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Treatment with *Cordyceps sinensis* enriches treg population in peripheral lymph nodes and delays type I diabetes development in NOD mice

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Cordyceps sinensis is a widely used Chinese traditional herb with a long history. In China *C. sinensis* is usually applied in the treatment of respiratory diseases, however, the efficacy of *C. sinensis* still lacks experimental evidence. Type I diabetes is a multi-factor related autoimmune disease caused by cellular-mediated destruction of insulin-producing pancreatic beta cells in the islets in human. We tested *C. sinensis* for its ability to work as an immune modulator in NOD mice, an animal model which mimicks the progression of type I diabetes in humans and found that treatment with *C. sinensis* extract could slow down disease development in NOD mice. Further research also suggested that treatment with *C. sinensis* extract increased the frequency of Treg cells and IFN-gama producing Th1 cells in peripheral lymph nodes. However, *C. sinensis* has no effect on the natural Treg cell differentiation in thymus.

1. Introduction

Type I diabetes is related to many factors such as genetics, environment such as virus infections and the abuse of medicine and drugs and diet. Genetic research have proved that self-antigen specific T cells occurring in thymus are a major reason for disease development (Bluestone and Bour-Jordan 2012). It has also become increasingly clear that disease progression results from a breakdown in the processes that maintain peripheral immune tolerance. The result of this process is localized tissue inflammation, islet dysfunction, and ultimately the destruction of pancreatic β cells due to concomitant defects in innate and adaptive immune responses (Thompson et al. 2012).

CD4 + CD25 + Treg cells were considered as the major controller in the development of several diseases including type I diabetes. Dysregulation of Treg functions in type I diabetes has been implicated as an important event in the early stage of disease development both in animal models and in humans. In Non-Obese-Diabetic (NOD) mice, which is the a widely used animal model for type I diabetes, Treg cells from old (about 16-week old) mice have less suppressing ability than those from younger mice (about 8-week old) (Gregori et al. 2003). However, it is worthy to pay attention that qualitative changes in Treg cell population have also been observed in clinical studies in human (Brusko et al. 2005; Keymeulen et al. 2005).

Cordyceps sinensis is a widely used Chinese traditional herb and firstly used about 2,000 years ago (Paterson 2012). In previous views, *C. sinensis* has a beneficial effect on cardiac arrhythmia, angina pectoris, liver diseases and diabetes. Clinical studies suggested that *C. sinensis* extract could be applied in the treatment of diabetes as an immune modulatory agent although its mechanism of action is unclear (Guo et al. 2007). Studies in animal models also reported that *C. sinensis* was effective against type II diabetes development in both genetic and strspetozotocin (STZ) induced diabetes mice and rats (Lo et al. 2004, 2006). Recent reports also indicate that *C. sinensis* has anti-hyperglycemic activities in type I diabetes (Zhang et al. 2008). Studies on chemical constituents of *C. sinensis* reported at least eight compounds; They were palmitic acid, erogosterol, ergosterol 5α,8α-peroside, cholesterol, β-sitosterol, caffeine, cereisterol, N-(2'-hydroxy-tetracosanoyl) -2-amino-1,3,4-trihydroxyoctadec-8E-ene (Li et al. 2003). Published works about FYT720, a small molecule which is isolated and modified from *C. sinensis* in NOD mice proved that treatment of FYT720 can prevent autoimmune diabetes in this mouse model and affected the lymphocytes circulating in peripheral blood (Yang et al. 2007). However, the physiological functions of these compounds are still unclear although studies about nutrition suggested that many compounds of mushroom have anti-diabetes activity (Pereral and Li 2011).

Although there are many reports about *C. sinensis* and its functions in different disease models, there is no report about its functions about Treg population in the type I diabetes model. Therefore, we performed our work with treating pre-diabetic NOD mice with *C. sinensis* to study its affects about Treg cell population. Our results indicate that *C. sinensis* has the potential to be applied in the treatment of type I diabetes.

2. Investigations and results

2.1. C. sinensis reduced the incidence of type I diabetes development in NOD mice

NOD mice are the most widely used animal model for type I diabetes and are generally considered as the most appreciated animal model for type I diabetes in human. It is well accepted

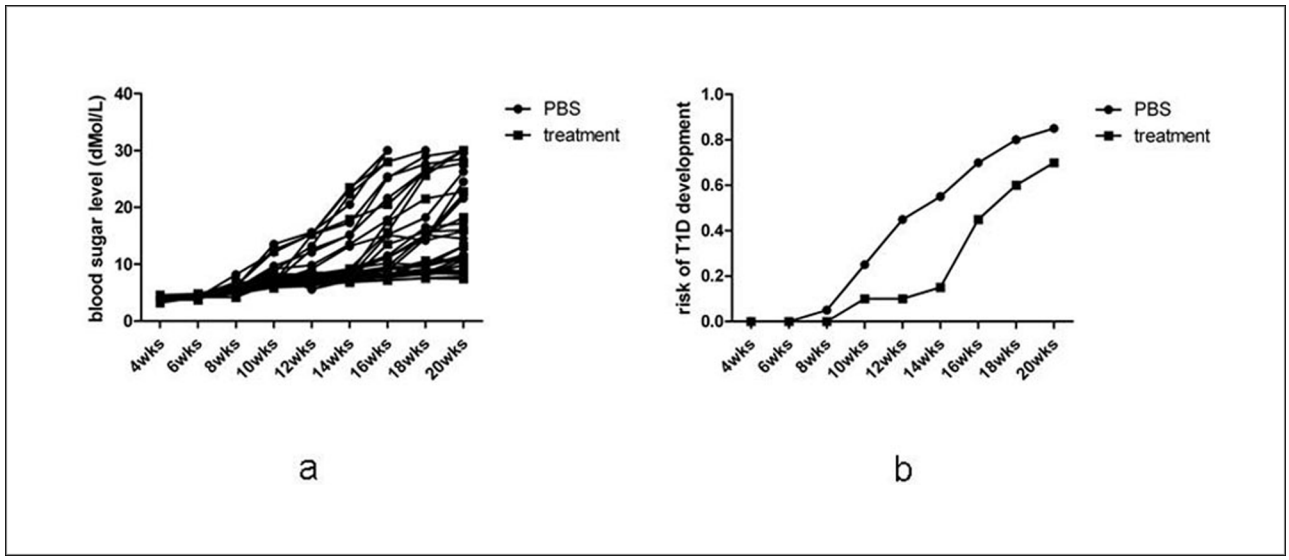

Fig. 1: Cumulative incidence of type I diabetes in NOD mice. Female NOD mice aged 4 weeks started to receive either 4 mg/kg/day *C. sinensis* abstract or 0.1 ml/day saline *via* oral gavage (n = 20 per group). Blood sugar level (a) every 2 week and (b) the risk of type I diabetes development (percentage) were shown each group. Observation was ended at 16 weeks after treatment initiated (at 20 weeks of age). $P < 0.05$ between two groups

that about 30% of female NOD mice become diabetic when they are 8 weeks old in SPF environment. The risk of disease increases with age and the incidence of type I diabetes in the old mice (more than 20 weeks) varies from 70% to 90%.

In our system, pre-diabetic female NOD mice (about 3∼4 weeks) were daily treated with *C. sinensis* extract (4 mg/kg) and the blood sugar level was tested every two weeks (Fig. 1). Process of disease development in the *C. sinensis* treatment group was delayed for more than 2 weeks. About 30% of NOD mice in PBS-treatment group became diabetic when whey were 10 weeks old, however, mice in the *C. sinensis* treatment group reached the same percentage when they were more than 14 weeks old. Furthermore, treatment with *C. sinensis* extract did not alleviate weight loss and other diabetes related symptoms in these diabetics mice (data not shown).

2.2. C. sinensis affected Treg cell population in peripheral tissues

Treg cells are the most important negative regulator in the development of type I diabetes. As previous works suggested, the risk of type I diabetes was closely related to the frequency of Treg cells in local tissues. Our previous results show that *C. sinensis* delayed type I diabetes development in NOD mice and we aimed to determine whether Treg cells were involved in this process.

We tested CD25 + Foxp3 + Treg cell population in both spleen and peripheral lymph nodes (Fig. 2a) in these female NOD mice who have been daily treated with *C. sinensis* extract for about 4 weeks and PBS-treated group as well, and found that the frequency of Treg cells in CD4 + T cells was enriched in the peripheral lymph nodes in the *C. sinensis* treated group compared to the control group (Fig. 2b). Frequency of Treg cell population in spleen was similar in *C. sinensis* treated and PBS control mice.

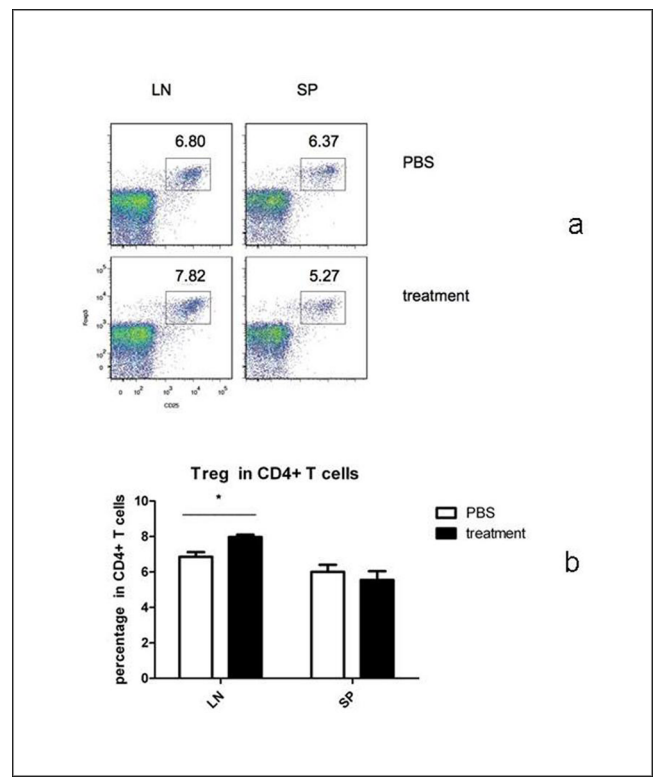

Fig. 2: Treg cell population percentage in NOD mice. Cells from peripheral lymph nodes and spleen from NOD mice treated with either *C. sinensis* abstract or PBS control. (a). Representative CD25/FoxP3 cytometry profiles of gated CD4 + TCRβ+ cells. (b).Mean percent CD25 + FoxP3 + in CD4 + T cells (mean ± SD, n = 4)

2.3. C. sinensis did not affect natural Treg cell population in thymus

In order to determine the source of the additional Treg cells in peripheral lymph nodes, whether they naturally occurred in thymus or not, we detected the natural Treg cell population in thymus of these NOD mice who have been daily treated with *C. sinensis* extract for about 4 weeks and PBS-treated group as well. Our results show that daily treatment of *C. sinensis* had no effect on the nTreg differentiation in thymus (Fig. 3).

2.4. C. sinensis recruited Th1 cell population in pancreas draining lymph node

Considering the fact that there was no such report about *C. sinensis* expanding Treg cells specifically in peripheral tissues but not in the spleen, we hypothesized that treatment with *C. sinensis* stopped T cells migration to central lymph organs such as

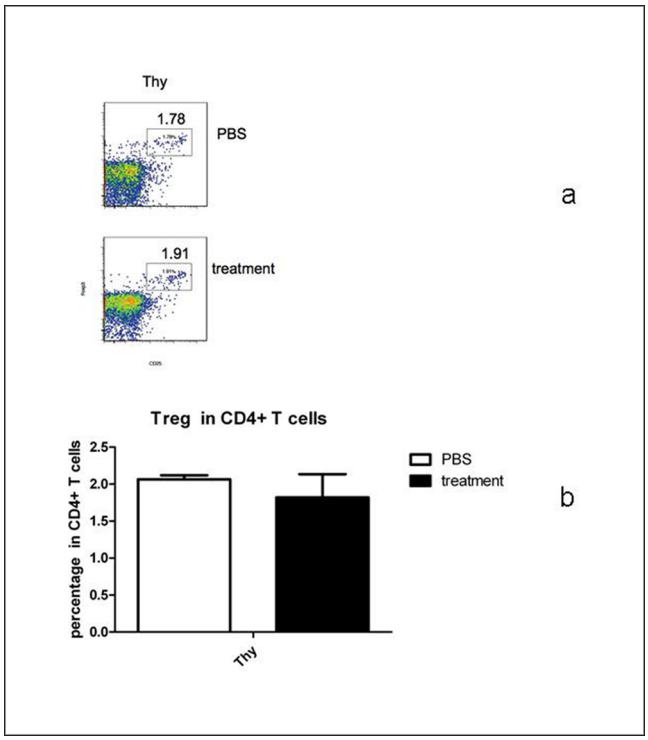

Fig. 3: Treg cell percentage in thymus of NOD mice. Thymus cells from NOD mice treated with either *C. sinensis* abstract or PBS control. (a). Representative CD25/FoxP3 cytometry profiles of gated CD4 + TCRβ+ cells. (b).Mean percent CD25 + FoxP3 + in CD4 + T cells (mean ± SD, n = 4)

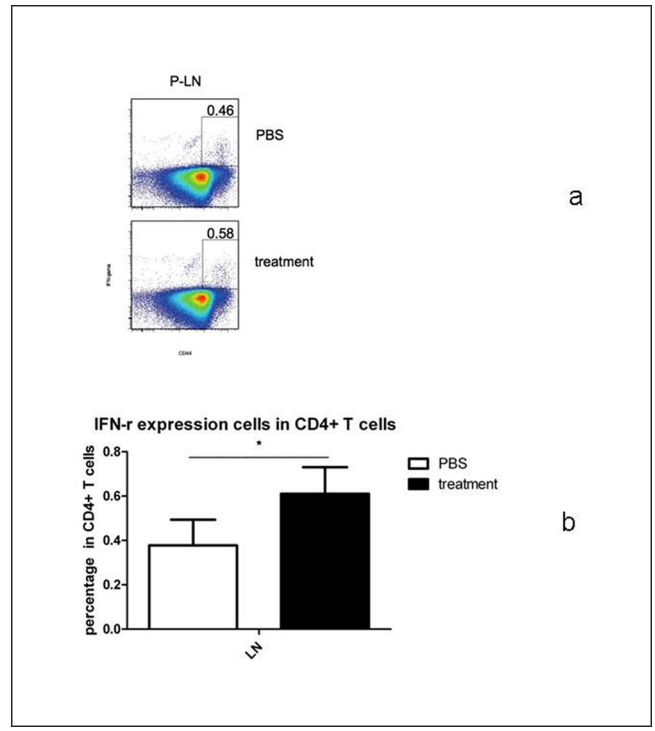

Fig. 4: IFN-gama producing Th1 cell percentage in pancreases draining lymph nodes of NOD mice. Lymph nodes cells from NOD mice treated with either *C. sinensis* abstract or PBS control were cultured *in vitro* for 4.5 hours in K medium with brefeldin A 2.5ug/ml. (a). IFN-gama + Th1 cells cytometry profiles of gated CD4 + TCRβ+ cells. (b).Mean percent CD44 + IFN-gama + in CD4 + T cells (mean ± SD, n = 4)

spleen and made them stay in the peripheral tissues *via* a non-T subsets specific pathway. To exam this hypothesis, we detected IFN-gama producing self-antigen reactivated CD4 + Th1 cell population in the pancreases draining lymph nodes in 12 week old NOD mice treated with *C. sinensis* extract or PBS control for 8 weeks (Fig. 4). The mice from the PBS-treated control group had big size pancreas draining lymph nodes and had more CD44 + activated CD4 + T cells (data not shown), according to the high blood sugar level. Frequency of INF-gama producing CD44 + CD4 + Th1 cells in the *C. sinensis* treatment group was also higher than in the PBS control group. However, the effect of *C. sinensis* on the other T cell subsets needs further examination.

3. Discussion

Type I diabetes is a multi-factor related chronic inflammatory disease. The incidence of type 1 diabetes has consistently increased worldwide during the last decades, especially in children and developed countries (Kay et al. 1991). Many environmental and genetic factors are related with the risk of type I diabetes in both human and animal models. Lymphocytes, including T cells, B cells and NK cells all play roles in the disease process (Gale 2002), however, as recent works have shown, the self-antigen (mostly beta antigen from islet insulin-producing beta cells) CTLs and the immune suppressor Treg cells are the most critical. Treg cells fuctions and whether they can contribute to this disease have been studied in the NOD model by several research groups (Gregg et al. 2004; Berzins et al. 2003) who found NOD have lower Treg frequency both in spleen and thymus than Bal b/c mice. However, studies about Treg cells differentiation in thymus did not show significant differences (Wu et al. 2002), which implies that the disorder of Treg cell population was caused by other factors, such as cytokines profile in local inflammatory tissues.

In our work, we treated prodiabetic NOD mice with *C. sinensis* extract and found that *C. sinensis* can delay type I diabetes development in NOD mice and reduce the risk of type I diabetes in old age mice significantly. However, *C. sinensis* cannot alleviate the symptoms of type I diabetes in this mice who have high blood sugar. Furthermore, we found that Treg cells population increased in peripheral lymph nodes and was reduced in the spleen. There is no report about *C. sinensis* can expanding Treg cells specifically in peripheral tissues but not in the spleen. We detected the natural Treg cell population in thymus and found that there was no significant difference between mice treated or not treated with *C. sinensis*, and hypothesized that *C. sinensis* can inhibit Treg cells migration from peripheral tissues to spleen. To test our hypothesis, we detected the Treg cell population in pancreas draining lymph nodes and found that the frequency of Treg cells also increased while comparing with NOD mice not treated with *C. sinensis*. Additional, we found that *C. sinensis* can also enrich IFN-gama producing Th1 cells in pancreas draining lymph nodes. The effects of *C. sinensis* on the other T cell subsets is unclear.

It is reported that Treg cells in NOD mice are dysfunctional and they cannot inhibit self-reactivity Th1 response efficiently (Mark and Bluestone 2005). And local enrichment of Treg increased the chances of Treg cells to meet the activated Th1 cells, which can offset the functions of these low-suppression Treg cells partially. Additional, the studies about the mechanism of FYT720 suggested that FTY720 can evoke redistribution of lymphocytes from circulation to secondary lymph tissues, leading to a reduction of circulating lymphocytes (Masubuchi et al. 1998). It seems that *C. sinensis* may contribute to a delay of type I diabetes in NOD mice by the similar pathway.

Even though much remains to be learned regarding the precise mechanism of *C. sinensis*, our present works suggest *C. sinensis* can delay type I diabetes development in NOD mice, which indicates that *C. sinensis* has the potential to be applied in preventing the development of type I diabetes in humans.

4. Experimental

4.1. Animals and reagents

NOD/LtJ mice were purchased from the Slac experimental animal centre (Shanghai) and housed in SPF environment. The experimental animal protocol met all regulations and standards and was approved by the Animal Care and Use Committee. NOD mice were randomized into 2 groups of 20 mice per group: (a) *C. sinensis* (4 mg/kg/day; treatment group) (b) PBS control group. *C. sinensis* powder was provided obtained from Jiminkexin Jiangxi, China. The methods to obtain the *C. sinensis* extract have been described previously (Zhang et al. 2005). In brief, 100 g *C. sinensis* powder was heated at 90 °C for 2 h in 500 ml distilled water and filtered. NOD mice were treated with *C. sinensis* extra 4 mg/kg/d *via* oral gavage.

4.2. Intracellular staining for Foxp3 and IFN-gama

For intracellular cytokine staining, cytokine secretion was blocked with brefeldin A, (GolgiPlug, BD Biosciences) for 4.5 h at 37 °C. Cells were then harvested, washed, and resuspended in staining buffer (0.5% BSA/PBS) containing GolgiPlug. Cells were fixed in 2% paraformaldehyde for 10 min and then washed and resuspended in staining buffer containing 0.5% saponin to permeabilize the cells. Ab binding for intracellular cytokines was conducted for 30 min at 4 °C and then were analyzed on a FACSCalibur flow cytometer (BD Biosciences). Cells were stained by FITC-conjugated anti-mouse TCRb (H57-597, ebioscience), Percp-Cy5.5-conjugated anti-mouse CD4 (GK 1.5, BioLegend), PE-conjugated anti-mouse CD25 (PC61.5, ebioscience), APC-conjugated anti-mouse Foxp3 (FJK-16 s, ebioscience) for Treg cells and Percp-Cy5.5-conjugated anti-mouse CD4 (GK 1.5, BioLegend), FITC-conjugated anti-mouse TCRb (H57-597, ebioscience), PE-conjugated anti-mouse IFN-γ (XMG1.2, BioLegend), APC-conjugated anti-mouse CD44 (IM7, BD Biosciences) for IFN-gama producing Th1 cells.

4.3. Statistical analysis

Data were analyzed using either Student's t test or ANOVA to compare the difference among groups. Numerical data are presented as the mean ± SEM. Statistical difference is considered to be significant at $P < 0.05$.

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