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Genistein modulation of streptozotocin diabetes in male B6C3F1 mice can be induced by diet

Tai L. Guo^{1,*}, Yunbiao Wang^{1,2}, Tao Xiong³, Xiao Ling⁴, and Jianfeng Zheng⁵

¹Department of Veterinary Biosciences and Diagnostic Imaging, College of Veterinary Medicine, University of Georgia, Athens, GA 30602-7382

²Key Laboratory of Wetland Ecology and Environment, Northeast Institute of Geography and Agroecology, Chinese Academy of Sciences, Changchun 130102, China

³College of Animal Science, Yangtze University, Jingzhou City, Hubei Province, China, 434025

⁴Institute for Food and Drug Control of Shandong Province, Jinan City, Shandong 250012

⁵Department of Pharmacology and Toxicology, Virginia Commonwealth University, Richmond, VA 23298-0613

Abstract

Diet and phytoestrogens affect the development and progression of diabetes. The objective of the present study was to determine if oral exposure to phytoestrogen genistein (GE) by gavage changed blood glucose levels (BGL) through immunomodulation in streptozotocin (STZ)-induced diabetic male B6C3F1 mice fed three different diets. These three diets were: NTP-2000 diet (NTP), soy- and alfalfa-free 5K96 diet (SOF) and high fat diet (HFD) with 60% of kcal from fat, primarily rendered fat of swine. The dosing regimen for STZ consisted of three 100 mg/kg doses (i.p.): the first dose was administered at approximately 2 weeks following the initiation of daily GE (20 mg/kg) gavage, and the second dose was on day 19 following the first dose, and the third dose was on day 57 following the first dose. In mice on the NTP diet, GE treatment decreased BGL with statistical significances observed on days 33 and 82 following the first STZ injection. In mice fed the HFD diet, GE treatment produced a significant decrease and a significant increase in BGL on days 15 and 89 following the first STZ injection, respectively. In mice fed the SOF diet, GE treatment had no significant effects on BGL. Although GE treatment affected phenotypic distributions of both splenocytes (T cells, B cells, natural killer cells and neutrophils) and thymocytes (CD4/CD8 and CD44/CD25), and their mitochondrial transmembrane potential and generation of reactive oxygen species, indicators of cell death (possibly apoptosis), GE modulation of neutrophils was more consistent with its diabetogenic or anti-diabetic potentials. The differential effects of GE on BGL in male B6C3F1 mice fed three different diets with varied

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*To whom correspondence should be addressed: Tai L. Guo, Ph.D., Department of Veterinary Biosciences and Diagnostic Imaging, College of Veterinary Medicine, University of Georgia, Athens, GA 30602-7382. Tel: (706) 542-1358, Fax: (706) 542-0051, tlguo1@uga.edu.

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phytoestrogen contents suggest that the estrogenic properties of this compound may contribute to its modulation of diabetes.

Keywords

genistein; diabetes; streptozotocin; immunomodulation; male B6C3F1 mice

Introduction

Immune dysregulation not only serves as a hallmark of type 1 diabetes (T1D), but also directly contributes to the pathogenesis of type 2 diabetes (T2D), β -cell dysfunction and insulin resistance (Dandona et al., 2004; Kristiansen and Mandrup-Poulsen, 2005). Depending on dose and other factors (e.g., diet), streptozotocin (STZ)-induced diabetes can be considered either T1D or T2D (Reed et al., 2000; Zhang et al., 2008; Gao et al., 2011; Muller et al., 2011; Shpilberg et al., 2012; Ramos-Rodriguez et al., 2013). The multiple low dose (MLD)-STZ-induced diabetes in mice that do not develop diabetes spontaneously is also a disease of immune origin since it is associated with a secondary autoimmune insulinitis following apoptotic injury of the pancreatic β -cells (Thomas-Vaslin et al., 1997; Ablamunits et al., 1999; Gao et al., 2013; Barbu-Tudoran et al., 2013; Yaochite et al., 2013), which confers this model the ability to detect nutrient or toxicant modulation of diabetes.

Diet affects various biological processes involving in the development of diabetes. The NTP-2000 open formula (NTP) is a defined, nonpurified wheat-based milk-free diet (Table 1; Rao et al., 2001; Chakir et al., 2005), and has been shown to increase the growth and survival of rodents (Rao et al., 2001; Rao and Crockett, 2003). The soy- and alfalfa-free 5K96 verified casein feed (SOF) is a natural ingredient diet that is formulated for using in experiments in which dietary estrogenic activity is a concern (Guo et al., 2014). High-fat diet from Harlan Laboratories (HFD; TD.06414 with 60% of kcal from fat, primarily rendered fat of swine) is commonly used for diet-induced obesity in mouse models, and STZ treatment in the presence of HFD has been shown to induce a T2D response, e.g., insulin resistance, hyperglycemia, and blood lipid disorder (Zhang et al., 2008). One objective of this study was to compare the effects of these three diets on STZ-induced diabetes in male B6C3F1 mice.

Contributions of estrogen to the development and progression of diabetes in males remain largely unknown. It has been shown that estrogen receptor (ER) α gene polymorphisms are associated with T2D and fasting glucose levels in male subjects (Meshkani et al., 2012; Linnér et al., 2013). The isoflavone genistein (GE; 4,7,4'-trihydroxyisoflavone), which is known to interact with ERs and act as an antioxidant, is a phytoestrogen found at high levels in soy products (Patisaul and Jefferson, 2010). GE exhibits weak estrogenic activity on the order of 10^{-2} to 10^{-3} compared to that of estradiol (Miksicek, 1994), but it is present in the body in concentrations (μ M) much higher than those of endogenous estrogens (Adlercreutz et al., 1993). It has been reported that GE might modulate disease outcome in male rats (Yang et al., 2011; Lee, 2006) and male mice (Fu et al., 2010) following chemical induction of diabetes; however, the exact mechanisms were unclear.

We hypothesized that exposure to the common soy phytoestrogen GE could modulate STZ-induced diabetes in male B6C3F1 mice fed the NTP, SOF or HFD diet through estrogen-related immunomodulation. The B6C3F1 mouse, a hybrid of male C3H/HeN and female C57BL/6J mice, was selected over randomly bred mice to decrease the variation between individual animal's responses and reduce the number of animals for each experiment, and yet have the vigor associated with the heterozygosity. This model has been widely used for studies of estrogenic effects (Papaconstantinou et al., 2003; Ng et al., 2006; Frawley et al., 2011). Furthermore, our studies on several strains of mice including non-obese diabetic (NOD), CD-1, C3H/HeN, C57BL/6J and B6C3F1 have suggested that MLD-STZ-induced diabetes in B6C3F1 mice was moderate, and has the potential to detect either protection or exacerbation of diabetes by various treatments (Zheng and Guo, 2009).

Materials and Methods

Animals and Animal Exposure

Male B6C3F1 mice were generated through mating male C3H/HeN with female C57BL/6J mice (Charles River Breeding Laboratories, Raleigh, NC). Mice arrived at 4–6 weeks of age, and were quarantined upon arrival. The mice were between 8 and 10 weeks of age prior to initiating the mating studies. Timed pregnant primiparous C57BL/6J mice were generated through housing two female C57BL/6J mice and one male C3H/HeN mouse in one cage for 24 h. Animal holding rooms were kept at 21–24°C and 40–60% relative humidity with a 12-h light/dark cycle. All animal procedures were conducted under an animal protocol approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee (IACUC).

Male offspring (72 in total) were used for this study, and were housed no more than 4 animals per cage in plastic cages with hardwood chip bedding and consumed NTP-2000 diet (Zeigler Bros, Inc., USA) and tap water from water bottles *ad libitum*. At approximately 4-wks of age, 24 mice were switched to the HFD diet (TD.06414; Harlan Laboratories, Madison, WI) and another 24 to the SOF diet (5K96, TestDiet, Richmond, IN), and the remaining mice were continued on the NTP diet. After 2 weeks, mice on each diet were divided into two groups and gavaged with either vehicle (VH) or GE (20 mg/kg) daily. Together, six groups were produced: (1) VHN: VH-treated mice on the NTP diet; (2) GEN: GE-treated mice on the NTP diet; (3) VHF: VH-treated mice on the SOF diet; (4) GEF: GE-treated mice on the SOF diet; (5) VHH: VH-treated mice on the HFD diet; and (6) GEH: GE-treated mice on the HFD diet.

Treatment with STZ

For STZ treatment, mice were injected (i.p.) with STZ (Sigma-Aldrich) solutions, prepared immediately before use, in 0.1 M citrate buffer (pH 4.5). The dosing regimen for STZ consisted of three 100 mg/kg doses: the first dose was given at approximately 2 weeks following the initiation of GE gavage, which was the period needed to produce a modulated immune response (Guo et al., 2007). The second dose was administered on day 19 following the first dose, and the third dose was on day 57 following the first dose. The goal was to generate a pre-diabetic state initially (Lebovitz, 1999; Ramos-Rodriguez et al., 2013), and

then, a diabetic disease. The vehicle groups (VHN, VHF and VHH) received the same amount of citrate buffer as detailed by the NIDDK Consortium for Animal Models of Diabetic Complications' (AMDCC) protocol (available from <http://www.amdcc.org>).

Measurement of blood glucose levels (BGL)

Monitoring of glycemic status was performed prior to the beginning of treatment to ensure that there were no significant differences between groups in BGL and no outlier animals. Thereafter, the animals were monitored for blood glucose changes every week. Non-fasting blood glucose was measured directly in small samples of venous blood (tail nick) using Accu-Chek Diabetes monitoring kit (Roche Diagnostics, Indianapolis, IN; Guo et al., 2014).

Bio-Plex Pro Diabetes Assay

Measurement of eight common metabolic biomarkers in sera was performed with the Bio-Plex Pro™ mouse diabetes immunoassay by following the manufacturer's instruction (Bio-Rad, Hercules, CA). Plates were run on a Bio-Plex MAGPIX™ Multiplex Reader with Bio-Plex anager™ MP Software (Luminex, Austin, TX). Each biomarker concentration was calculated as pg/ml. The levels of sensitivity in this panel were 0.64, 4.31, 0.59, 0.5, 68.29, 5.07, 2.98 and 184.89 pg/ml for ghrelin, gastric inhibitory polypeptide (GIP), glucagon-like peptide-1 (GLP-1), glucagon, insulin, leptin, plasminogen activator inhibitor type 1 (PAI-1) and resistin, respectively.

Spleen and thymus collection and single-cell suspension preparation

Spleen and thymus were removed from mice and placed in 3 ml Earle's Balanced Salt Solution (EBSS) with HEPES (GibcoBRL, Grand Island, NY). Cell suspensions were prepared by pressing the spleen between the frosted ends of two microscope slides into a 60 × 15-mm petri dish. The slides were washed with the buffer using a pasture pipette, and then single-cell suspensions were placed into 4-ml plastic test tubes. Splenocytes and thymocytes were then centrifuged at 300 × g for 5 min and resuspended in 3 ml RPMI 1640 (GibcoBRL, Grand Island, NY) complete medium with 10% fetal bovine serum (Hyclone, Logan, Utah). The cell count was determined using a ZBII Coulter counter in the presence of ZAP-OGLOBIN II lytic reagent (Coulter Corp., Miami, FL).

Flow cytometric analysis of splenic T cells, T cell subsets, B cells, neutrophils, natural killer (NK) cells and thymocytes

The respective cell types were labeled with an appropriate monoclonal antibody (mAb) conjugated with a fluorescent molecule for visualization. For splenic T cell enumeration, a fluorescein isothiocyanate (FITC) conjugated anti-mouse CD3e mAb (diluted 1:80; BD PharMingen, San Diego, CA) was used. For splenic B cell enumeration (Figure 1A), FITC conjugated anti-mouse IgM mAb (diluted 1:80) was used (BD PharMingen). For splenic CD8⁺ and CD4⁺ cells (Figure 1B), a FITC conjugated mAb specific for the CD8a (Ly-2) marker and phycoerythrin (PE) conjugated anti-CD4 (BD PharMingen) were used. Anti-mouse NK1.1 with PE was used to label splenic NK cells (CD3⁺NK1.1⁺; Figure 1C). For splenic neutrophils (Gr-1⁺Mac-3⁻; Figure 1D), FITC conjugated mAb for Gr-1 and PE conjugated anti-Mac-3 were used. FITC conjugated rat anti-mouse CD24 and PE conjugated

rat anti-mouse CD11a were obtained from BD Pharmingen and eBioscience (San Diego, CA), respectively. In addition to CD4 and CD8 (Figure 1E), CD44-PE and CD25-FITC were also used to label thymocytes (Figure 1F). Isotype-matched irrelevant antibodies were used as controls. Following the addition of the reagents, the cells were incubated at 4°C in the dark for 30 minutes. The cells were washed and enumeration performed on a Becton Dickinson FACScan Flow Cytometer in which log fluorescence intensity was read and a forward scatter threshold high enough to eliminate red blood cells. Five thousand cells were counted for each sample. Absolute cell numbers were calculated using the percentage of cells and the total cell counts.

Analysis of Ψ_m and ROS generation

Splenocytes and thymocytes (1×10^6 cells/ml) were stained for 15 min with 40 nM 3,3'-dihexyloxacarbocyanine (DiOC₆(3); Life Technologies, Grand Island, NY) and 2 μ M hydroethidine (Life Technologies) to measure mitochondrial transmembrane potential (Ψ_m) and reactive oxygen species (ROS) generation, respectively (Guo and White, 2010). Following excitation at 488 nm (250 mW), emission was monitored through a 530/30 nm bandpass filter for DiOC₆(3) and 575/26 nm bandpass filter for ethidium; logarithmic amplification was used to detect the fluorescence. Viable cells were characterized as a discrete population exhibiting bright DiOC₆(3) fluorescence and virtually no ethidium fluorescence (DiOC₆(3)^{bright}Eth^{dim}). A decline in Ψ_m was shown by a decrease in DiOC₆(3) fluorescence (DiOC₆(3)^{dim}); ROS generation was evidenced by an increase in ethidium fluorescence (Eth^{bright}). The contour plot for such analysis has been published previously (Guo and White, 2010).

Statistical Analysis

To determine the type of analysis to be used, the Bartlett's Test for homogeneity was conducted. The software used was JMP Pro 10. Homogenous data were analyzed using a one-way analysis of variance, and the Dunnett's t test was used to determine differences between the control and experimental groups. Non-homogenous data were analyzed using a nonparametric analysis of variance and the Wilcoxon Rank Test to determine differences between the vehicle control group and exposure groups. A group was considered statistically significant from the control group if $p < 0.05$.

Results

Body weight and blood glucose levels in STZ-treated male B6C3F1 mice on three different diets

To determine how diets affected the growth and BGL following STZ treatment, three different diets (NTP, SOF and HFD) were included in this study, and the VH groups were used for comparison. The body weights of VHH mice were greater than both VHN and VHF mice starting at approximately two weeks of the study (Figure 2A). No differences in body weights were observed between VHN and VHF mice at any time points evaluated during the study.

Following the injection of first STZ and prior to the third STZ, VHN mice had higher BGL than VHF mice. Significant changes were observed on days 6, 15 and 26 after the first STZ injection (Figure 2B). Following the second STZ and prior to the third STZ, VHH mice had the highest BGL among the three groups (Figure 2B). After the third STZ injection, there were sharp increases in BGL in all three diet groups; however, the BGL started declining with time in VHH mice at approximately two weeks following the third STZ injection, and significant differences were reached at two time points when compared to either VHN mice (days 82 and 96 after the first injection of STZ) or VHF mice (days 89 and 96 after the first injection of STZ).

Effect of GE on the body weight, organ weights and blood glucose levels in STZ-treated male B6C3F1 mice on three different diets

GE treatment had no significant effects on the body weights in mice fed either the NTP (Figure 3A) or SOF diet (Figure 3C). For mice fed the HFD diet, GE treatment significantly decreased the body weight in the period between injections of the first and third STZ (Figure 3E).

In mice fed the NTP diet, there were slight decreases in BGL in GE-treated mice (GEN) when compared to VHN mice with statistical significances observed on days 33 and 82 after the first STZ injection (Figure 3B). In mice fed the SOF diet, although the BGL in GE-treated mice (GEF) were numerically lower than VH mice (VHF) following the third injection of STZ, none of the changes reached the levels of statistical significance (Figure 3D). In mice fed the HFD diet, a significant decrease and a significant increase in BGL were observed in GE-treated mice (GEH) on days 15 and 89 following the first STZ injection when compared to VHH mice, respectively (Figure 3F).

For organ weights (Table 2), VHF mice had smaller liver (absolute weight) than both VHN and VHH mice. Due to the difference in body weights, relative liver weights of VHN mice were greater than that of VHH mice. Although the relative weights of lungs and kidneys of both VHN and VHF mice were greater than that of VHH mice, there were no significant differences in the absolute weights of these two organs among the three VH groups (Table 2). When compared to VHH mice, VHN and VHF mice had smaller thymuses (absolute weight), although there were no significant differences among them when the data were expressed as relative weight. There were no differences in the weights of either spleen or pancreas among these three VH groups no matter when the data were expressed as the absolute or the relative value (Table 2).

In mice fed the NTP diet, GE treatment significantly decreased both the absolute and relative weights of liver and spleen when compared to VHN mice (Table 2); however, no significant effects on either the absolute or the relative weights of lungs, thymus, kidneys and pancreas were observed. In mice fed the SOF diet, GE treatment significantly decreased the relative weights of both liver and kidneys when compared to VHF mice (Table 2). No significant differences were observed in other organs no matter whether the data were expressed as the absolute weights or the relative weights (Table 2). For mice on the HFD diet, GE treatment had no significant effects on either the absolute or relative weights of liver, spleen, lungs, thymus, kidneys and pancreas (Table 2).

Effects of GE on splenocyte differentials and the surface marker expression in STZ-treated male B6C3F1 mice on three different diets

Immune responses play an important role in MLD-STZ-induced diabetes (Thomas-Vaslin et al., 1997; Ablamunits et al., 1999; Gao et al., 2013; Barbu-Tudoran et al., 2013; Yaochite et al., 2013). Thus, flow cytometric analysis of splenocyte differentials was undertaken. The data were expressed as both percent and absolute values, and significant differences among VH mice on three different diets were noted as follows: For percent values (Figure 4), VHN mice had a lower %CD4⁺CD8⁻ T cells than VHF mice (Figure 4A); VHF mice had a lower %IgM⁺ B cells than VHH mice (Figure 4C); VHH mice had a lower %NK (NK1.1⁺CD3⁻) cells than both VHN and VHF mice (Figure 4D); and VHN mice had a higher %neutrophils (Gr-1⁺MAC-3⁻) than both VHF and VHH mice (Figure 4E). For absolute values (Table 3), VHF mice had a lower number of CD4⁺CD8⁺ T cells than VHH mice; VHH mice had more IgM⁺ B cells than both VHN and VHF mice; and VHN mice had more neutrophils than both VHH and VHF mice (Table 3).

To determine if GE alteration of BGL was related to its immunomodulatory effects, the splenocyte differentials (both absolute and relative) were further evaluated. In mice on the NTP diet, GE treatment significantly increased %CD4⁺CD8⁺ T cells (Figure 4B) and decreased both the percentage (Figure 4E) and absolute number of neutrophils (Gr-1⁺MAC-3⁻; Table 3). In mice on the SOF diet, GE treatment significantly increased %CD4⁺CD8⁻ T cells (Figure 4A) and the number of CD4⁺CD8⁺ T cells (Table 3), and both the percentage and the number of IgM⁺ B cells (Figure 4E and Table 3); however, %NK cells was decreased (Figure 4D). In mice on the HFD diet, GE treatment had no significant effects on any of the cells evaluated when the data were expressed as either the percent value (Figure 4) or absolute value (Table 3).

By staining cell surface markers of CD24 and the surface integrin subunit CD11a, splenocytes could be separated into three distinctive populations (Figure 5A): CD11a⁺CD24⁻ (upper left quadrant), CD11a⁺CD24⁺ (upper right quadrant), and CD11a⁻CD24⁺ (lower right quadrant). In addition to the percentages, the mean fluorescence intensities (MFI) of CD11a and CD24 of these three populations were compared among the different diet groups, and further evaluated for their modulation by GE. Differences among VH mice on the three different diets were mainly observed in the CD11a⁺CD24⁻ cells with significantly lower percentage in VHH mice than both VHN and VHF mice (Figure 5C). A slight, albeit significant, difference between VHN and VHF mice was observed for the MFI of CD11a on CD11a⁺CD24⁻ cells (Figure 5B). GE modulation was observed in mice on the NTP diet in which the CD11a expression by CD11a⁺CD24⁻ cells and the percentage of CD11a⁻CD24⁺ cells were significantly decreased when compared to the corresponding control (Figure 5B and 5D).

Effects of GE on thymocyte differentials in STZ-treated male B6C3F1 mice on three different diets

Two sets of cell surface markers were employed to determine thymocyte differentials. Thymocytes were first stained for CD4 and CD8 markers. When compared to VHN mice, both VHF and VHH mice had lower %CD4⁺CD8⁻ (Figure 6A) and %CD4⁻CD8⁻

thymocytes (Figure 6C). VHH mice, but not VHF mice, also had significantly lower %CD4⁺CD8⁺ thymocytes than VHN mice (Figure 6D). Correspondingly, both VHF and VHH mice had higher %CD4⁺CD8⁺ thymocytes than VHN mice (Figure 6B). GE treatment only decreased %CD4⁺CD8⁺ thymocytes when mice were maintained on the NTP diet (Figure 6A). When the data were expressed as absolute values, there was a significant difference between VHN and VHH mice in the number of CD4⁺CD8⁺ thymocytes (Table 3). However, GE treatment had no significant effects on these thymocyte subsets in any of the diet groups (Table 3).

The expression of CD44 and CD25 by thymocytes was also determined by flow cytometric analysis. When compared to VHN mice, %CD44⁺CD25⁺ and %CD44⁺CD25⁺ thymocytes were lower in both VHF and VHH mice (Figure 7A, 7B). Additional differences in VHH mice included higher %CD44⁺CD25⁺ (Figure 7C) and lower %CD44⁺CD25⁺ thymocytes when compared to VHN mice (Figure 7D). When the data were expressed as absolute values, there were no significant differences among these groups (Table 3).

In mice on the NTP diet, GE treatment had no significant effects on any of these thymocyte subsets when the data were expressed as either percent (Figure 7) or absolute values (Table 3). The effects of GE in mice on the HFD diet were similar to those in mice on the SOF diet: GE treatment significantly decreased %CD44⁺CD25⁺ (Figure 7A) and %CD44⁺CD25⁺ (Figure 7B), and increased %CD44⁺CD25⁺ thymocytes (Figure 7C). GE had no effects on %CD44⁺CD25⁺ thymocytes in either of the diet groups (Figure 7D). When the data were expressed as absolute values, there were no significant differences between vehicle and GE-treated groups in any of the diet groups except for a decrease of CD44⁺CD25⁺ thymocytes in GEF mice when compared to VHF group (Table 3).

Effects of GE on Ψ_m and ROS generation by splenocytes and thymocytes in STZ-treated male B6C3F1 mice on three different diets

It has been reported that splenocytes from STZ-induced diabetic rats exhibited more apoptotic features than the control animals including disruption of mitochondrial membrane potential (Manna et al., 2010). In addition to its estrogenic effects, GE also possesses antioxidant activities (Kim and Lim, 2013). To shed some light the mechanisms underlying the modulatory effect of diets and GE on the development of diabetes, the Ψ_m and ROS generation by splenocytes and thymocytes were evaluated. DiOC₆(3)^{dim}Eth^{dim} cells has been shown to be early apoptotic cells while DiOC₆(3)^{dim}Eth^{bright} cells late apoptotic cells (Castedo *et al.*, 1995).

Splenocytes from VHH mice were more prone to spontaneous cell death (possibly apoptosis), which was reflected by the finding that less than 2% cells were viable (DiOC₆(3)^{bright}Eth^{dim}, Figure 8A) and approximately 90% cells were DiOC₆(3)^{dim}Eth^{bright} (possibly in the stage of late apoptosis; Figure 8C) after incubation for 24 h at 4°C followed by 4 h at 37°C. When compared to VHN mice, which had approximately 13% viable cells (Figure 8A) and 62% DiOC₆(3)^{dim}Eth^{bright} cells (Figure 8C), a significant decrease in %viable cells and a significant increase in %DiOC₆(3)^{dim}Eth^{bright} cells were observed in VHH mice.

GE treatment did not significantly affect the apoptotic potential of splenocytes in mice maintained on either the NTP or HFD diets. In mice on the SOF diet, VHF mice had approximately 19% viable cells (Figure 8A) and 66% DiOC₆(3)^{dim}Eth^{bright} cells (Figure 8C); GE treatment decreased % viable cells and increased % DiOC₆(3)^{dim}Eth^{bright} cells significantly. Neither the diet nor GE affected % DiOC₆(3)^{dim}Eth^{dim} cells (possibly early apoptotic) significantly (Figure 8B).

In contrast to splenocytes, thymocytes from VHH mice (approximately 68% viable cells, 16% DiOC₆(3)^{dim}Eth^{dim} cells and 16% DiOC₆(3)^{dim}Eth^{bright} cells) were more resistant to spontaneous cell death than that from VHN mice (approximately 19% viable cells, 26% DiOC₆(3)^{dim}Eth^{dim} cells and 54% DiOC₆(3)^{dim}Eth^{bright} cells) and VHF mice (approximately 46% viable cells, 20% DiOC₆(3)^{dim}Eth^{dim} cells and 34% DiOC₆(3)^{dim}Eth^{bright} cells) after incubation for 24 h at 4°C followed by 2.5 h at 37°C (Figure 8D, 8E, 8F). Thymocytes from VHF mice also exhibited less cell death than VHN mice as reflected by higher % viable cells and lower % DiOC₆(3)^{dim}Eth^{dim} cells and % DiOC₆(3)^{dim}Eth^{bright} cells.

GE treatment decreased the cell death of thymocytes in mice maintained on both the NTP and HFD diets, which manifested as a significant increase in viable cells (Figure 8D) and a significant decrease in DiOC₆(3)^{dim}Eth^{bright} cells (Figure 8F). GE treatment in mice on the SOF diet, however, did not alter the percentages of respective apoptotic populations significantly.

Discussion

Many attempts have been made to identify environmental factors that influence the development of diabetes in humans, but the results have not always been consistent. Analysis of nutrient components of diabetes-promoting, standard cereal-based diets, such as NTP-2000, showed that the major diabetes-promoting ingredients were wheat, soy, and milk protein sources (Scott, 1996). However, it has been reported that the soy phytoestrogen GE reduces hyperglycemia and islet cell loss in alloxan-induced diabetic male Sprague-Dawley rats (Yang et al., 2011) and in STZ-induced diabetic male C57BL/6J mice (Fu et al., 2010; Elmarakby et al., 2011). In addition, supplementation of GE and isolated soy protein increased the plasma insulin level and decreased the hemoglobin A1c level in STZ-induced diabetic male Sprague-Dawley rats (Lee, 2006). Our previous study has also suggested that oral administration of GE reduced the incidence and increased the time to onset of T1D in genetically susceptible female NOD mice when fed a soy- and alfalfa-free 5K96 diet (Guo et al., 2014). In this study, we explored the underlying immunological mechanisms using STZ-induced diabetic male B6C3F1 mice. Although the present diet-related effects on BGL were small, these results are in agreement with other reports of GE in male animals (Lee, 2006; Fu et al., 2010; Elmarakby et al., 2011; Yang et al., 2011), where modulation depended on diets.

The NTP-2000 diet has been selected for the National Toxicology Program's toxicology and carcinogenesis studies since 1995 (Rao et al., 1997). Soybean and fish meals are the major sources of protein, corn and wheat products are the major sources of carbohydrates, soy oil

and corn oil are the major sources of fat, and alfalfa meal, oat hulls and purified cellulose are the major sources of fiber in this diet. The SOF diet 5K96 is based on the NIH-31 formula, except that casein replaces the protein contributed by soy and alfalfa, and soy oil is replaced by corn oil, to preclude phytoestrogens that might be present. Among the three diet groups, the highest BGL were observed in mice on the HFD diet between the second and third STZ injections, which was likely due to HFD-induced increases in BGL. HFD-mediated hyperglycemia in the presence of low dose of STZ has been reported (Reed et al., 2000; Fricovsky et al., 2012). However, following the third STZ injection, VHH mice had the lowest BGL. The reasons for these changes are unknown, but likely related to the food components including the fatty acid profiles (e.g., n-6, n-3 polyunsaturated fatty acids and saturated fatty acids) in these diets.

Although GE treatment reduced BGL in male B6C3F1 mice that were maintained on the NTP diet, the biological significance was unclear. The expression profiles of eight biomarkers involved in the regulation of glucose metabolism and obesity were measured using the Bio-Plex Pro mouse diabetes 8-plex immunoassay, and they included ghrelin, GIP, GLP-1, glucagon, insulin, leptin, PAI-1 and resistin. GE treatment had no significant effects on any of these biomarkers (data not shown). For mice on the HFD diet, GE treatment decreased body weights prior to the third STZ injection and BGL prior to the second STZ injection. These periods represent the pre-diabetic stage or early stages of T2D (Ramos-Rodriguez et al., 2013). However, these presumable beneficial effects were lost following the third STZ injection in these HFD-fed mice, suggesting that higher dose of STZ might have overridden the effects of GE. Therefore, further study of the protective roles of GE in early stages of T2D is warranted (Kim et al., 2006).

Myeloperoxidase activity was found to be 3-fold higher in the pancreas following MLD-STZ treatment (Mabley et al., 2003), and our previous studies have shown that MLD-STZ treatment induced an increase of splenic neutrophils in B6C3F1 mice (Zheng and Guo, 2009). In diabetic patients, hyperglycemia has also been linked to neutrophil dysfunction (Alba-Loureiro et al., 2007). Defects in neutrophil apoptosis, and increases in neutrophil oxidative respiratory burst activity and production of IL-8, IL-1 β , TNF- α and IL-1 α may contribute to the chronic inflammation observed in these patients (Karima et al., 2005; Hatanaka et al., 2006; Hand et al., 2007; Hanses et al., 2011). Interestingly, mice on the NTP diet had the highest BGL, and also exhibited the highest number of splenic neutrophils among the three diet groups in this study. Moreover, GE treatment decreased both the percentage and absolute number of neutrophils, and also decreased BGL in mice fed the NTP diet. Additional studies of neutrophil contribution in MLD-STZ-induced diabetes will not only help identify the mechanisms underlying this disease but also shed light on GE modulation of diabetes.

Barouch et al. (2000) have also shown that the expression of CD11a by neutrophils was increased in diabetic male Long-Evans rats. In our study, mice on the NTP diet had the highest expression of CD11a by the CD11a⁺CD24⁻ splenocytes among the three diet groups. Moreover, GE treatment decreased the expression of CD11a by CD11a⁺CD24⁻ splenocytes, and also decreased BGL in mice fed the NTP diet. Thus, further study of CD11a⁺CD24⁻ splenocyte identity and the CD11a expression by neutrophils will be of importance.

However, such effects of GE were absent when mice were maintained on either the SOF diet or HFD diet. In addition, GE modulation of other immune parameters was also diet-dependent. While GE treatment increased T cells in mice on both the NTP and SOF diets, only mice on the SOF diet exhibited an increase in B cells and a decrease in NK cells. Mice on the HFD diet did not show any changes in the splenic parameters examined. In our previous study with male rats maintained on SOF diet (Guo et al., 2002), GE treatment also increased both T cells and B cells, and decreased NK cells. These consistent findings between mice and rats highlighted the importance of immunomodulatory role of GE.

HFD-induced apoptosis has been reported to occur in various tissues or organs including liver (Wang et al., 2008), brain (Moraes et al., 2009), heart (Ballal et al., 2010), kidneys (Chung et al., 2012), and enterocytes (Gniuli et al., 2008). Although more evidence using different detection methods, such as TUNEL, Annexin V staining and caspase assay is needed, splenocytes from the HFD-fed mice in our study seemed to exhibit more apoptotic features than other groups including decreased Ψ_m and increased ROS generation; however, thymocytes from VHH mice were more resistant to cell death (possibly apoptosis; The order of cell death potential of thymocytes was: NTP > SOF > HFD), which was consistent with the observation that mice on the HFD diet had the biggest thymus among the VH groups maintained on three different diets. This is an unexpected finding, and the reasons for the apparent differential effects of HFD on the cell death of splenocytes (increases) and thymocytes (decreases) are unknown. Moreover, GE treatment increased cell death of splenocytes with significant changes observed in SOF mice, while it decreased cell death of thymocytes with significant changes in mice on both the HFD and NTP diets. Although the significance of these modulations is currently unclear, it is interesting to note that the cell death potential of thymocytes seemed to be positively related to the BGL.

Thymocytes pass through a series of stages of development: $CD4^-CD8^- \rightarrow CD4^+CD8^+ \rightarrow CD4^+CD8^-$ or $CD4^-CD8^+$ thymocytes. The $CD4^-CD8^-$ thymocytes are further divided into four stages: $CD44^+CD25^- \rightarrow CD44^+CD25^+ \rightarrow CD44^-CD25^+ \rightarrow CD44^-CD25^-$ (Haks et al., 1999). GE treatment had more effects on $CD44^+CD25^-$ than $CD4^+CD8^+$ subsets, suggesting that GE might affect the early development of thymocytes. Among the thymocyte subsets evaluated, mice on the HFD diet had a similar pattern to mice on the SOF diet, although VHH mice had the lowest $%CD4^-CD8^+$ and $%CD44^-CD25^+$ subsets in these three diet groups. Thymus has been demonstrated to be the major organ where estrogen exerts its immunomodulatory effects (Luster et al., 1984; Erbach and Bahr, 1991; Selvaraj et al., 2005). The concentrations of both phytoestrogen GE and daidzein were found to be below the limit of detection in SOF diet (0.5 ppm; Guo et al., 2002) and presumably low in the HFD diet, while the NTP diet contained 5% soybean meal and 7.5% alfalfa meal (the total concentrations of phytoestrogens ranged between 95 and 134 mg/kg; Thigpen et al., 1999; Degen et al., 2002). In this study, VHN mice had higher BGL than VHF mice during the entire study period with significant differences observed following the injection of first STZ and prior to the third STZ. These differences were more likely due to a decrease in the BGL in mice on the SOF diet rather than an increase in the BGL in mice on the NTP diet because all these mice were on the NTP diet in their whole life. It is interesting to speculate that perinatal phytoestrogen exposure may contribute to the differential effects observed between these diets.

Thymus has been shown to be sensitive to both acute and chronic stress (Everds et al., 2013). However, there were no significant differences in thymic and body weights between STZ treatment only and VHN groups (data not shown), suggesting that our gavage procedure posed minimal stress to mice. Therefore, the effects of GE observed in this study should represent the chemical itself. For a 4-month-old infant who consumes soy formula as directed by the manufacturers, approximately 6–11 mg/kg body weight of isoflavones can be obtained (Setchell et al., 1997). The 20 mg/kg dose used in this study therefore was minimally above present exposure levels in humans on a mg/kg-basis. This amount of GE in a mouse is much lower than a clinical human treatment dose (approximately 100 mg/day) in terms of milligram per square meter of body surface, which usually gives more accurate interspecies extrapolation (Hodgson, 1997). Additionally, the serum level of GE (1.4–7.5 μ M) in mice that have been fed 1000 ppm GE-containing diet (~80 mg/kg body weight) was equivalent to that in men who received 100 mg GE/day (Djuric et al., 2001; Yellayi et al., 2002; Bhandari et al., 2003), and in infants on soy formula (Cao et al., 2009).

In summary, we observed in this study that oral exposure to GE at a physiologically relevant dose slightly, but significantly, reduced BGL in diabetic male B6C3F1 mice on the NTP diet and in pre-diabetic mice on the HFD diet. Although GE treatment affected both T cells and B cells, GE modulation of neutrophils may be more related to the anti-diabetic and diabetogenic potentials of this compound. Estrogen has been reported to delay neutrophil apoptosis, and increase its degranulation and oxidative stress markers (Molloy et al., 2003; Chiang et al., 2004). Various ERs including ER α , ER β , non-classical ER and G-protein-coupled receptor 30 have been identified (Molero et al., 2002; Filardo et al., 2007; Holladay et al., 2010), and GE interacts with them with different affinity. Additionally, GE can affect the activities of various enzymes (Wiegand et al., 2009). Further study of the molecular mechanisms underlying the modulatory effect of GE on diabetes, that specifically focus on neutrophils, are needed to understand contributions of estrogenic and enzyme inhibitory activities (e.g., tyrosine kinase inhibition) to dysregulated glucose homeostasis, and the safety issues associated with ingestion of this compound.

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Abbreviations used

BGL	blood glucose levels
EBSS	Earle's Balanced Salt Solution
GE	genistein
HFD	high fat diet
NK cells	natural killer cells
NOD	non-obese diabetic

NTP	NTP-2000 open formula
SOF	soy- and alfalfa-free 5K96 verified casein feed
STZ	streptozotocin
T1D	type 1 diabetes
T2D	type 2 diabetes

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Highlights

1. Diets affected streptozotocin-induced diabetes in male B6C3F1 mice.
2. Genistein modulation of streptozotocin diabetes can be induced by diet.
3. Genistein modulation of neutrophils is associated with blood glucose levels.

Figure 1.

Representative dot plots of flow cytometric analysis of (A) splenic IgM⁺ B cells, (B) splenic CD4 and CD8 T cells, (C) splenic NK (NK1.1⁺CD3⁻) cells (lower right quadrant), (D) splenic neutrophils (Gr-1⁺MAC-3⁻; lower right quadrant), (E) CD4CD8 thymocytes, and (F) CD44CD25 thymocytes.

Figure 2.

Effects of different diets on body weight (A) and blood glucose levels (B) in streptozotocin-induced diabetic male B6C3F1 mice following gavage with vehicle (25 mM Na₂CO₃) daily. Animals were treated as described in the Materials and Methods, and their body weights and blood glucose levels measured weekly. VHN: VH-treated mice on the NTP diet; VHF: VH-treated mice on the SOF diet; VHH: VH-treated mice on the HFD diet. N = 11–12. *a*, *p* < 0.05 between VHN and VHF; *b*, *p* < 0.05 between VHN and VHH; *c*, *p* < 0.05 between VHF and VHH.

Figure 3.

Effect of genistein on body weight (A, C, E) and blood glucose levels (B, D, F) in male B6C3F1 mice on three different diets following treatment with STZ (100 mg/kg; 3X). VHN: VH-treated mice on the NTP diet; GEN: GE-treated mice on the NTP diet; VHF: VH-treated mice on the SOF diet; GEF: GE-treated mice on the SOF diet; VHH: VH-treated mice on the HFD diet; GEH: GE-treated mice on the HFD diet. N = 9–12. *, $p < 0.05$ when compared to corresponding VH controls.

Figure 4.

Genistein affected the percentages of splenic CD4⁺CD8⁻ T cells (A), CD4⁻CD8⁺ T cells (B), IgM⁺ B cells (C), NK (NK1.1⁺CD3⁻) cells (D), and neutrophils (Gr-1⁺MAC-3⁻; E) in male B6C3F1 mice on three different diets following treatment with STZ (100 mg/kg; 3X). Animals were treated, and flow cytometric analysis of splenocytes performed as described in the Materials and Methods. VHN: VH-treated mice on the NTP diet; GEN: GE-treated mice on the NTP diet; VHF: VH-treated mice on the SOF diet; GEF: GE-treated mice on the SOF diet; VHH: VH-treated mice on the HFD diet; GEH: GE-treated mice on the HFD diet. N = 6. *a*, *p* < 0.05 between VHN and VHF; *b*, *p* < 0.05 between VHN and VHH; *c*, *p* < 0.05 between VHF and VHH. *, *p* < 0.05 when compared to corresponding VH controls.

Figure 5.

Genistein affected splenocyte differentials and surface marker expression in male B6C3F1 mice on three different diets following treatment with STZ (100 mg/kg; 3X). (A) A representative contour plot (CD11a vs. CD24) of flow cytometric analysis; (B) The mean fluorescence intensities (MFI) of CD11a by CD11a⁺CD24⁻ cells; (C) %CD11a⁺CD24⁻ cells, and (D) %CD11a⁻CD24⁺ cells. Animals were treated, and flow cytometric analysis performed as described in the Materials and Methods. VHN: VH-treated mice on the NTP diet; GEN: GE-treated mice on the NTP diet; VHF: VH-treated mice on the SOF diet; GEF: GE-treated mice on the SOF diet; VHH: VH-treated mice on the HFD diet; GEH: GE-treated mice on the HFD diet. N = 6. *a*, *p* < 0.05 between VHN and VHF; *b*, *p* < 0.05

between VHN and VHH; $c, p < 0.05$ between VHF and VHH. *, $p < 0.05$ when compared to corresponding VH controls.

Figure 6.

Genistein affected the percentages of CD4⁺CD8⁻ thymocytes (A), CD4⁺CD8⁺ thymocytes (B), CD4⁻CD8⁻ thymocytes (C), and CD4⁻CD8⁺ thymocytes (D) in male B6C3F1 mice on three different diets following treatment with STZ (100 mg/kg; 3X). Animals were treated, and flow cytometric analysis of thymocytes performed as described in the Materials and Methods. VHN: VH-treated mice on the NTP diet; GEN: GE-treated mice on the NTP diet; VHF: VH-treated mice on the SOF diet; GEF: GE-treated mice on the SOF diet; VHH: VH-treated mice on the HFD diet; GEH: GE-treated mice on the HFD diet. N = 6. *a*, *p* < 0.05 between VHN and VHF; *b*, *p* < 0.05 between VHN and VHH. *, *p* < 0.05 when compared to corresponding VH controls.

Figure 7.

Genistein affected the percentages of CD44⁺CD25⁻ thymocytes (A), CD44⁺CD25⁺ thymocytes (B), CD44⁻CD25⁻ thymocytes (C), and CD44⁻CD25⁺ thymocytes (D) in male B6C3F1 mice on three different diets following treatment with STZ (100 mg/kg; 3X). Animals were treated, and flow cytometric analysis of thymocytes performed as described in the Materials and Methods. VHN: VH-treated mice on the NTP diet; GEN: GE-treated mice on the NTP diet; VHF: VH-treated mice on the SOF diet; GEF: GE-treated mice on the SOF diet; VHH: VH-treated mice on the HFD diet; GEH: GE-treated mice on the HFD diet. N = 6. *a*, *p* < 0.05 between VHN and VHF; *b*, *p* < 0.05 between VHN and VHH. *, *p* < 0.05 when compared to corresponding VH controls.

Figure 8.

Genistein affected the Ψ_m and ROS generation of splenocytes (A, B, C) and thymocytes (D, E, F) in male B6C3F1 mice on three different diets following treatment with STZ (100 mg/kg; 3X). The percentages of viable (A, D), DiOC₆(3)^{dim}Eth^{dim} (possibly early apoptotic; B, E), and DiOC₆(3)^{dim}Eth^{bright} cells (possibly late apoptotic; C, F) were determined by staining with fluorescent dyes DiOC₆(3) and hydroethidine after incubation of splenocytes for 24 h at 4°C followed by 4 h at 37°C, and of thymocytes for 24 h at 4°C followed by 2.5 h at 37°C as described in the Materials and Methods. VHN: VH-treated mice on the NTP diet; GEN: GE-treated mice on the NTP diet; VHF: VH-treated mice on the SOF diet; GEF: GE-treated mice on the SOF diet; VHH: VH-treated mice on the HFD diet; GEH: GE-treated mice on the HFD diet. N = 6. a, *p* < 0.05 between VHN and VHF; b, *p* < 0.05 between VHN and VHH; c, *p* < 0.05 between VHF and VHH. *, *p* < 0.05 when compared to corresponding VH controls.

Table 1

Macronutrient composition of the experimental diets

Diets	<u>Amount by weight (%)</u>			Additional Information
	Protein	Carbohydrates	Fat	
NTP-2000 open formula (NTP)	14.6	53.5	8.2	Open formula, nonpurified wheat-based milk-free rodent diet (Zeigler Bros, Inc., Gardners, PA)
5K96 verified casein feed (SOF)	22.0	66.6	11.4	Casein based soy- and alfalfa-free diet; less than 1.0 ppm total isoflavones (aglycone equivalents of genistein, daidzein and glycitein; TestDiet, Purina Mills, St. Louis, MO)
TD.06414 high fat diet (HFD)	18.4	21.3	60.3	Using primarily rendered fat of swine. The composition of the fat: 37% saturated, 47% mono-unsaturated, and 16% poly-unsaturated (Harlan Laboratories, Madison, WI)

Table 2

Effects of genistein on organ weights (both absolute and relative weights) in streptozotocin-induced diabetic male B6C3F1 mice fed three different diets

Groups	Thymus	Lung	Pancreas	Spleen	Kidneys	Liver
VHN	mg 55.6 ± 3.4	263.5 ± 10.9	175.8 ± 7.1	112.2 ± 7.4	636.9 ± 20.0	2071.6 ± 65.4
	% 0.15 ± 0.01	0.73 ± 0.03	0.49 ± 0.03	0.31 ± 0.01	1.76 ± 0.04	5.73 ± 0.15
GEN	mg 64.3 ± 4.0	259.8 ± 9.7	157.1 ± 9.2	93.9 ± 3.1 *	600.7 ± 25.2	1816.2 ± 63.3 *
	% 0.18 ± 0.01	0.74 ± 0.04	0.44 ± 0.03	0.26 ± 0.01 *	1.68 ± 0.06	5.11 ± 0.20 *
VHF	mg 56.7 ± 3.4	260.1 ± 12.8	163.4 ± 10.6	110.4 ± 9.8	644.7 ± 26.3	1846.1 ± 59.8 ^d
	% 0.16 ± 0.01	0.72 ± 0.02	0.42 ± 0.05	0.31 ± 0.03	1.80 ± 0.06	5.21 ± 0.26
GEF	mg 66.2 ± 4.6	269.4 ± 12.0	172.2 ± 11.9	95.8 ± 5.0	626.5 ± 27.7	1729.4 ± 71.8
	% 0.18 ± 0.01	0.72 ± 0.02	0.46 ± 0.03	0.25 ± 0.01	1.66 ± 0.03 *	4.60 ± 0.15 *
VHH	mg 76.8 ± 5.1 ^{b, c}	282.1 ± 12.3	180.2 ± 10.6	129.6 ± 7.7	645.1 ± 17.2	2038.1 ± 100.1 ^c
	% 0.18 ± 0.02	0.65 ± 0.02 ^{b, c}	0.42 ± 0.03	0.31 ± 0.03	1.52 ± 0.10 ^{b, c}	4.75 ± 0.27 ^b
GEH	mg 84.8 ± 6.6	309.9 ± 14.6	203.1 ± 10.2	118.2 ± 5.7	659.7 ± 26.0	2320.4 ± 113.8
	% 0.18 ± 0.01	0.68 ± 0.03	0.44 ± 0.02	0.26 ± 0.01	1.44 ± 0.05	5.04 ± 0.18

Male B6C3F1 mice were gavaged daily with vehicle (25 mM Na₂CO₃) or genistein (20 mg/kg) for up to 130 days. VHN: VH treatment on the NTP diet; GEN: GE treatment on the NTP diet; VHF: VH treatment on the SOF diet; GEF: GE treatment on the SOF diet; VHH: VH treatment on the HFD diet; GEH: GE treatment on the HFD diet. N = 9–12.

^a *p* 0.05 between VHN and VHF;

^b *p* 0.05 between VHN and VHH;

^c *p* 0.05 between VHF and VHH;

* *p* 0.05 between VH and GE treatments on the same diet.

Table 3

Effect of genistein on the number of splenocyte and thymocyte subsets in streptozotocin-induced diabetic male B6C3F1 mice fed three different diets

Groups	VHN	GEN	VHF	GEF	VHH	GEH
Splenocyte Differentials ($\times 10^6$)						
CD4 ⁺ CD8 ⁻	11.3 $\pm$ 2.9	10.2 $\pm$ 4.2	9.7 $\pm$ 4.0	17.9 $\pm$ 2.2	19.1 $\pm$ 3.3	17.6 $\pm$ 2.3
CD4 ⁺ CD8 ⁺	6.2 $\pm$ 1.1	6.0 $\pm$ 1.3	4.3 $\pm$ 1.5	8.2 $\pm$ 0.8 [*]	9.6 $\pm$ 1.3 ^c	8.2 $\pm$ 0.9
IgM ⁺	34.4 $\pm$ 7.5	28.8 $\pm$ 4.9	24.7 $\pm$ 9.7	66.5 $\pm$ 9.9 [*]	89.4 $\pm$ 12.8 ^{b, c}	80.8 $\pm$ 10.0
NK1.1 ⁺ CD3 ⁻	2.8 $\pm$ 0.7	1.4 $\pm$ 0.7	2.1 $\pm$ 0.9	1.5 $\pm$ 0.4	1.4 $\pm$ 0.6	0.4 $\pm$ 0.1
Gr-1 ⁺ MAC-3 ⁻	4.1 $\pm$ 0.7	0.8 $\pm$ 0.3 [*]	0.6 $\pm$ 0.3 ^a	0.6 $\pm$ 0.3	1.3 $\pm$ 0.8 ^b	0.2 $\pm$ 0.1
Total Cells	102.6 $\pm$ 28.6	62.0 $\pm$ 20.9	66.7 $\pm$ 27.4	93.2 $\pm$ 10.8	130.4 $\pm$ 19.4	93.4 $\pm$ 12.3
Thymocyte Differentials ($\times 10^6$)						
CD4 ⁺ CD8 ⁻	8.7 $\pm$ 1.4	6.1 $\pm$ 0.6	7.9 $\pm$ 0.7	6.9 $\pm$ 0.5	8.0 $\pm$ 0.9	7.8 $\pm$ 0.1
CD4 ⁺ CD8 ⁺	50.6 $\pm$ 7.9	41.6 $\pm$ 5.1	58.1 $\pm$ 7.3	54.8 $\pm$ 6.1	63.6 $\pm$ 6.3	70.6 $\pm$ 3.1
CD4 ⁺ CD8 ⁺	2.7 $\pm$ 0.5	2.1 $\pm$ 0.2	2.3 $\pm$ 0.2	1.9 $\pm$ 0.2	2.1 $\pm$ 0.3	2.0 $\pm$ 0.1
CD4 ⁺ CD8 ⁻	10.9 $\pm$ 1.8	11.3 $\pm$ 2.2	7.4 $\pm$ 1.0	6.7 $\pm$ 1.3	6.3 $\pm$ 0.9 ^b	7.1 $\pm$ 0.7
CD44 ⁺ CD25 ⁻	2.9 $\pm$ 0.5	2.4 $\pm$ 0.3	2.5 $\pm$ 0.2	2.0 $\pm$ 0.2	2.5 $\pm$ 0.2	2.0 $\pm$ 0.2
CD44 ⁺ CD25 ⁺	1.9 $\pm$ 0.4	1.3 $\pm$ 0.3	1.3 $\pm$ 0.2	0.8 $\pm$ 0.1 [*]	1.4 $\pm$ 0.1	1.1 $\pm$ 0.1
CD44 ⁺ CD25 ⁻	64.8 $\pm$ 9.2	54.9 $\pm$ 7.3	69.2 $\pm$ 8.2	65.6 $\pm$ 6.2	74.0 $\pm$ 7.2	82.5 $\pm$ 2.6
CD44 ⁺ CD25 ⁺	3.3 $\pm$ 1.0	2.5 $\pm$ 0.5	2.9 $\pm$ 0.4	1.9 $\pm$ 0.1	2.1 $\pm$ 0.2	1.9 $\pm$ 0.1
Total Cells	73.0 $\pm$ 11.0	61.1 $\pm$ 7.5	75.8 $\pm$ 8.8	70.3 $\pm$ 6.4	80.0 $\pm$ 7.6	87.5 $\pm$ 2.8

Male B6C3F1 mice were gavaged daily with vehicle (25 mM Na₂CO₃) or genistein (20 mg/kg) for up to 130 days. VHN: VH treatment on the NTP diet; GEN: GE treatment on the NTP diet; VHF: VH treatment on the SOF diet; GEF: GE treatment on the SOF diet; VHH: VH treatment on the HFD diet; GEH: GE treatment on the HFD diet. N = 6.

^a *p* 0.05 between VHN and VHF;

^b *p* 0.05 between VHN and VHH;

^c *p* 0.05 between VHF and VHH.

* *p* 0.05 between VH and GE on the same diet.