Endocrine (2019) 66:494–502
https://doi.org/10.1007/s12020-019-02081-x

ORIGINAL ARTICLE

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Effects of glucagon-like peptide-1 analog liraglutide on the systemic inflammation in high-fat-diet-induced mice

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Received: 28 January 2019 / Accepted: 3 September 2019 / Published online: 21 September 2019

Abstract

Objective Metabolic syndrome is a chronic-metabolic disease caused by a variety of factors, including high peripheral blood insulin levels and insulin resistance. It has been reported that GLP-1 could regulate insulin resistance. It is not known whether and how GLP-1 protects from fat-induced inflammation and immune changes. We investigated if GLP-1 alters the populations of fat-induced inflammation and immune cells and the related mechanism.

Methods We obtained obese C57BL/6J mice by feeding them high-fat food, then treated the obese mice with GLP-1+ high-fat diet (G + Hi), normal chow diet (Nor), or high-fat diet (Hi) ($n = 20$ for each group) for 8 weeks. The GLP-1 receptor−/− B6 group were fed with HFD for 8 weeks (GLP-1R KO + Hi). In vivo and in vitro experiments were conducted on mice immune cells to investigate the effects of GLP-1 on the changes of the immune components and functions in obesity.

Results We found that GLP-1 could efficiently change the CD4+ T subsets and level of cytokines in high-fat-induced mice by GLP-1 receptor. Further, these changes were correlated with a reduction in fat content and serum lipid level. Interestingly, GLP-1 could enhance the function of Tregs in vitro.

Conclusion Our data showed that GLP-1 has an important role in shaping the CD4+ T population in high-fat-diet-induced mice by GLP-1 receptor, possibly providing a new target for the treatment of metabolic syndrome.

Keywords Obesity · Immune · GLP-1 · Metabolic syndrome

What is already known about this subject?

GLP-1 helps defend against metabolic syndrome, including hyperlipidemia, hypertension, insulin resistance, and increased adiposity.

Metabolic syndrome shows significant changes in the immune system components.

What does this study add?

GLP-1 altered the immune components and changed the CD4+ T subsets and Treg function in obesity. By knocking out GLP-1 receptor in B6, we found the GLP-1 could regulate metabolic disorders by GLP-1R.

GLP-1 has an important role in shaping the CD4+ T population in high-fat-diet-induced mice, which may provide a new target for the treatment of metabolic syndrome.

Introduction

Glucagon-like peptide-1 (GLP-1) is produced in the intestinal epithelial endocrine L-cells by differential processing of proglucagon. GLP-1 is released in response to meal intake, stimuli, and molecular mechanisms [1, 2]. The main functions of GLP-1 are to stimulate insulin secretion and to inhibit glucagon secretion, thereby contributing to limited postprandial glucose excursions. GLP-1 also inhibits gastrointestinal motility and secretion. Because of these actions, GLP-1 is currently being evaluated for the therapy

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of obesity and type 2 diabetes. Decreased secretion of GLP-1 may contribute to the development of obesity [3]. GLP-1 could function via combining with the glucagon-like peptide-1 receptor (GLP-1R) [4–7].

Metabolic syndrome is a group of obesity-related metabolic abnormalities that increase an individual's risk of developing type 2 diabetes and cardiovascular disease. Metabolic syndrome is characterized by the large volume and increased number of adipose cells, resulting in the higher content of visceral fat deposition. The basic change of metabolic syndrome is the immune system components, especially in adipose tissue, skeletal muscle, liver, pancreas, the intestinal tract, cardiovascular system, and peripheral circulation. The functional polarization of CD4+ T subsets has been shown to participate in the early development of chronic inflammation more than B cells, macrophages, and dendritic cells [7]. A balanced ratio of CD4+ T subsets is more important in the development of obesity and other multisystem autoimmune diseases. In systemic lupus erythematosus, the immune system also showed systemic inflammation and imbalance [8].

Here, we showed that GLP-1 helps defend against metabolic syndrome, mainly including insulin resistance and increased adiposity. Further, food restriction prevented obesity but not insulin resistance in the high-fat-diet-induced mice. Interestingly, along with the change of CD4 + T subsets, the function of CD4+ CD25+ Foxp3+ T cells was altered by GLP-1.

Material and methods

Animal models

Eighty-four-week-old male C57BL/6J mice were purchased from Jackson Lab [8]. All mice were housed in specific pathogen-free (SPF) conditions in a 12-h dark/light cycle. All mice received irradiated food ad libitum and were housed in individually ventilated filter cages (SPF). The Institutional Animal Care and Use Committee at Shandong University approved the use of mice in this study.

After 1 week of adaptive feeding, 20 mice were randomly chosen to be fed with a regular chow diet as the control group (CTRL group, 10% calories from fat, purchased from KeAoXieLi Co., Ltd, Beijing, China), whereas 60 were fed a high-fat diet (HFD group, 60% calories from fat, purchased from Botai Hongda Biotechnology Co., Ltd, Beijing, China).

Animal processing

In the 8th week of the high-fat diet, we separated the obesity mice (HFD) into three groups: G + Hi (GLP-1+ high-fat diet) group, Nor (normal chow diet) group, and Hi (high-fat diet) group ($n = 20$ for each group). The GLP-1 analog Liraglutide injection was bought from Novo Nordisk A/S. GLP-1 (100 μg/(kg.d)) was intraperitoneally injected daily into the G + Hi group for 8 weeks. Then we generated GLP-1R−/− B6 mice by bred from either heterozygous breeding (GLP-1R± B6 × GLP-1R± B6) or homozygous breeding (GLP-1R−/− B6 × GLP-1R−/− B6 or GLP-1R +/+ B6 × GLP-1R+/+ B6). We gave GLP-1R group with HFD for 8 weeks (GLP-1R KO + Hi). The body fat mass of the mice ($n = 5$ for each group) was detected by MRI (Signa Excite HD 1.5 T, USA) while the mice were anesthetized. Serum leptin, triglyceride (TG), and total cholesterol (TC) were detected with an automatic biochemistry analyzer (COBAS INTEGRA 800, Switzerland).

Glucose and insulin tolerance tests (GTT and ITT)

For GTT, mice were fasted for 12 h. Blood glucose was measured at 0, 15, 30, 60, and 120 min after i.p. injection of 2 g/kg body weight of dextrose. ITT was performed by fasting the mice for 4 h followed by i.p. injection of 1 U/kg body weight of insulin (Novolin, Novo Nordisk, Princeton, NJ, USA). Blood glucose was measured at 0, 15, 30, 60, 90, and 120 min. All blood glucose measurements were done using an Accucheck glucometer (Roche Diagnostics, Indianapolis, IN, USA).

Flow cytometry staining and intracellular staining

Lymphocytes were harvested from the spleen and visceral fat. For Fc Blocking antibody (15 min, 4 °C) 2–5 × 106 cells were incubated prior to staining for antibodies to CD3, CD4, CD8α, CD25, Foxp3, B220, TCRbeta, and CD44, together with a viability dye (all from BioLegend), all for 30 min at 4 °C. For intracellular staining, 5×10^6 cells were stimulated with PMA (50 ng/mL, Sigma) and Ionomycin (500 ng/mL, Sigma) in the presence of 1 μL/mL Golgi-PlugTM (BD) at 5×10^6/mL for 4 h. Post stimulation, cells were stained as above. Cells were then washed, fixed (20 mins at RT), and permeabilized using eBioscienceTM Intracellular fixation and permeabilization buffer set. Cells were then incubated with an Fc Blocking antibody (15 min, 4 °C) prior to staining for antibodies to IL-4 and IL-17. The flow cytometer used was a BD Aria III (Becton and Dickinson Co., Ltd, USA).

Cell isolation

Lymphocytes were harvested from spleen and visceral adipose tissue. Tregs were isolated from spleen using the MACSTM Mouse Treg Selection Kit (Miltenyi Biotec GmbH) following the manufacturer's protocol. CD4+

T cells were negatively isolated through incubating splenocytes with Anti-Biotin Microbeads using magnetic selection with LD columns. CD4 + CD25+ T cells were then isolated with CD25+ antibody using magnetic selection with an MS columns. The purity was routinely 90–95% as verified by flow cytometry.

Cell culture and CSFE staining

For 3 days, 100,000 purified CD4+ CD25+ T cells and CD4− T cells (reactive T cells) were separately stimulated with α-CD3 and α-CD28 (final concentration 1:300), with CSFE incubation added. After 72 h, cell culture supernatants were collected. The CSFE labeled CD4− T cells were co-cultured with CD4CD25+ T cells for 72 h. Proliferation was determined by CSFE incorporation using a flow cytometer.

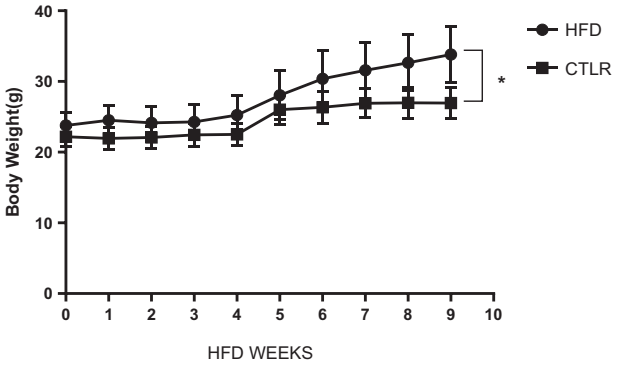

Fig. 1 Significant difference in the body weight of high-fat diet group (HFD) and control diet group (CTLR)

ELISA

IFN-γ, IL-22, and IL-17 were detected in serum using the Mouse ELISA MAXTM Standard kit (BioLegend) as directed.

Results

High-fat diet altered CD4+ T populations

To simulate the effect of GLP-1 on metabolic syndrome, we obtained high-fat-diet-induced mice. By feeding with a high-fat diet for 8 weeks, the body weight of HFD group mice increased significantly compared with CTLR group mice (Fig. 1). Investigations into populations of CD4+ T cells identified changes with the high-fat diet. Here, we found an increase population of CD4+ IFN-γ+ T cell (Th1) and CD4+ IL-17a+ T cell (Th17) subsets in spleen and vat (Fig. 2a–d) in HFD mice. As these populations increased, we found a reduction in the CD4+ IL-4 T cell (Th2) and CD4+ CD25+ T cells (Treg) in spleen and vat (Fig. 2b, c) in HFD mice. We found no significant phenotypic differences in macrophages or dendritic cells (data not shown). Interestingly, a study assessing murine spleen T cells found that high-fat-diet-induced obesity could cause an imbalance of immune cell subsets both in T cells and APC [9]. The mechanism referred to insulin resistance and chronic

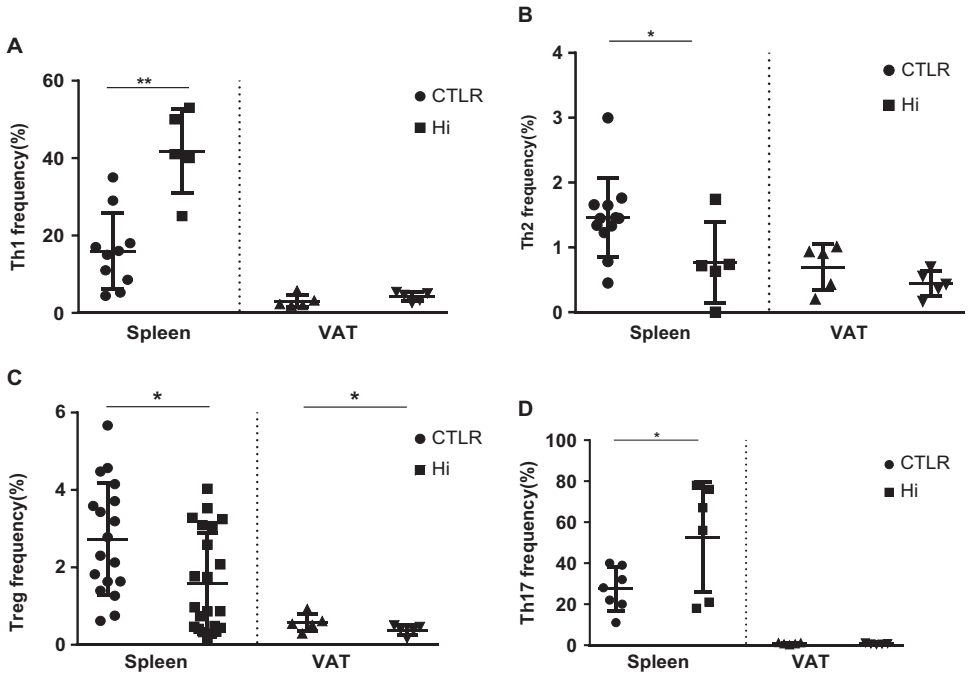

Fig. 2 An increase population in CD4+ IFN-γ+ T cells (Th1) (**a**), CD4+ IL-17a+ T cells (Th17) (**d**) subsets in spleen was observed by high-fat food diet for 8 weeks. A reduction in the CD4+ IL-4 T cells (Th2) (**b**), CD4+ CD25+ T cells (Treg) (**c**) in spleen and vat by high-fat food diet for 8 weeks

inflammation in visceral adipose tissue, liver, and muscles. Therefore, we hypothesized whether one target could produce more anti-inflammation cytokines and immune cells to affect obesity.

GLP-1 altered the ratio and number of inflammatory Th cells

GLP-1 upregulated the ratio and number of anti-inflammatory T cells by controlling the level of inflammatory cytokines. After 8 weeks of GLP-1 injection in HFD mice, we found an increased population of CD4+ IL-4 T cells (Th2) and CD4+ CD25+ T cells (Treg) in spleen cells and vat (Fig. 3b–d) in the G + Hi group. Together, we found a decrease in the population of CD4+ IFN-γ+ T cells (Th1) and CD4+ IL-17a+ T cell (Th17) subsets in spleen cells and vat (Fig. 3a–c) in the G + Hi group. However, the normal food intervention for HFD (Nor group) is not significantly different from the G + Hi group. Therefore, this data suggest that GLP-1 could regulate the immune subsets independent of the influence of diet.

Given that we identified changes in the CD4+ T populations (Fig. 2), we wanted to confirm whether these changes were in accordance with the changes in the cytokines or the changes in the function of immune cells. Therefore, we investigated the serum inflammation cytokines. We found that IL-17a, IFN-gamma, and IL-22 were significantly reduced by GLP-1 in the G + Hi group. However, IL-17a, IFN-gamma, and IL-22 were not significantly down-regulated in the Nor group. While the proportions of the CD4+ T subsets were different among G + Hi, Nor, CTLR, and Hi group mice, the altered cytokines and Treg function were capable of modifying proportional differences.

GLP-1 enhanced the function of Tregs

Having identified alterations in the CD4+ T compartment, we considered whether GLP would also alter the function of T subsets, especially Treg subsets. We assessed Treg proliferation co-cultured with CD8+ T cells labeled with CSFE and found that regardless of diet factor, GLP-1 had decreased proliferative capabilities (Fig. 4). Again, we investigated the secretion of cytokines in the serum and found that IL-17a, IFN-gamma, and IL-22 from G + Hi group mice, independent of diet factor, secreted lower levels of inflammation cytokines than the Nor, CTLR, and Hi group mice (Fig. 5a–c). Even stimulating the Treg cells alone with anti-CD3 and anti-CD28 resulted in proliferation at lower concentrations and lower IL-17a, IFN-gamma, and IL-22 in G + Hi Treg cells. This data confirmed that GLP-1

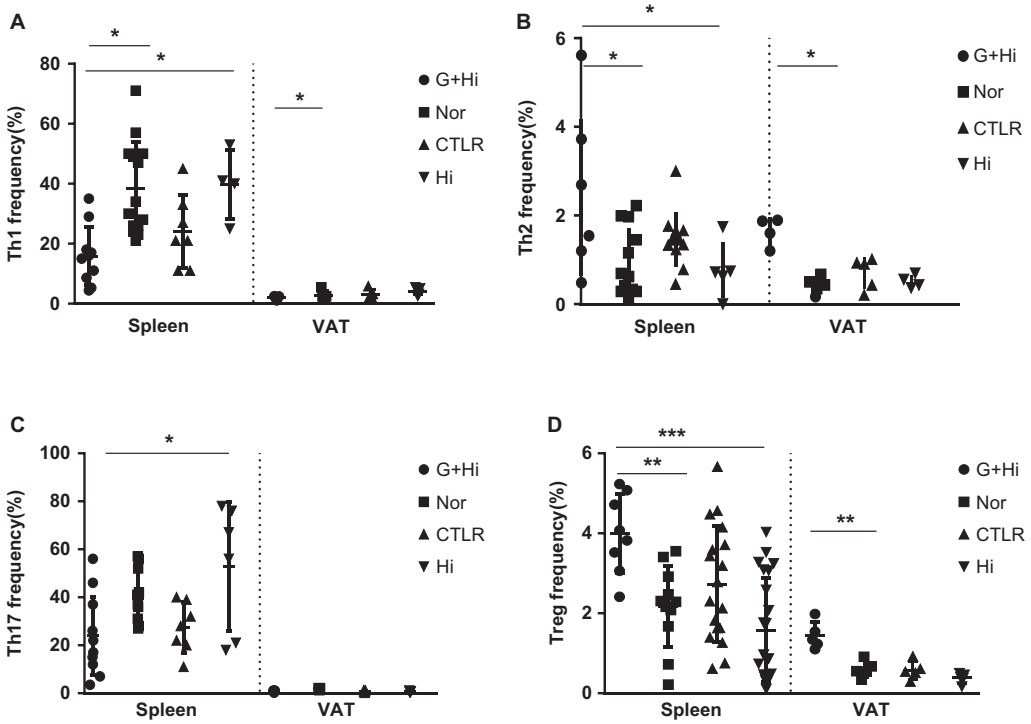

Fig. 3 An increased population of CD4+ IL-4 T cells (Th2) (**b**) and CD4+ CD25+ T cells (Treg) (**d**) in spleen and vat was found in GLP-1 treated obese mice for 8 weeks together with normal food (G + Hi group). A reduction population in CD4+ IFN-γ+ T cells (Th1)(A) and CD4+ IL-17a+ T cells (Th17) (**c**) subsets in spleen and vat were investigated in G + Hi group. There is no significant difference in Th1, Th2, Th17, and Treg subsets in obese mice treated with normal food. G + Hi represents the obese C57BL/6J mice treated with GLP-1; Nor represents normal chow diet; Hi represents high-fat diet ($n = 20$ for each group) for 8 weeks

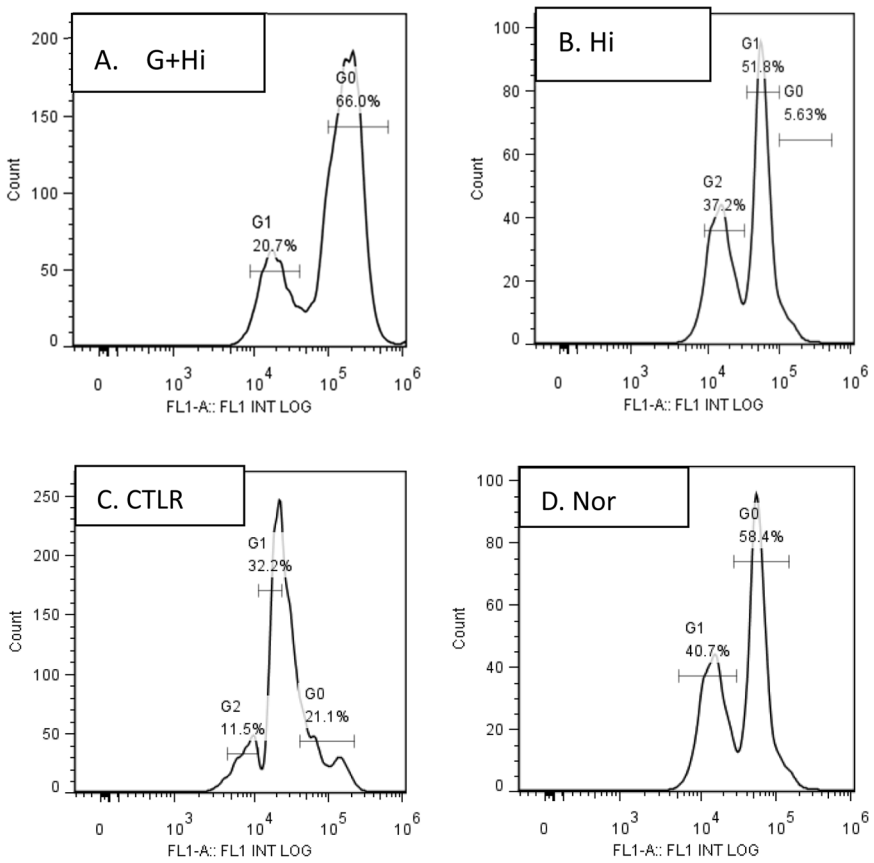

Fig. 4 A decreased proliferative capability of G + Hi Tregs was found in the proliferation of CD8+ T cells labeled with CFSE, co-cultured with Tregs from different groups. G + Hi represents the obese C57BL/6J mice treated with GLP-1; Nor represents normal chow diet; Hi represents high-fat diet ($n = 20$ for each group) for 8 weeks

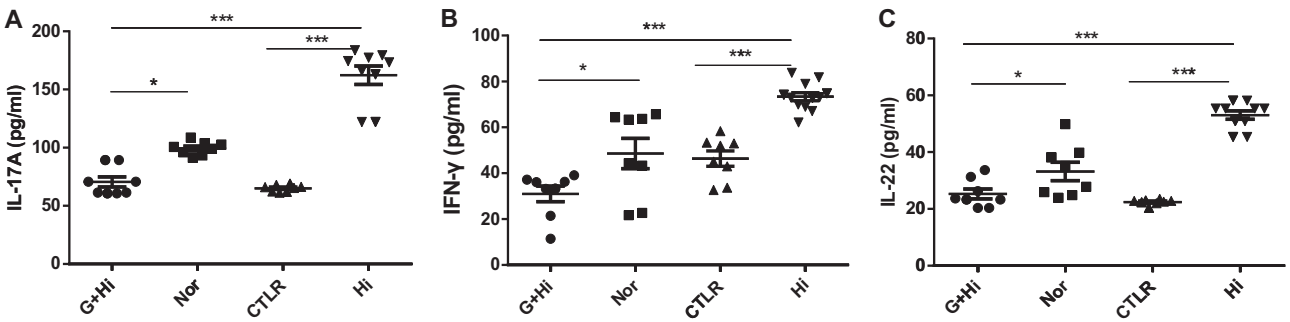

Fig. 5 Significant differences in secretions of IL-17a, IFN-gamma, and IL-22 were revealed in G + Hi group mice. G + Hi represents the obese C57BL/6J mice treated with GLP-1; Nor represents normal chow diet; Hi represents high-fat diet ($n = 20$ for each group) for 8 weeks

could reduce IL-17a, IFN-gamma, and IL-22 independently of diet but that the secretion of cytokines from these cells was dependent on GLP-1.

GLP-1 altered the systemic metabolism disorder by GLP-1R

Having identified changes in the CD4+ T cell populations, we determined if there were any differences in systemic metabolic disorder. GTT and ITT were conducted in male mice on five groups before harvesting mice. G + Hi group has better glucose intolerance than Nor, CTLR, and Hi groups. After injected for 15 min, the blood glucose of G + Hi upregulated straightly and went down at 30 min (Fig. 6a). Similarly, G + Hi insulin resistance had a significant decrease in insulin sensitivity compared with other groups (Fig. 6b). Similarly, GLP-1R KO + Hi had an obvious impaired insulin resistance and glucose sensitivity

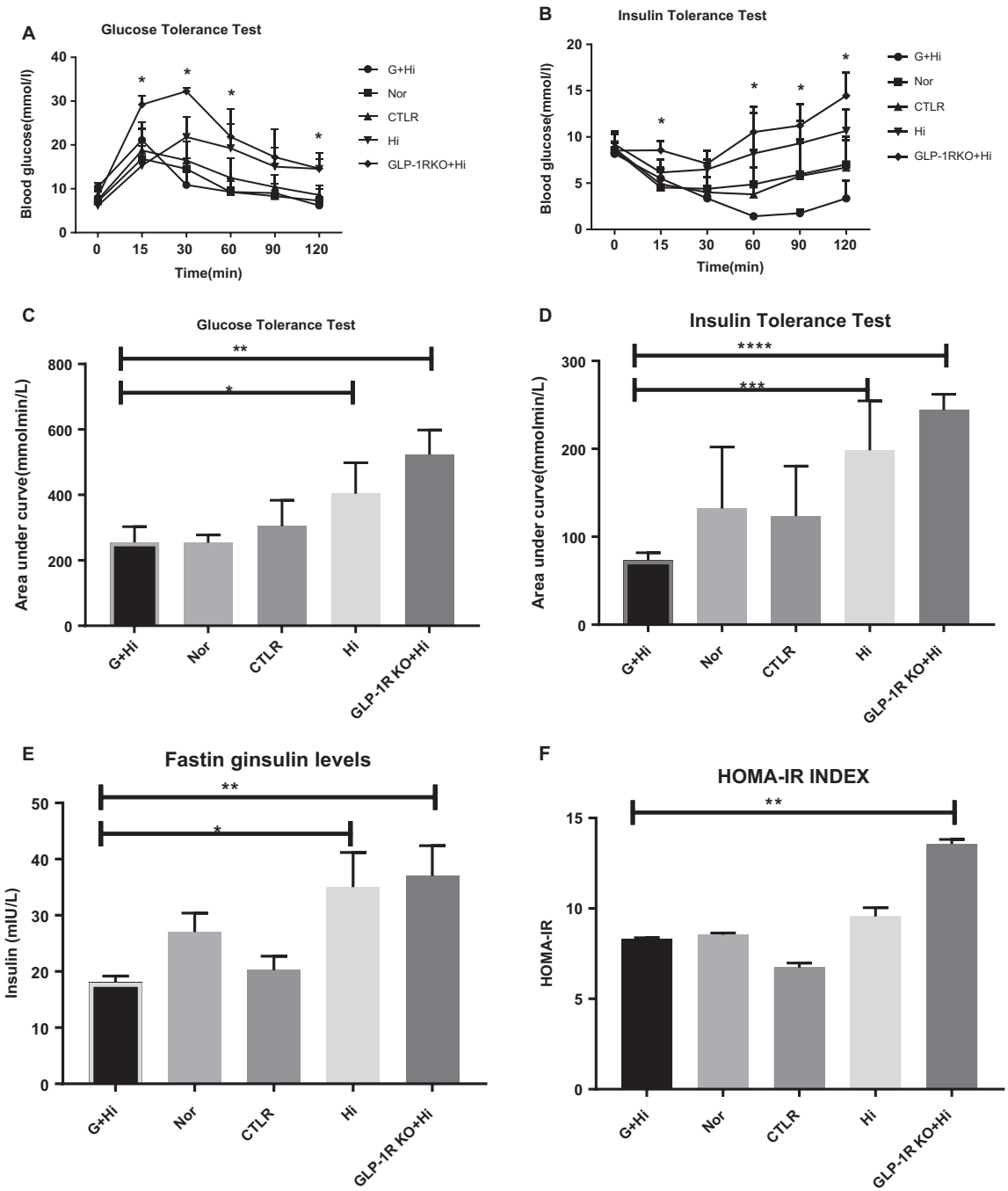

Fig. 6 Glucose and insulin tolerance tests in male mice. GTT and ITT were performed as described in the material and methods. The data were obtained from 11–13 male mice. **a** G + Hi and GLP-1R KO + Hi were significantly different from Nor, CTLR, and Hi groups in 15, 30, 60, and 120 min (*$P < 0.05$); **b** G + Hi and GLP-1R KO + Hi were significantly different from Nor, CTLR, and Hi in 15, 60, 90, and 120 min (*$P < 0.05$); **c** In area under the curve of glucose in GTT, G + Hi was significantly different from Hi and GLP-1R KO + HI groups (*$P < 0.05$); **d** In area under the curve of glucose in ITT, G + Hi was significantly different from Hi and GLP-1R KO + HI groups (*$P < 0.05$); **e** G + Hi showed the lower fasting insulin level compared with Hi and GLP-R KO + Hi group (*$P < 0.05$). **f** Homa-IR index showed G + Hi was significantly lower than Hi and GLP-1R KO + Hi group (*$P < 0.05$). G + Hi represents the obese C57BL/6J mice treated with GLP-1; GLP-1R KO + Hi represents GLP-1 receptor knocking out C57BL/6J mice fed with high-fat diet; Nor represents normal chow diet; Hi represents high-fat diet ($n = 20$ for each group) for 8 weeks

at 30 min point (Fig. 6a, b). The area under the curve also showed the similar insulin resistance and glucose sensitivity in G + Hi group (Fig. 6c, d). On other way, GLP-1R KO + Hi showed highest fasting insulin level compared with G + Hi group. And Homa-IR index showed GLP-1R KO + Hi had significantly different to G + Hi group (Fig. 6e, f).

We tested the serum level of leptin, TG, and TC. Here, we found a reduction of leptin in the G + Hi group independently of diet. We also observed a significant difference in TG and TC by GLP-1 (Table 1). Interestingly, by MRI, we found that GLP-1 induces less visceral fat mass compared with HFD, and this is independent of diet (Table 2 and Fig. 7). This suggests that the mass of fat may be associated with the regulation of immune cells. Thus, GLP-1 may be capable of reducing fat tissue and improving the disorder of systematic metabolism.

Discussion

The increasing incidence of metabolic syndrome is widely thought to result from nutrient excess due to the increased food consumption and/or reduced levels of physical activity [9–13]. Humanity is facing an epidemic of interrelated metabolic diseases collectively referred to as metabolic syndrome, the hallmarks of which include hyperglycemia, hyperlipidemia, insulin resistance, obesity, and hepatic steatosis. Such nutrient excess results in obesity and may activate the immune system, resulting in chronic pro-inflammatory kinase cascades that desensitize the metabolic response to insulin. GLP-1 and its pancreatic receptors are important for the control of body weight and blood glucose levels. A previous study also showed that GLP-1 played a physiological role in the regulation of ingestion homeostasis [14].

Previous work has shown that GLP-1 is important in the loss of body weight [7, 15]. However, most studies have focused on the role of GLP-1 in adipose tissue but few have investigated the response and alterations to immune cells [16]. Therefore, we investigated the role of GLP-1 in shaping the immune cells in a high-fat-diet-induced mouse model [3, 9, 10, 15–24]. Here, we found that GLP-1 was responsible for altering the CD4+ T subsets and functions, particularly in regards to altered IL-17, IFN-gamma, and IL-

Table 1 A significant reduction of leptin and TC and TG in G + Hi group was found

Group	Leptin (ng/ml)	*F*	^{1}P	^{2}P	TG (mmol/l)	*F*	^{1}P	^{2}P	TC (mmol/l)	*F*	^{1}P	^{2}P
G + Hi	15.03 ± 4.05	19.088	0		0.573 ± 0.15	5.15	0.004		2.36 ± 0.58	29.787	0	
CTLR	13.90 ± 2.84			0.512	0.87 ± 0.29			0.014	2.73 ± 0.13			0.230
Hi	25.82 ± 0.86			0.000	0.97 ± 0.31			0.001	4.64 ± 0.99			0.000
Nor	19.27 ± 2.76			0.021	0.67 ± 0.15			0.351	2.40 ± 0.33			0.837

G + Hi represents the obese C57BL/6J mice treated with GLP-1; Nor represents normal chow diet; Hi represents high-fat diet ($n = 20$ for each group) for 8 weeks

Table 2 GLP-1 induced visceral fat mass compared with HFD but independent on diet factor by MRI

Group	Total fat content (g)					MRI FAT Mass (mm^3)
	Mesenteric	Epidi	Perirental	Subscapular	Subcutaneous	
G + Hi	0.29 ± 0.19	0.69 ± 0.27	0.80 ± 0.89	0.22 ± 0.06	0.33 ± 0.22	72259.8 ± 4674.39
CTLR	0.49 ± 0.52	0.59 ± 0.16	0.74 ± 0.60	0.26 ± 0.10	0.34 ± 0.15	102522 ± 4418.75
Nor	0.45 ± 0.49	0.86 ± 0.52	0.54 ± 0.29	0.28 ± 0.12	0.59 ± 0.51	113239.8 ± 4771.26
Hi	0.99 ± 1.09	1.34 ± 0.41	1.29 ± 0.75	0.91 ± 0.52	1.79 ± 0.27	208523 ± 5241.3

G + Hi represents the obese C57BL/6J mice treated with GLP-1; Nor represents normal chow diet; Hi represents high-fat diet ($n = 20$ for each group) for 8 weeks

Epidi epididymisadipose

Fig. 7 GLP-1 induced visceral fat mass compared with HFD but independent on diet factor by MRI. G + Hi represents the obese C57BL/6J mice treated with GLP-1; Nor represents normal chow diet; Hi represents high-fat diet ($n = 20$ for each group) for 8 weeks

22 secretion. Interestingly, the effect of GLP-1 on CD4+ T subsets is independent of normal diet. Likewise, treatment with GLP-1 protected the HFD mice 100% from developing obesity. It was shown later that the effects of GLP-1 treatment were mediated through CD4+ T subsets, whereby Th1 and Th17 subsets were reduced in GLP-1-treated mice in spleen and vat; the population of Th2 and Treg subsets were increased in GLP-1 treated mice in spleen and vat. Importantly, most of the immune subsets changes altered in visceral fat tissues and spleen. Interestingly, in our study, the cytokines producing subsets (IL-17a, IL-4, and IFN-γ) were altered in GLP-1-treated mice compared with control mice. Furthermore, these pro-inflammation CD4+ T subsets produced more IL-17, IFN-gamma, and IL-22. These findings in relation to GLP-1 are novel, and thus may provide an alternative treatment option for metabolic syndrome [25]. To investigate the further mechanism, we generated GLP-1R−/− B6 mice. The insulin and glucose results referred that GLP-1 could affect the metabolic disorders by GLP-1 receptors. After knocking out the GLP-1 receptor in B6 mice, GLP-1R KO + Hi had similar insulin tolerance in 15, 30, and 60 min like Hi group. The glucose tolerance was also similar to Hi group in 15, 30, and 60 min. All these data showed that GLP-1 could improve metabolic disorders by GLP-1 receptor.

We also found that GLP-1 could enhance the function of CD4+ CD25+ T cells by co-culturing them with CD8+ T subsets. For stimulating cells needs more than 10,000 cells, we choose proliferate cells from spleen not vat. Through the co-culture of CD4+ CD25+ T cells and CD8+ T subsets in the presence of anti-CD3 and anti-CD28 stimulation, we were able to show that GLP-1 enhances the capability of anti-inflammation. Furthermore, to investigate if GLP-1 can affect the metabolic status, we found that glucose intolerance, insulin sensitivity had been significantly regulated by GLP-1; serum lipid and leptin had remarkable reduction in mice treated with GLP-1. Interestingly, when GLP-1 was used to treat obesity in mice, the fat mass was altered significantly, which demonstrated the function of loss of body weight by GLP-1.

Given that GLP-1 is involved in sensing and promoting an immune response to obesity, we wanted to confirm the changes and mechanism of GLP-1 in T cells. We hypothesize that GLP-1 can affect the function and the production of cytokines by CD4+ T cells via GLP-1 receptors on the surface of T cells. Therefore, this suggested that GLP-1 is important in shaping CD4+ T populations, as previous studies have also suggested in a non-autoimmune mouse model.

In summary, we reported that GLP-1 is important for systemically shaping the CD4+ T populations in high-fat-diet-induced mice. GLP-1 played a vital role in production and function of cytokines by Tregs. We also found that GLP-1 is not dependent on the normal diet. Furthermore, GLP-1 can promote the reduction of lepin, serum lipids, and fat mass. Given our findings, it may be likely that GLP-1 may provide a new target for modulating CD4+ T populations in the treatment of metabolic syndrome.

Acknowledgements This work was supported by grants from the Key Research and Development Program of Shandong Province (No. 2016GSF201012) and the Natural Science Foundation of Shandong Province (No. BS2015YY011) and the National Natural Science Foundation (No. 81500631).

Authors' contributions LS conceived the project. SS and LS designed the experiments. SS analyzed the data and conducted experiments. SS wrote and edited the manuscript.

Compliance with ethical standards

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

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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