the end of study by 230.59% compared with Control 0 Week (Figure 5 A). Protein levels of FoxO1 and p-FoxO1 exhibited the similar changes (Figure 5 B,C), suggesting that FoxO1 might be a target of exenatide in regulating Th17 and Treg cells. Furthermore, our previous study has estimated that protective effect of GLP-1 was mediated by PI3K/Akt/FoxO1 signaling pathway (Shao et al. 2014). Therefore, we detected the levels of PI3K/Akt/FoxO1 and their respective phosphorylated forms in Th17 and Treg cells in vitro. It was found that palmitate exposure decreased PI3K, Akt, FoxO1 and their phosphorylation levels compared with control group in Th17 cells (Figure 5 D,E). And such decrease could be completely corrected by exenatide intervention. Similar results were observed in Treg cells as well (Figure 5 F,G). Taken together, these findings suggested that exenatide suppressed the differentiation of Th17 cells while promoted Treg development, which may be partly mediated by PI3K/Akt/ FoxO1 pathway.

Discussion

The recognition of chronic low-grade inflammation in the development of obese T2DM has been paid increasing attention. Our previous study has discussed that Th17/Treg imbalance may be involved in this inflammatory process (Wang et al. 2018). Although exenatide was widely applied as hypoglycemic agent in T2DM, its immuno-inflammatory regulation properties are being recognized gradually (Alicic et al. 2021; Lee et al. 2016). However, studies on the effect and underlying mechanisms of exenatide on Th17/Treg balance in T2DM are limited. Our present study demonstrated that exenatide significantly suppressed the proportion of Th17 cells but enhanced Treg cells both in vivo and in vitro under the context of obese T2DM, which may be mediated by PI3K/Akt/FoxO1 pathway.

It is known that function of pancreatic β cells is gradually impaired by excessive glucose (glucotoxicity) and fatty acids (lipotoxicity) under the context of obese T2DM (Shao et al. 2014). Additionally, chronic low-grade inflammation could result in a marked accumulation and impaired function of various immune cells including macrophages and B cells in pancreas, which can cause β-cell dysfunction as well (Eguchi et al. 2017; Ying et al. 2019). Consistently, our current study observed decreased HOMA-β cell and increased insulitis as the progress of T2DM (Fig. 1), suggesting that islet inflammation may be involved in β-cell dysfunction in T2DM. Although the related molecular mechanism is not fully elucidated, several factors are referred to participate in this process, including adipose tissue macrophages infiltration, endoplasmic reticulum (ER) stress and FFA-induced lipopolysaccharide (LPS) receptor toll-like receptor (TLR)4 activation (Zeyda et al. 2009). In addition, T cells-mediated immunity was identified to be intertwined with metabolic disorders (Lee et al. 2018). Dalmas et al. reported that an increased frequency of effector T cells (Teffs) promoted glycemic deterioration in obese T2DM patients (Dalmas et al. 2014), while an increase in Treg frequencies restores insulin sensitivity and prevents diabetic nephropathy (Eller

et al. 2011). In this study, we demonstrated that the peripheral frequency of Th17 cells increased but Treg proportion reduced markedly following the development of diabetes (Fig. 2), which is consistent with data from clinical studies conducted in obese or T2DM patients (Ip et al. 2016; Yuan et al. 2017). Furthermore, Reinert-Hartwall et al. demonstrated that the upregulation of the IL-17 and Th1/Th17 plasticity involved in impaired β-cell function in T1DM (Reinert-Hartwall et al. 2015). These findings disclose a clue that the imbalance of Th17/Treg may contribute to the β-cell dysfunction and the progression of T2DM.

Infiltration of Th17 cells in pancreas was validated in NOD mice, which was associated with pathogenesis of T1DM (Bellemore et al. 2016; Martin-Orozco et al. 2009). However, there is no research that investigate the infiltration of Th17 and Treg in pancreas under the context of T2DM. Of note, our study identified the migration of Th17 cells from the islet periphery into intra-islet, which may contribute to severer insulitis and β cell failure. A previous study from Grieco and colleagues have demonstrated that Th17 immunity aggravated insulitis by inducing β-cell apoptosis and exacerbating chemokines expression, the latter may in turn augment the attraction of invading immune cells in T1DM (Grieco et al. 2014). Similarly, Honkanen et al. also demonstrated that IL-17 inhibited the mRNA expression of antiapoptotic gene and enhanced cytokines-induced proapoptotic effects in human islet cells (Honkanen et al. 2010). Howbeit, the present study identified that the level of IL-17 expression in pancreas was decreased as the progression of T2DM, which was contradictory to the findings of circulating Th17 cells. This may suggest tissue-specific changes during disease progression, in which different Th17 responses have been identified in different tissues in obesity (Cavallari et al. 2016). Furthermore, it is recognized that Th17 cells could switch to other T helper cell programs under certain cytokine milieu (Martin-Orozco et al. 2009; Stadhouders et al. 2018). Martin-Orozco et al. identified that Th17 cells could promote pancreatic inflammation in NOD mice, but only induce T1DM upon conversion into interferon-γ (IFN-γ) producers (Martin-Orozco et al. 2009). Accordingly, we assume that the pathogenicity of Th17 transdifferentiation may be also involved in pancreas under the context of obese T2DM, which may explain the lower level of IL-17 + cells in pancreas.

In the current study, Foxp3 + Tregs were undetectable in pancreas. Similarly, Willcox and colleagues analyzed postmortem pancreatic samples from 16 T1DM patients, with Foxp3 + Tregs detectable only in a single patient (Willcox et al. 2009). Study from Nti et al. also reported that the number of Tregs were decreased to undetectable levels in the pancreatic lymph nodes of the untreated hyperglycemic NOD mice (Nti et al. 2012). Deficiency of Tregs may result in the progress of inflammation and diabetes. A previous study from Watts et al. reported that depletion of Foxp3 + Tregs precipitates destructive β-cell autoimmunity in NOD.DEREG (‘depletion of regulatory T cell’) mouse model (Watts et al. 2021). Conversely, transfer of Tregs could largely prevent Teffs-induced diabetes development in NOD mice (Sprouse et al. 2018). Accordingly, these data suggested that the lack of Treg cells may be one of the possible causes for the development of islet inflammation in T2DM.

Nowadays, GLP-1RA is widely implemented in therapy for weight control and T2DM (Meier 2012). Moreover, in vitro, in vivo, and clinical studies over the last decades have collectively demonstrated that GLP-1 and GLP-1RAs have beneficiary effects on preservation of β-cell function. Our results showed that

exenatide intervention remarkably ameliorated HOMA-β, which confirmed its protective action on β-cell function. It is considered that weight loss could contribute to the recovery of β-cell function (Taylor et al. 2018). Of note, in our study, although less body weight gain was observed in exenatide-treated mice, such effect could only persist for 3 weeks (Fig. 1). Thus, we assumed that the preservation of β-cell function by short-term exenatide intervention may be independent of the weight loss (Shao et al. 2014).

There are various direct protective effects of GLP-1 on pancreatic β cells, including inhibition of glucolipotoxic ER stress, regulation of transcription factors and signaling molecules that is implicated in β-cell proliferation, and prevention of cell apoptosis mediated by the induction of anti-apoptotic proteins such as Bcl-2 and Bcl-xl (Lee et al. 2014). Interestingly, beyond these mechanisms, the anti-inflammatory properties of GLP-1RA have been gradually identified (Lee et al. 2016). In the current study, exenatide intervention alleviated the severity of insulitis, which could partially explain the improvement of islet β-cell function. The underlying mechanisms of GLP-1 on immuno-inflammatory regulation are not quite understood. Most of related studies were performed in autoimmune diseases. Moschovaki et al. demonstrated that GLP-1RA could protect mice from a nondiabetic, T-cell-dependent glomerulonephritis model by inhibiting the proliferation of Th1 and Th17 cells (Moschovaki Filippidou et al. 2020). A study from Chiou and colleagues considered that GLP-1RA could modulate the differentiation of encephalitogenic Th1/Th17, providing mechanistic insight on T cells regulation in ameliorating experimental autoimmune encephalomyelitis by GLP-1 (Chiou et al. 2019). Furthermore, co-culture with exenatide reduced the levels of IL-1β, IL-2, IL-17 and IFN-γ in human islet supernatants (Cechin et al. 2012), disclosing that Th17 cell may be involved in GLP-1 related immuno-inflammatory modulation under diabetes context as well. On the other hand, GLP-1RAs are found to increase the frequency of Tregs in NOD mice (Xue et al. 2008) and high-fat-diet-induced obesity mice (Sha et al. 2019). Consistently, our findings demonstrated that the imbalance of Th17/Treg in peripheral blood were obliterated by exenatide supplementation under the context of obese T2DM. Accordingly, it is speculated that the beneficial effect of GLP-1RA on islet inflammation and β-cell function may be partially mediated by regulating the proliferation of Th17 and Treg cells.

Inconsistent with the findings from peripheral blood, the frequency of IL17 + T cells in pancreas showed insignificant difference between control and exenatide-treated mice. According to immunofluorescence analysis, at the end of the study, IL17 + T cells mainly invaded in the center of the islets in controls, but gathered around peri-islet in exenatide group. It seems that exenatide treatment prevented or attenuated the infiltration of IL-17 + Th17 cells into pancreatic islets rather than altering the level of Th17 cells. It has been demonstrated that migration and infiltration of immunocytes into inflamed islets are an essential component of the immune response of β-cell destruction (Khodabandehloo et al. 2016). In this regard, exenatide is assumed to prevent the development of destructive insulitis, partially through inhibiting intra-islet infiltration of Th17 cells. It is recognized that IL-17 + T cells are expressed chemokine (CC motif) receptor 6 (CCR6) (Honkanen et al. 2010). The expression of CCR6 ligand, chemokine (C-C motif) ligand 20 (CCL20) could be exacerbated by IL-17 in islets of T1DM (Grieco et al. 2014). CCR6 and CCL20 play an important role in migration and infiltration of Th17 cells at inflamed tissues (Singh et al. 2014). A study from Lee and colleagues demonstrated that resveratrol treatment reduced the severity of insulitis by

inhibiting CCR6-CCL20-mediated Th17 cell migration from peripheral lymphoid organs to pancreas (Lee et al. 2011). Furthermore, Bang-Berthelsen et al. reported that GLP-1RA liraglutide could alleviate colonic inflammation partially by downregulating CCL20 levels in a colitis mouse model (Bang-Berthelsen et al. 2016). Therefore, exenatide may block Th17 migration into islet by regulating Th17-associated chemokines, which need to be evidenced in obese T2DM in further study. However, Foxp3 + Tregs were still undetectable in pancreas in exenatide-treated group. Previous study found that exendin-4 could cause an increasing trend of Treg number in lymph nodes of NOD mice, although such change was insignificant (Drucker et al. 2008). Therefore, the immune-regulatory effects of exenatide on the promotion of Tregs may be weak in pancreas of T2DM. It is speculated that larger dose and longer intervention of exenatide may make the effect obvious.

The PI3K/Akt pathway is essential for the development of T cells (Juntilla et al. 2008) and often dysregulated in various inflammatory disorders (Li et al. 2020; Stylianou et al. 2011). One mechanism by which PI3K/Akt regulate T cell differentiation is through the modulation of FoxO1 (Hedrick et al. 2012). In the context of CD4 + T cell differentiation, FoxO1 controls Foxp3 promoter activity and are critical for the differentiation and function of Tregs (Ouyang et al. 2010). In addition, previous study has demonstrated that Foxo1 could act as a potent anti-inflammatory control switch acting on suppression of ROR-γT-induced Th17 differentiation program (Laine et al. 2015). Although various studies have highlighted the specific role of FoxO1 in T cell biology (Hedrick et al. 2012), it remains unclear whether FoxO1 presides the regulation of Th17/Treg differentiation in the context of diabetes. In the current study, a decreasing tendency of FoxO1 was observed along with elevated Th17 cells and reduced Tregs following the progress of diabetes. In addition, declined levels of PI3K, Akt, and FoxO1 were observed under lipotoxic stress in vitro, indicating that PI3K/Akt/FoxO1 pathway may be involved in the differentiation and proliferation of Th17 and Treg cells.

The regulatory role of GLP-1RAs mediated by PI3K/Akt/FoxO1 signaling has been implicated in multiple disease process (Chen et al. 2019; Shao et al. 2014). Our previous study has demonstrated that liraglutide protected β-cell function under lipotoxic stress via PI3K/Akt/FoxO1 pathway (Shao et al. 2014). In the current study, the decreased phosphorylation of PI3K/Akt/FoxO1 could be completely canceled by exenatide intervention accompanied by the restoration of Th17/Treg balance. Moreover, all the effects of exenatide on CD4 + T cells proliferation were prohibited when AS1842856 was pre-incubated. All these findings disclose that exenatide may regulate the differentiation of Th17 and Treg cells through PI3K/Akt/FoxO1 pathway in the context of obese T2DM.

Conclusions

Our results indicate that exenatide could protect β-cell function in db/db mice partially by alleviating islet inflammation via restoring the balance of Th17/Treg. In addition, PI3K/Akt/FoxO1 pathway was involved in this process, disclosing a potential mechanism exenatide-related immuno-inflammatory modulation in obese T2DM.

Abbreviations

Akt
Protein kinase B
CCR6
Chemokine (CC motif) receptor 6
CCL20
Chemokine (C-C motif) ligand 20
ER
Endoplasmic reticulum
FFA
Free fatty acid
FoxO1
Foxrkhead box protein O1
Foxp3
Forkhead box P3
HE
Hematoxylin and eosin
GLP-1
Glucagon-like peptide-1
GLP-1RA
Glucagon-like peptide-1 receptor agonist
HOMA-β
Homoeostasis model assessment of β-cell function
HOMA-IR
Homoeostasis model assessment of insulin resistance
IL-17
Interleukin-17
IFN-γ
Interferon-γ
LPS
Lipopolysaccharide
OGTT
Oral glucose tolerance test
PA
Palmitate
PBMCs
Peripheral blood mononuclear cells
PI3K
Phosphoinositide 3-kinase

T1DM
Type 1 diabetes mellitus
T2DM
Type 2 diabetes mellitus
Teffs
Effector T cells
TGF-β
Transforming growth factor-β
Th17 cell
T helper 17 cell
TLR4
Toll-like receptor 4
Treg cell
Regulatory T cell.

Declarations

Ethics approval and consent to participate

All animal care and experimental protocols were approved by the Institutional Animal Care and Use Committee of Tongji Medical College.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors have declared that no conflict of interest exists.

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Author contributions

Conception, design and critical revision: SS. Interpretation of the results and article writing: XQ. Data analysis: ZX and LT. All authors read and approved the final manuscript.

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Figures

Figure 1

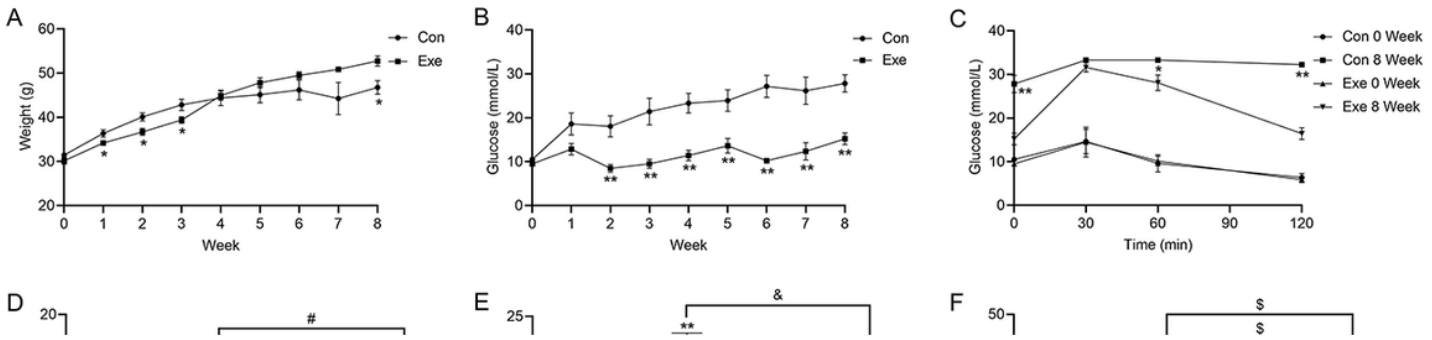

Figure 1

Time course changes of body weight (A) and blood glucose (B) in control (Con) and exenatide-treated (Exe) db/db mice. OGTT (C), fasting insulin (D), HOMA-IR (E), HOMA-β (F), representative HE micrographs of pancreatic insulitis (G), and the percentage of insulitis scores of pancreatic islets (H) in Con and Exe group before and after 8-week treatment. Data are presented as mean±SEM, n=8 in each group; ** P<0.01,* P0.05 vs control (A,B), control 8 week (C), or control 0 week (D,E,F); & P<0.01, # P<0.05 vs control 8 week (D,E,F); $ P>0.05. Scale bar=50 μm.

OGTT, oral glucose tolerance test; HOMA-IR, homeostasis model assessment of insulin resistance; HOMA-β, homeostatic model assessment of β cell function.

Figure 2

Peripheral frequency of Th17/Treg in db/db mice via flow cytometry. (A) Representative flow cytometric plots of Th17 cells from control (Con) and exenatide (Exe) groups at week 0 and week 8. (B) Statistical analysis of the percentage of Th17 cells in control and exenatide groups. (C) Representative flow cytometric plots of Treg cells with CD4-FITC, CD25-PE and Foxp3 staining. (D) Statistical analysis of the

percentage of Treg cells. Data are presented as means±SEM; n=8 in each group; ** *P*<0.01, * *P*<0.05 vs control 0 week; & *P*<0.01, # *P*<0.05 vs control 8 week; $ *P*>0.05.

Figure 3

Detection of Th17 cells in pancreas from control (Con) and exenatide (Exe) groups at week 0 and week 8. (A) mRNA levels of IL-17 by real-time PCR. mRNA quantities were calculated as a ratio to the level of GAPDH mRNA in each sample. Data are shown as the relative expression ratio to Control 0 Week. Results are expressed as means±SEM, n=8 in each group. (B) Representative image of IL-17 by Western blotting. ACTIN was used as a loading control. (C) Gray value analysis of Western blotting. Data are shown as the relative expression ratio to Control 0 Week and expressed as means±SEM, n=8 in each group. (D) Representative immunofluorescence of pancreatic sections using anti-IL-17 staining (red), anti-insulin staining (green) and DAPI staining (blue), scale bar=20 μm. **P*<0.05 vs control 0 week; $ *P*>0.05.

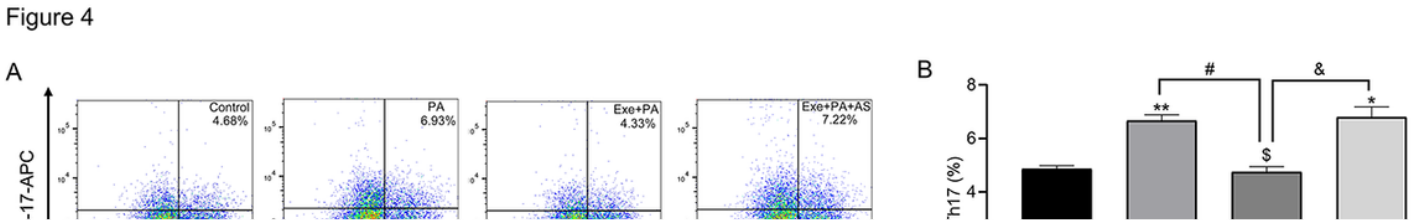

Figure 4

Frequency of Th17/Treg via flow cytometry in vitro. Naïve CD4+T cells collected from C57BL/6J mice were cultured under Th17/Treg-inducing conditions with palmitate (PA), exenatide plus palmitate (Exe+PA) and exenatide, palmitate plus FxoO1 inhibitor AS1842856 (Exe+PA+AS). (A) Representative flow cytometric plots of Th17 cells from different groups. (B) Statistical analysis of the percentage of Th17 cells in different groups. (C) Representative flow cytometric plots of Treg cells with CD4-FITC, CD25-PE and Foxp3 staining. (D) Statistical analysis of the percentage of Treg cells. Data are presented as means±SEM of three independent experiments. ***P*<0.01, **P*<0.05 vs control group; # *P*<0.05 vs PA group; & *P*<0.05 vs Exe+PA group; $ *P*>0.05.

Figure 5

Effects of exenatide on Th17/Treg proliferation via FoxO1 signaling. (A) mRNA levels of FoxO1 in PBMCs from control (Con) and exenatide (Exe) groups detected by real-time PCR. mRNA quantities were calculated as a ratio to the level of GAPDH mRNA in each sample. Data are shown as the relative expression ratio to Con 0 Week. Results are expressed as means±SEM, n=8 in each group. (B) Representative image of Foxo1 and phosphor (p)-FoxO1 in PBMCs from Con and Exe groups detected by Western blotting. ACTIN was used as a loading control. (C) Gray value analysis of Western blotting. Data are shown as the relative expression ratio to Con 0 Week and expressed as means±SEM, n=8 in each group. (D) Representative image of total PI3K, Akt, FoxO1 and p-PI3K, p-Akt, p-FoxO1 in Th17 cells in vitro detected by Western blotting. Th17 cells were treated with palmitate (PA), exenatide plus palmitate (Exe+PA) and exenatide, palmitate plus FxoO1 inhibitor AS1842856 (Exe+PA+AS). (E) Gray value analysis of western blotting in Th17 cells. (F) Representative image of total PI3K, Akt, FoxO1 and p-PI3K, p-Akt, p-FoxO1 in Treg cells in vitro detected by Western blotting. Treg cells were treated with PA, Exe+PA and Exe+PA+AS. (G) Gray value analysis of western blotting in Treg cells. Data are shown as the relative expression ratio to Control. Three independent experiments were performed and results are expressed as means±SEM. ** P<0.01, * P<0.05 vs control 0 week (A,C), or control group (E,G); & P<0.01, # P<0.05 vs control 8 week (A,C), or PA group (E,G).

Supplementary Files

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- TableS1.doc