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Dietary Flavonol Epicatechin Prevents the Onset of Type 1 Diabetes in Nonobese Diabetic Mice

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ABSTRACT: Type 1 diabetes (T1D) is an autoimmune disease characterized by the selective destruction of pancreatic β-cells. Although successful islet transplantation provides a promising treatment, high cost, lack of donor organs, immune-mediated destruction of transplanted islets, and side effects from immunosuppressive drugs greatly limit its uses. Therefore, the search for novel and cost-effective agents that can prevent or ameliorate T1D is extremely important to decrease the burden of T1D. In this study, we discovered that epicatechin (EC, 0.5% in drinking water), a flavonol primarily in cocoa, effectively prevented T1D in nonobese diabetic (NOD) mice. At 32 weeks of age, 66.7% of control mice had overt diabetes, whereas only 16.6% of EC-treated mice became diabetic. Consistently, EC mice had significantly higher plasma insulin levels but lower glycosylated hemoglobin concentrations compared to control mice. EC had no significant effects on food or water intake and body weight gain in NOD mice, suggesting that EC's effect was not due to alterations in these variables. Treatment with EC elevates circulating anti-inflammatory cytokine interleukin-10 levels, ameliorates pancreatic insulitis, and improves pancreatic islet mass. These findings demonstrate that EC may be a novel, plant-derived compound capable of preventing T1D by modulating immune function and thereby preserving islet mass.

KEYWORDS: *epicatechin, flavonol, NOD mice, type 1 diabetes, islets*

INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disease characterized by T-cell-mediated destruction of pancreatic β-cells, leading to insulin deficiency.[1] T1D is the primary form of diabetes in children, with 95% of childhood diabetes being T1D.[2] It is estimated that 70 000 children under 15 years old develop T1D worldwide annually, and the incidence is growing by 3−5% each year.[3] Although successful islet transplantation provides a promising approach for T1D treatment, high cost from islet transplantation, lack of sufficient donor organs, ongoing immune-mediated destruction of transplanted islets, and side effects from the immunosuppressive drugs greatly limit the widespread use of this procedure.[4] Thus, the search for novel and cost-effective agents that can prevent or treat T1D could be an important strategy to decrease the burden from this disease.

A recent epidemiological study found that people living on the San Blas Islands, who are used to consuming a large quantity of flavanol-rich cocoa beverage daily, have a considerably lower incidence of ischemic heart disease, stroke, and diabetes compared to those who live on the mainland of Panama.[5] Interestingly, these differences disappeared when San Blas islanders migrated to Panama City, where the quantity of cocoa beverage consumption is considerably reduced.[6] These data suggest that cocoa may exert health-promoting effects. While the specific cocoa components primarily responsible for these actions are not known, several lines of evidence show that cocoa-derived flavanols, a subtype of flavonoids, may potentially exert various health benefits. Collectively, data from past studies demonstrate that flavanol isolates from cocoa can improve vascular function,[7−12] inhibit platelet activation and aggregation,[13] suppress the production of various inflammatory molecules,[14−20] increase the secretion of anti-inflammatory cytokines IL-4[18] and IL-5[19] and transforming growth factor (TGF)-β[20] from cultured human immune cells, improve the expression of antioxidant enzymes,[21−24] reduce blood cholesterol levels,[23−25] and acutely reduce blood pressure in healthy people,[26] but it had no significant effect on hyperglycemia in type 2 diabetic mice.[27] In addition, some studies reported that cocoa flavanols may have antioxidant properties and scavenge reactive oxygen species.[28−30] The major flavanols present in cocoa are epicatechin (EC), catechin, and procyanidin oligomers.[31] After cocoa consumption, EC is found to be the primary (>96%) flavanol in human circulation, with the plasma concentration reaching over 6 μM after the intake of cocoa, while other forms of flavanols including procyanidin dimer and catechin are less than 1% and 3%, respectively.[32] Consistent with this finding, a recent study provides evidence that EC primarily mediates the beneficial effects of flavanol-rich cocoa in humans.[9]

In the development of T1D, inflammation plays a critical role. The infiltration of inflammatory cells into the islets and subsequent insulitis are hallmarks of the pathogenesis of T1D. Activated T-cells and macrophages release several proinflammatory cytokines, such as interleukin-1β (IL-1β), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α), which are believed to be important mediators leading to β-cell destruction in T1D.[33−38] IL-4 was reported to prevent insulitis and T1D by potentiating T helper 2 (Th2) cell function,[39] and TGF-β can inhibit the expression of IFN-γ, TNF-α, and IL-1β in T-cells, macrophages, NK cells, and B-cells.[40]

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In this study, we investigated whether dietary intake of EC can modulate immunity and prevent the onset of T1D using nonobese diabetic (NOD) mice, a most widely used spontaneous T1D model that shares a variety of characteristics of patients with T1D.[41] We provided evidence for the first time that dietary supplementation of EC can preserve functional β-cell mass and prevent the onset of T1D in NOD mice.

MATERIALS AND METHODS

Chemicals. EC was purchased from Sigma-Aldrich (purity >90% by HPLC, St. Louis, MO, USA); AIN 93G diet was supplied by Dyet, Inc. (Bethlehem, PA, USA); glucometer and strips were from Kroger Inc. (Cincinnati, OH, USA); glycosylated hemoglobin (HbA1c) assay kit was purchased from Henry Schein, Inc. (Melville, NY, USA); insulin ELISA kits were from Mercodia Inc. (Winston-Salem, NC, USA); and cytokine array kits were purchased from Quansys Biosciences (West Logan, UT, USA).

Mice and Experimental Design. Four-week-old female NOD/LtJ mice were obtained from Jackson Laboratory (Bar Harbor, ME, USA). Mice were fed a standard rodent diet *ad libitum* and housed in an environmentally controlled (23 ± 2 °C; 12 h light/dark cycle) animal facility. Mice were randomly divided into two groups (n = 12) and given either 0% or 0.5% EC in drinking water. We chose this dose because our recent study showed that 0.5% EC provided in drinking water is very effective in exerting a number of beneficial effects in obese diabetic mice without causing toxicity.[42] On the basis of allometric scaling,[43] this dose of EC is equivalent to daily consumption of 250 g of typical dark chocolate containing 6% EC.[44] To ensure the stability of EC, the stock compound was stored at −80 °C and the water bottle was sealed and kept away from light. Fresh EC was made and provided to mice every other day with the same batch of EC throughout the study. Food intake and body weight were measured biweekly, and water intake was recorded every three days. Nonfasting blood glucose levels were measured in blood from the tail vein every 3−5 weeks using a glucometer. During the whole period of treatment, the general clinical condition and mortality of the mice were monitored daily. Euthanasia of animals was independently assessed by a veterinarian according to AAALAC guidelines. Mice with body weight less than 25% of their original body weight were euthanized by inhalation of CO_2, and their blood and tissues were collected and included for biochemical analysis. The animal protocol for this study was approved by the Institutional Animal Care and Use Committee at Virginia Tech.

Intraperitoneal Glucose Tolerance Test. For the glucose tolerance test, mice at 31 weeks of age (n = 5/group) were fasted for 12 h and then injected intraperitoneally with a single bolus of glucose (2 g/kg body weight).[45] Blood glucose was measured at time points of 0, 5, 15, 30, 60, and 120 min after glucose administration.

Fasting Plasma Insulin and HbA1c Measurements. At the end of the experiment, mice were fasted overnight and anesthetized for collecting blood samples. Blood HbA1c levels were measured using an assay kit, and plasma insulin concentrations were measured using an ELISA kit.

Pancreatic Islet Mass and Insulitis Evaluations. Pancreata were removed after mice were euthanized and immediately fixed in 10% neutral buffered formalin and embedded in paraffin. Tissue sections 500 μm apart from each other were deparaffinized, hydrolyzed, and stained with hematoxylin. The relative islet area was determined using point counting stereology as described previously.[46,47] Briefly, a 100-square-grid reticle (1 cm^2) was used to count points over islet tissue using an Olympus BX51 microscope. The area occupied by islets was divided by total area of pancreatic tissue on the slide to determine the relative percentage of islet area. Pancreatic islet mass was calculated by multiplying the relative islet area by the total pancreatic weight. Insulitis was scored as follows according to previously published methods:[48,49] score 0 = no lymphocytic infiltration, score 1 = peri-insulitis (less than 20% infiltration), score 2 = 20−50% infiltrated islet, score 3 = 50−80% infiltrated islet, and score 4 = more than 80% infiltration. Five sections were scored for each mouse, and 12 mice from each group were evaluated in this study.

Plasma Cytokine Measurements. Cytokines from serum were tested using a mouse cytokine array kit (Quansys Biosciences West Logan, UT, USA), including IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12p70, monocyte chemoattractant protein-1 (MCP-1), IFN-γ, TNF-α, macrophage inflammatory protein-1α (MIP-1α), granulocyte macrophage colony-stimulating factor (GMC-SF), and RANTES.

Statistical Analysis. Data were analyzed by one-way or two-way repeated measures ANOVA where appropriate. Significant differences between treatments were analyzed using Student's t test. The logrank test was applied to compare the survival distributions of the control and EC-treated groups. Data of immune cell infiltration into islets were subjected to the nonparametric Mann−Whitney U test. Differences were considered significant at $p < 0.05$.

RESULTS

Dietary Supplementation of EC Prevents T1D in NOD Mice. In the present study, we tested if EC has a beneficial effect on T1D by using NOD mice as animal models. We found that EC (0.5% in drinking water) effectively prevented the T1D onset in NOD mice (Figure 1A). At the age of 32 weeks, 8 out of 12 mice (66.7%) in the control group developed overt diabetes (nonfasting blood glucose more than 250 mg/dL), while only 2 out of 12 mice (16.7%) in the EC-treated group became diabetic

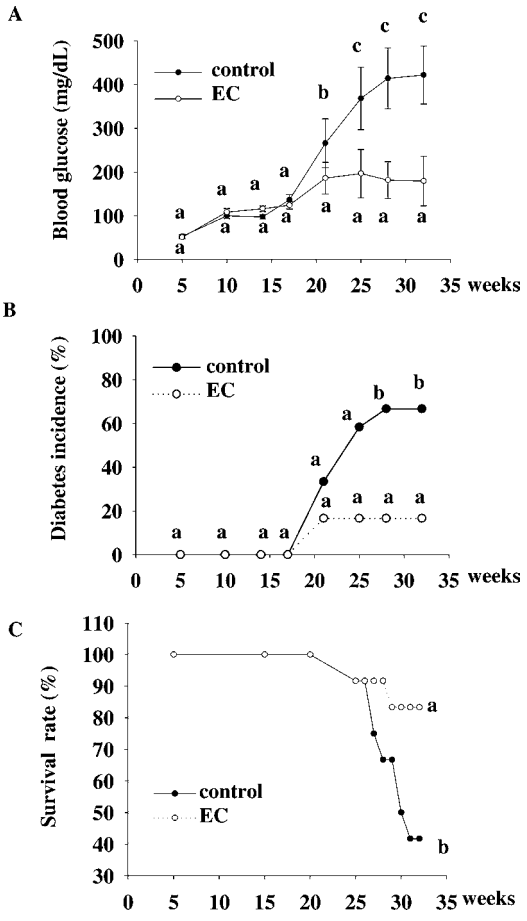

Figure 1. EC prevents the onset of diabetes in NOD mice. Five-week-old NOD-LtJ female mice were given 0.5% EC in drinking water. Aged-matched control mice were given regular water. Nonfasting blood glucose (A), the incidence of diabetes (B), and survival rate (C) were recorded. Data are expressed as mean ± SE (A) or as a percentage (B, C). Means without a common letter differ (n = 12 mice/group), $p < 0.05$.

Table 1. EC Has No Effect on Food and Water Intake and Body Weight in Nondiabetic NOD Mice

	water intake (mL/d/mouse)		food intake (g/d/mouse)		body weight (g)	
age (wk)	control	EC	control	EC	control	EC
5	2.92 (0.09)[a]	3.02 (0.00)	2.36 (0.01)	2.47 (0.06)	14.7 (0.2)	15.1 (0.2)
7	3.90 (0.07)	3.46 (0.02)	2.44 (0.01)	2.45 (0.02)	20.6 (0.2)	20.5 (0.2)
9	3.38 (0.10)	3.23 (0.23)	2.37 (0.01)	2.31 (0.04)	21.1 (0.2)	20.6 (0.2)
11	2.92 (0.09)	3.31 (0.28)	2.73 (0.04)	2.84 (0.05)	22.2 (0.2)	21.4 (0.2)
13	3.42 (0.12)	3.58 (0.33)	2.87 (0.05)	2.88 (0.05)	22.8 (0.2)	22.0 (0.2)
15	2.96 (0.18)	3.11 (0.41)	2.78 (0.03)	2.76 (0.06)	22.5 (0.2)	22.5 (0.2)
17	3.92 (0.33)	3.47 (0.24)	2.89 (0.02)	2.98 (0.09)	23.4 (0.2)	23.5 (0.3)

[a]Data are mean (±SE).

(p = 0.04, Figure 1B). Consistent with these data, EC treatment promoted survival of diabetic mice (83.3% in EC group vs 41.7% in the control) (Figure 1C). All mice used for calculating mortality rate, which were healthy before the onset of diabetes, gradually developed severe hyperglycemia and subsequently lost a significant amount of body weight, suggesting that these mice died because of diabetes and its complications, which was evaluated and confirmed by a veterinarian. EC treatment did not alter food and water intake as well as body weight of NOD mice before they became diabetic (Table 1), suggesting that the preventive effects of EC on diabetes in NOD mice is not due to alternations of these variables. We did not record these variables when mice started developing overt diabetes, because food and water intake and body weight are influenced by diabetes and thus greatly significantly changed after mice become overt diabetic. Therefore, it is impossible to differentiate the effects of EC and diabetes on these parameters after the onset of diabetes. However, we recorded all animal body weights at the time when mice died from diabetes or were euthanized at the end of experiment, which show that EC-fed mice had significantly higher body weight than control mice (25.2 ± 3.8 vs 19.8 ± 4.7), which is largely due to a significantly lower incidence of diabetes in the EC group than that in the control group, consistent with data showing that EC prevented or delayed the onset of diabetes in NOD mice.

EC Improves Glucose Tolerance and HbA1c Levels. We performed glucose tolerance tests on mice that were still alive at 31 weeks of age. Treatment with EC lowered blood glucose by about 50% (ANOVA, $p < 0.05$) during the first 60 min of the glucose tolerance test (Figure 2), which, however, could be largely due to the improved fasting blood glucose by EC treatment. To our surprise, blood glucose levels after 1 h of glucose administration were not significantly different between the two groups. To further confirm the antidiabetic effect of EC in NOD mice, we measured blood levels of HbA1c, which reflect an average of blood glucose over a period of two to three months.[50] Consistently, HbA1c concentrations were significantly lower in EC-treated mice as compared to those in the control mice (5.2 ± 0.36 vs 7.4 ± 0.76, p = 0.02) (Table 2).

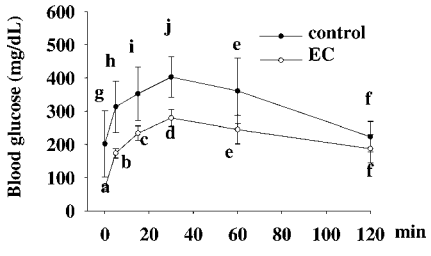

Figure 2. EC improves glucose tolerance in NOD mice. Overnight-fasted mice were injected intraperitoneally with a bolus of glucose (2g/kg body weight), followed by measurements of blood glucose at 0, 5, 15, 30, 60, and 120 min after glucose injection. Data are expressed as mean ± SE (n = 5 mice/group). Means without a common letter differ, $p < 0.05$.

Table 2. EC Lowers HbA1c and Increases Plasma Insulin Levels[a]

	HbA1c (%)	plasma insulin (μg/L)
control	7.4 (0.76)	0.129 (0.03)
EC	5.2 (0.36)[b]	0.392 (0.06)[b]

[a]Data are mean (±SE). [b]$p < 0.05$ (n = 9 and 12 mice in control and EC groups, respectively).

EC Treatment Increases Plasma Insulin Levels and Pancreatic Islet Mass. To determine if the improved blood glucose results from increased insulin secretion, we measured and compared plasma insulin levels in control mice and EC-treated mice. Mice treated with EC had significantly higher plasma insulin levels (0.392 ± 0.06 μg/L vs 0.129 ± 0.03 μg/L, p = 0.01) as compared with those in untreated mice (Table 2). Consistent with this result, EC treatment significantly improved pancreatic islet mass (1.5 ± 0.15 mg vs 0.9 ± 0.24 mg, p = 0.025) (Figure 3).

EC Improves Insulitis and Increases Plasma IL-10 and IL-12 Levels. In both T1D patients and NOD mice, invasion of pancreatic islets by immune cells and subsequent islet inflammation (insulitis) primarily causes β-cell destruction. Therefore, we evaluated whether the antidiabetic action of EC is associated with decreased insulitis. We observed that the degree of lymphatic infiltration into islets from EC-treated mice was lower than that in control mice. EC-fed mice had a significantly higher proportion of immune cell-free islets (p = 0.02) but fewer islets with clear infiltration (Figure 4), which is in agreement with the decreased occurrence of diabetes in EC-treated mice. We then measured an array of immune regulatory cytokines and chemokines (IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12, MCP-1, IFN-γ, TNF-α, MIP-1α, GMCSF, and RANTES) in the circulation. EC-treated mice increased plasma IL-10 (p = 0.000002) and IL-12 (p = 0.005) levels, while other cytokines and chemokines were not affected (Figure 5).

DISCUSSION

T1D is an insulin-deficient disease caused by T-cell-mediated autoimmune destruction of pancreatic β-cells. In the present study, we found that EC supplemented in drinking water 0.5% (w/v) can effectively prevent the onset of diabetes and

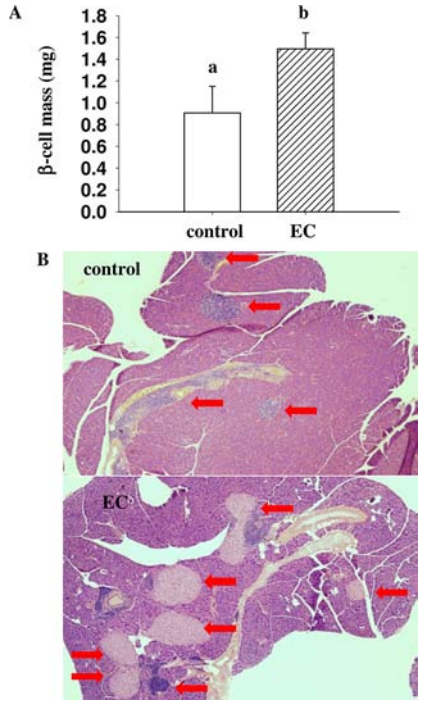

Figure 3. EC supplementation improves pancreatic islet mass in NOD mice. Islet mass was calculated by multiplying pancreas weight by relative β-cell area on tissue slides (n = 12). Images shown are representative pancreas section from control and EC-treated mice (A). Pancreatic islets are identified by arrows (B). Data are expressed as mean ± SE. Means without a common letter differ, $p < 0.05$.

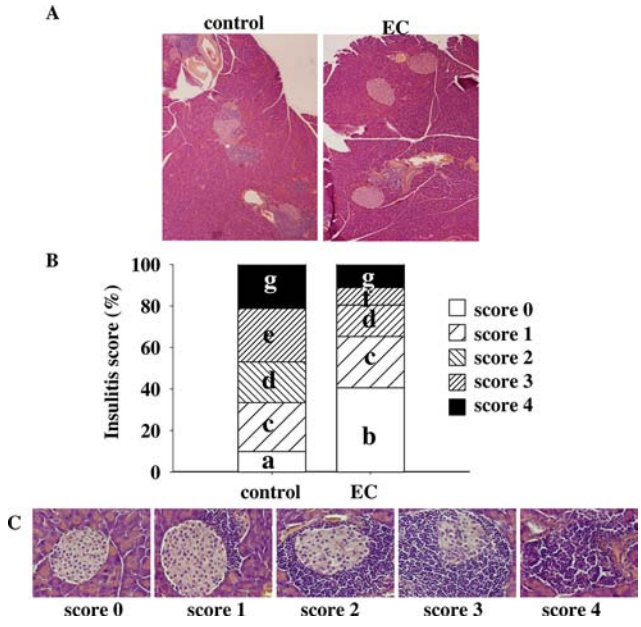

Figure 4. EC treatment ameliorates pancreatic islet insulitis. Pancreatic sections were stained with hematoxylin and assessed for insulitis as described in the Materials and Methods section. Representative histological sections of pancreas from control and EC-treated mice (A), insulitis scores (B), and representative images for different grades of insulitis (C) are shown. Twelve mice in each group and 5 sections per mouse were scored. Means without a common letter differ, $p < 0.05$.

subsequently promoted survival of NOD mice. Consistently, EC treatment significantly improved glucose tolerance and lowered HbA1c levels, which were associated with increased circulating

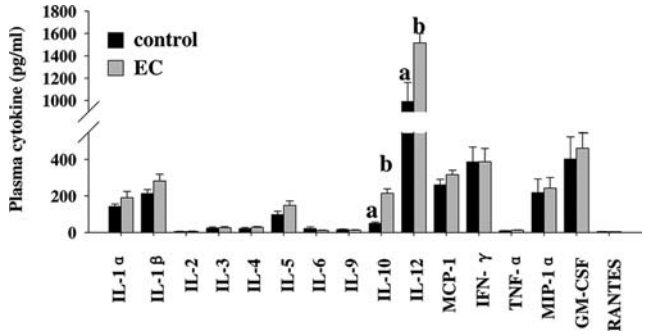

Figure 5. Supplementation of EC increases plasma IL-10 and IL-12 levels. Blood was drawn from fasted mice, and plasma samples were used for measurements of various cytokines, including IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-9, IL-10, IL-12, MCP-1, IFN-γ, TNF-α, MIP-1α, GM-CSF, and RANTES (n = 9 mice in control group, n = 12 in EC group). Means without a common letter differ, $p < 0.05$.

insulin levels, improved insulitis, and preserved pancreatic islet mass. These findings provide evidence for the first time that EC may be a natural agent that can protect pancreatic islets from autoimmune-mediated destruction and thereby prevent the development of T1D.

In the present study, long-term treatment with EC had no effect on food and water intake and body weight gain of nondiabetic NOD mice, suggesting that this compound likely has no significant toxic effect and that an antidiabetic effect of EC is not a secondary action whereby it modulates these variables in mice. Indeed, our recent study demonstrated that long-term dietary treatment with EC at this dose has an array of beneficial effects without causing toxicity in obese diabetic mice.[42] Consistently, it was reported that dietary intake of 1.5 g/kg/day of EC did not induce any toxicological relevant changes in rats (48).

Blood glucose levels are tightly regulated through the coordinated actions of several organs. The intestine is the first key organ involved in glucose homeostasis, where carbohydrate in the meal is digested and released glucose is transported to the circulation.[51] While catechins were reported to suppress intestinal absorption of glucose in rodents,[52] which could contribute to postprandial glycemic control and body weight gain, the antidiabetic action of EC in NOD mice should be primarily ascribed to its ultimate protection of functional pancreatic islets, as demonstrated by significantly larger β-cell mass in EC-treated mice. Consistently, mice given EC had significantly higher circulating insulin levels compared to those in the controls. Furthermore, EC treatment improved fasting blood glucose levels and intraperitoneal glucose tolerance, which largely reflects a direct response of β-cells to circulating glucose.[53−55]

While the pathogenic mechanisms and T-cell-mediated autoimmune process that destroy pancreatic β-cells in T1D are complex and are still not fully defined,[56−59] it is clear from past studies that the infiltration of immune cells into the islets and subsequent insulitis are hallmarks of the pathogenesis of T1D. Activated T-cells and macrophages produce pro-inflammatory cytokines, such as IL-1β, IFN-γ, and TNF-α, which are believed to be important mediators leading to β-cell destruction in T1D.[33−38] In the present study, we found that EC intake significantly reduced immune cell infiltration in the islets. This finding demonstrates that the preservation of functional β-cell mass by dietary intake of EC is likely mediated through

preventing immune cell infiltration and thereby β-cell destruction. To further examine whether EC targets the immune systems to modulate immunity in NOD mice, we measured the circulating levels of inflammation-related cytokines, which are indicators of immune cell activity. EC had no effect on most of the cytokines tested in this study. Paradoxically, we observed that EC increased plasma levels of pro-inflammatory cytokine IL-12, which was reported to enhance T1D development.[60] However, this effect of EC appears to be moderate. On the contrary, plasma IL-10 levels in EC-treated mice were 6.8-fold higher compared to those in untreated mice. IL-10 is an anti-inflammatory cytokine that is primarily secreted by Th2 cells and regulatory T-cells.[61,62] Previous studies demonstrated that administration of IL-10 or IL-10 gene transfer prevents insulitis and diabetes in NOD mice,[63−66] suggesting that IL-10 may have an important role in the control of T1D development. On the basis of these observations, it is reasonable to speculate that the effect of EC on insulitis and thereby T1D onset may be mediated by stimulating IL-10 production, which warrants further investigation.

While data from this study suggest that the antidiabetic effect of EC might be due to modulation of immunity, thereby protecting islets from immune cell-mediated destruction of pancreatic β-cells, the underlying mechanism for this action by EC is still unclear. It is well recognized that oxidative stress may play a potential role in the initiation of chronic inflammation and various degenerative diseases including diabetes, which is always associated with a decline in antioxidant levels in a number of tissues. EC is considered a potent free radical scavenger at pharmacological doses,[67] and its biological effects are frequently attributed to a presumably antioxidant activity. At physiologically relevant doses however, EC displayed a very low ability to scavenge free radicals.[68] Our recent study showed that dietary intake of epigallocatechin gallate (EGCG), a similar antioxidant primarily present in green tea, also prevented T1D in NOD mice.[69] Interestingly, unlike EC, which prevented insulitis and therefore protected islets from immune cell-mediated destruction, EGCG did not prevent immune cell infiltration into pancreatic islets but may directly promote islet survival.[69] These findings suggest that the preventive effect of EC in the pathogenesis of T1D could be primarily mediated via antioxidant-independent mechanisms.

In conclusion, we provided evidence for the first time that dietary supplementation of EC can prevent the onset of T1D in NOD mice. This protective effect is likely due to EC prevention of islets from immune cell-infiltration-mediated destruction of pancreatic islets, thereby preserving functional β-cell mass. However, further studies are still needed to define the underlying mechanism for this action by EC, which will provide the basis for further preclinical and clinical trials to evaluate its preventive and therapeutic potential for T1D.

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Notes

The authors declare no competing financial interest.

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