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Garcinia kola treatment exhibits immunomodulatory properties while not affecting type 1 diabetes development in an experimental mouse model

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Abstract

Objective: T cells orchestrate an inflammatory response that destroys pancreatic insulin-producing β cells during the development of autoimmune type 1 diabetes (T1D). *Garcinia kola* Heckel (GK) is a plant widely exploited in West African traditional medicine. Some of the therapeutic effects of GK nut's extract (GKE) have been suggested to be due to its anti-inflammatory potential. Since GKE has never been investigated in a T1D experimental model, nor in the T cells' context, we aimed to determine whether GKE exhibits antidiabetic properties and affects T cells by its anticipated anti-inflammatory action.

Methods: The effect of aqueous GKE (aGKE) ingestion, 100 mg/kg daily by drinking water over the period of 6 weeks, has been tested in a low-dose streptozotocin-induced (LDSTZ) mouse model of autoimmune T1D. T cells were studied *in vitro* and *in vivo* in mice treated by aGKE.

Results: The results showed that aGKE treatment, which started a week before induction of disease, neither delayed the development of T1D, nor reduced glycemia severity. Interestingly, aGKE treatment did affect T cells and their function, significantly decreasing the frequency of helper (T_H) and cytotoxic (T_C) T cells, while elevating the levels of pro-inflammatory cytokines, TNF-α, IL-6, and IFN-γ, and suppressing IL-2.

Conclusion: In conclusion, our results did not confirm the antidiabetic property of GKE, while suggesting its therapeutic exploration in T_H2-dependent pathologies that benefit from an aggravated T_H1 response, such as allergies.

Keywords

Garcinia kola extract, Type 1 diabetes, Low-dose streptozotocin (LDSTZ) T1D, T cells, cytokines

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Introduction

Type 1 diabetes (T1D) is a T cell-dependent disease characterized by the autoimmune destruction of the pancreatic insulin-producing β cells with a consequent complete lack of insulin and an occurrence of hyperglycemia. While the insulin administration provides a foundation for the current treatment strategy, disease onset prevention

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would be the desired goal in the fight against T1D. Despite recent advances in preventative efforts, such as immunotherapy with manipulation of T cells, these strategies demonstrate varying degrees of effectiveness, toxicity, and usefulness, remaining a prominent challenge in drug research and development.[1,2]

Experimental murine models have been used for studying autoimmune diabetes. T1D induced by low-doses of streptozotocin in mice—LDSTZ model—exhibits similarities in T cell involvement, cytokine disbalance, and the inflammatory lesion of pancreatic islets with a human disease.[3,4] T cells populations and their respective cytokines, such as the cytotoxic (T_C), T-helper (T_H)1 and T_H17 cells, play a pathogenic role in T1D, in contrast to protective action of T_H2 and regulatory T cells (T_{reg}).[5–7]

Garcinia kola Heckel (GK) has been widely used in African traditional medicine because of its claimed health benefits ranging from antibacterial, antiviral, and antiparasitic to hypoglycemic, neuroprotective and antiasthmatic, just to name a few. Besides its use in West African folkloric medicine, GK nut has been chewed daily as a cultural staple believed to prevent a plethora of diseases.[8–10] Pharmaceutical activities of GK nut and its extracts (GKEs) have been widely attributed to kolaviron (KV), which represents a mixture of biflavonoids.[8,9,27] Some of the therapeutic effects of GKE have been suggested to be due to the antioxidative, radical scavenging, and anti-inflammatory properties.[11–14] Whereas the antidiabetic effects of GK have never been studied in T1D, daily chewing of GK nut as a potential T1D preventative measure might serve like an attractive, safe alternative to current immunotherapy efforts.

This study investigated whether the aqueous GKE (aGKE) treatment, using a delivery method closest to the humans' chewing of GK nuts, suppresses disease onset and severity in a mouse model of T1D. We hypothesized that aGKE administration, if impacting T1D development, would affect T cell populations or their function.

Material and methods

GKE preparation and liquid-chromatography mass spectrophotometry (LC-MS) analysis

GK seeds were obtained from Dr Oladele Gazal, St Cloud State University, purchased in Ijebu-Ode, Ogun State, Nigeria. The seeds were verified in the Department of Forestry and Wildlife Management, Federal University of Agriculture, Abeokuta, Ogun State, Nigeria. GK seeds were dried and grounded into a powder. A stock aGKE solution of 40 mg/mL was prepared via aqueous extraction described by Ogunmoyole et al.[14] A 1:5 *w:v* ratio of GK seed powder was added to autoclaved water, followed by 48 h of maceration using a magnetic stirring plate (Thermo Fisher) at 4°C. This solution was filtered and dried for 72 h at 37°C. The filtrate was collected, weighed, and the appropriate amount of autoclaved water was added to make a stock solution at 40 mg/mL. The aGKE stock solution was divided into aliquots and stored in a −20°C freezer for further use. In order to assure that our extraction method produces consistent results, three separate extraction procedures were performed using described protocol, and respective aGKE samples were sent to the University of Iowa (https://chem.uiowa.edu/research/resources/mass-spectrometry-facility) for liquid-chromatography mass spectrophotometry (LC-MS) analysis. The following active compounds, originally described by Iwu et al.,[27] were confirmed in our aGKE samples: *Garcinia* biflavonoid (GB) 1, GB-2, and kolaflavonone.

Mice

C57BL/6J (B6) mice were originally purchased from The Jackson Laboratory (Bar Harbor, ME). They were bred at St Cloud State University vivarium. Male B6 mice were used for the experiments described in this manuscript. They were kept in NexGen Lo-Profile cages (Allentown Inc., Allentown, NJ) in the room with a 12-h light/dark cycle, 22°C temperature, and 40–60% relative humidity. B6 mice had ad libitum access to their food (AIN-93G Rodent Diets, Harlan, Indianapolis, IN) and autoclaved water. Mice were euthanized by CO_2 asphyxiation. A resource equation method for sample size determination and justification of animal numbers was used. St Cloud State University Institutional Animal Care and Use Committee approved all the protocols and procedures performed on experimental mice (Protocol ID #5–98).

Experimental design

aGKE treatment and monitoring of T1D development. Seven-week-old B6 male mice were randomly divided into GKE treatment and control groups. A pilot experiment was performed in which water intake was determined in mice of the same sex, age and weight as the experimental mice, following the Boston University protocol "Adding the novel compound to the drinking water and documentation of fluid intake" (https://www.bu.edu/researchsupport/compliance/animal-care/working-with-animals/additives-to-the-drinking-water-for-rats-and-mice-iacuc/). Then, the aGKE was added in a dose of 100 mg/kg, based on mice weight, and the water intake was determined. It was necessary to assure that fluid intake of aGKE-enriched water was not diminished and that animals did not become dehydrated because of any new taste, smell, or other factor altering the drinking water.

GKE treatment groups received 100 mg/kg aGKE daily in their drinking water throughout the entire experimental

period of 6 weeks. The control groups for aGKE treatment received just drinking water. At 8 weeks of age, mice started receiving STZ injections over the 5 days. Glycemia and body weight measurements were taken during the following time points, starting on day 3 before STZ administration, and continuing bi-weekly from day 8 (since a rise in glycemia levels was previously observed in this experimental model at that time point[4]) to day 30 post the initial STZ injection. Additional experiments were performed in which healthy mice (not STZ-administered) were treated by aGKE in drinking water for the same period of 6 weeks.

T cell studies

The LDSTZ-administered and aGKE-exposed mice were euthanized on days 11 and 30 post-initial STZ injection to investigate GKE's effects on T cells during the T1D development, by studying splenocyte counts, viability, T cell proliferation, T cell subsets, and their cytokine production. An additional set of experiments was performed to study the same immune parameters in healthy (not STZ-administered) mice treated by aGKE.

Induction of T1D

T1D was chemically induced in mice by five intraperitoneal (i.p.) injections of 40 mg/kg STZ (Sigma-Aldrich, St Louis, MO). STZ was dissolved in 0.05 M citrate buffer (pH 4.5), and injected to control and aGKE-treated 8-weeks-old B6 male mice, as described previously.[4,6]

Blood glucose and body weight measurements

To determine a blood glucose level, a drop of 0.6 µL tail vein blood was placed onto a strip and measured by Accu-Chek Aviva glucose meter (Roche Diagnostics, Indianapolis, IN). This method does not require anesthesia, allowing a precise determination of glycemia with a minimal physiologic disturbance of a mouse.[15] The body weight was recorded at the same time as blood glucose was sampled. Diabetes was determined after a mouse exhibited two repeated measurements of 250 mg glucose/dL or higher.

Preparation of splenocytes

Splenocytes were prepared from the isolated spleens as described previously.[6,16] In brief, a spleen was forced through a 70-µm nylon mesh strainer (BD Falcon, San Jose, CA); the resulting suspension was then treated with ACK Lysis Buffer (NH_4CL 8.29 g/L, $KHCO_3$ 1.0 g/L, EDTA Na_2 $2H_2O$ 0.0375 g/L; Lonza BioWhittaker, Walkersville, MD) to remove erythrocytes, and washed three times using phosphate-buffered saline (PBS, pH 7.5). Trypan blue (Lonza BioWhittaker) exclusion was utilized to count cells in a hemocytometer and determine cell viability.

T cell proliferation assay

Splenocytes obtained from aGKE-treated and control mice were suspended in RPMI-1640 medium containing 1U penicillin/ml, 100 µg streptomycin/ml, and 10% fetal calf serum (FCS) (Sigma) at the concentration of $4x10^5$ cells/100 µL. Concanavalin A (ConA) (Sigma) was added at 3 µg/mL, and the cells were cultured for 72 h at 37°C under 5% CO_2. Proliferation was quantified using an Alamar Blue colorimetric assay (Invitrogen, Grand Island, NY), and the optical densities measured following the manufacturer's instructions by the ELISA plate reader (GeneMate, Kaysville, UT), as described previously.[4]

Splenocyte staining and flow cytometry

The aliquots of 10^6 splenocytes, isolated from each mouse, were suspended in a buffer [0.1% NaN3, 1% FCS in PBS (pH 7.4)], exposed to appropriate antibodies, and incubated at 4°C for 30 min in the dark. Thereafter, cells were washed, 10,000 events acquired by FACSCalibur flow cytometer, and analyzed using CellQuest Pro software (BD Biosciences, San Diego, CA), as previously described.[4,16] For quantification of immune cell markers, such as CD4 (T_H), CD8 (T_C), CD3 (T cells), CD4/CD25 (T_{reg}), CD45RB220 (B cells), CD335 (NK cells) and CD11b (macrophages), the following fluorochrome-labeled antibody clones were used: peridinin chlorophyll-a protein (PerCP)-conjugated anti-CD4 (clone RM4–5), fluorescein isothiocyanate (FITC)-conjugated anti-CD8 (clone 53–6.7), allophycocyanin (APC)-conjugated anti-CD25 (clone 3C7), phycoerythrin (PE)-conjugated anti-CD3 (clone 145–2C11), APC-conjugated anti-CD45RB220 (clone RA3–6B2), FITC-conjugated anti-CD335 (clone NKp36), and PerCP-conjugated anti-CD11b (clone M1/70) (all from BD Biosciences).

Cytokine measurement

The levels of interleukin (IL)-2, IL-4, IL-6, IL-10, IL-17A, interferon (IFN)-γ, and tumor necrosis factor (TNF)-α were determined in the supernatants of 48-hr-cultured splenocytes stimulated by ConA using a commercially available cytokine kit (mouse cytometric bead assay $T_H1/T_H2/T_H17$ kit, BD Biosciences), and analyzed by FCAP Array software (SoftFlow, New Brighton, MN).[4,16] The sensitivity level of studied cytokines was as following: 0.1 pg IL-2/ml, 0.03 pg IL-4/ml, 16.8 pg IL-10/ml, 0.8 pg IL-17 A/ml, 0.5 pg IFN-γ/ml, and 0.9 pg TNF-α/ml.

In vitro *GKE treatment*

Splenocytes obtained from non-treated healthy B6 mice were cultured and stimulated by ConA (as described previously) in the presence of aGKE. Serial dilutions of aGKE were prepared starting at 1000 µg/ml down to 2 µg/mL. Cells were cultured for 72 h and 48 h, and T cell proliferation and cytokine production were quantified, respectively, as described above.

Statistical analysis

For assessment of *in vivo* obtained data between the experimental groups, such as diabetes incidence and glycemia/body weight levels, the statistical software JMP's survival analysis and log-rank test (SAS Institute Inc.), and one-way analysis of variance (ANOVA) with repeated measures were used, respectively. Data obtained from *in vitro* experiments, evaluating T cell proliferation and cytokine levels, were analyzed performing ANOVA and Wilcoxon method for each comparison using JMP statistical software. For all other results, in which *ex vivo* obtained data were evaluated (immunophenotyping, T cell proliferation, and cytokine profiles), a Student's t-test was performed; a p-value < 0.05 was considered as statistically significant.

Results

aGKE exhibits immunomodulatory effects on T cells in vitro

Previous studies demonstrated *in vitro* anti-inflammatory potential of GKE using the monocytes/macrophage cell lines.[17–19] Since there are no studies on the effects of GKE on T cells, we asked whether aGKE would affect T cell function *in vitro* by assessing their mitogen-induced proliferation capacity and cytokine profiles. Five experiments were performed, in which aGKE was added in the concentration range from 0 µg/mL to 1000 µg/mL. Figure 1 Illustrates that the addition of aGKE did not affect ConA-induced T cell proliferation (Figure 1(a)) while influencing the production of some cytokines (Figures 1(b)–(h)). A statistically significant increase of TNF-α and IL-6 levels were observed with an addition of the highest concentrations of aGKE (500 µg/mL and 1000 µg/mL, respectively) (Figures 1(d) and (f)), while IL-17 level was markedly increased even by the addition of lower aGKE concentrations (Figure 1(c)). Interestingly, there was a prominent drop in IL-2 obtained by exposure to a low range of aGKE concentrations (2 µg/mL to 125 µg/mL) (Figure 1(h)). A trend in increased IL-10 (Figure 1(b)) and suppressed IL-4 production (Figure 1(g)) was observed by the addition of aGKE to the mitogen-stimulated T cell cultures, whereas IFN-γ levels seemed to be unaffected (Figure 1(e)).

In vivo *aGKE treatment does not affect disease incidence and glycemia levels in LDSTZ model of T1D*

Based on the observed immunomodulatory effect of aGKE on T cells *in vitro*, we further tested its impact on T1D development and severity *in vivo*. B6 males were exposed to aGKE in a dose of 100 mg/kg daily through their drinking water during a period of 6 weeks. The aGKE treatment started 1 week before the induction of diabetes by LDSTZ administration and lasted for another 4 weeks (30 days) after the first STZ injection. aGKE-treated mice did not lose their body weights in comparison to control mice (Figure 2(c)). In agreement with body weight data, there were no clinical signs of toxicity or mortality observed in mice that drank aGKE. As illustrated in Figure 2(a), aGKE treatment did not affect diabetes development compared to controls ($p > 0.05$). Figure 2(b) shows that the average glycemia in aGKE-treated mice was not different from the control group. Thus, these results indicate that aGKE treatment affects neither LDSTZ-induced diabetes development nor its severity.

In vivo aGKE treatment decreases splenic T cell populations while potentiating a pro-inflammatory cytokine profile during T1D development in LDSTZ mouse model

Considering T cell-dependent nature of T1D development, and conflicting data obtained in our *in vitro* (suggesting immunomodulation) and *in vivo* experiments (unaffected diabetes incidence), we proceeded to study the effects of *in vivo* aGKE treatment on T cell subpopulations and T cell function during the development of experimental T1D. The same dose of aGKE, 100 mg/kg daily, and the administration route was used. Mice were sacrificed, and their spleens removed for analysis on day 11 (early time point) and day 30 (late time point) post-induction of disease by STZ. Figures 3(a) and (b) show that cell numbers and viability were not affected by aGKE treatment on day 11. When T cells' function was assessed, a trend of reduced mitogen-induced T cell proliferation was observed (without reaching a statistical significance) (Figure 3(c)). The flow cytometric analysis (Figure 3(d)) revealed a significantly lower percentage of $CD3^+$ T cells and T cell subpopulations, including $CD4^+$, $CD8^+$, and $CD4^+CD25^+$ cells, with a significant increase of B cells, compared to values associated with control mice. The populations of macrophages and NK cells were not affected (data not shown). An analysis of cytokine profiles showed a significant increase of IFN-γ, TNF-α and IL-6, accompanied by a

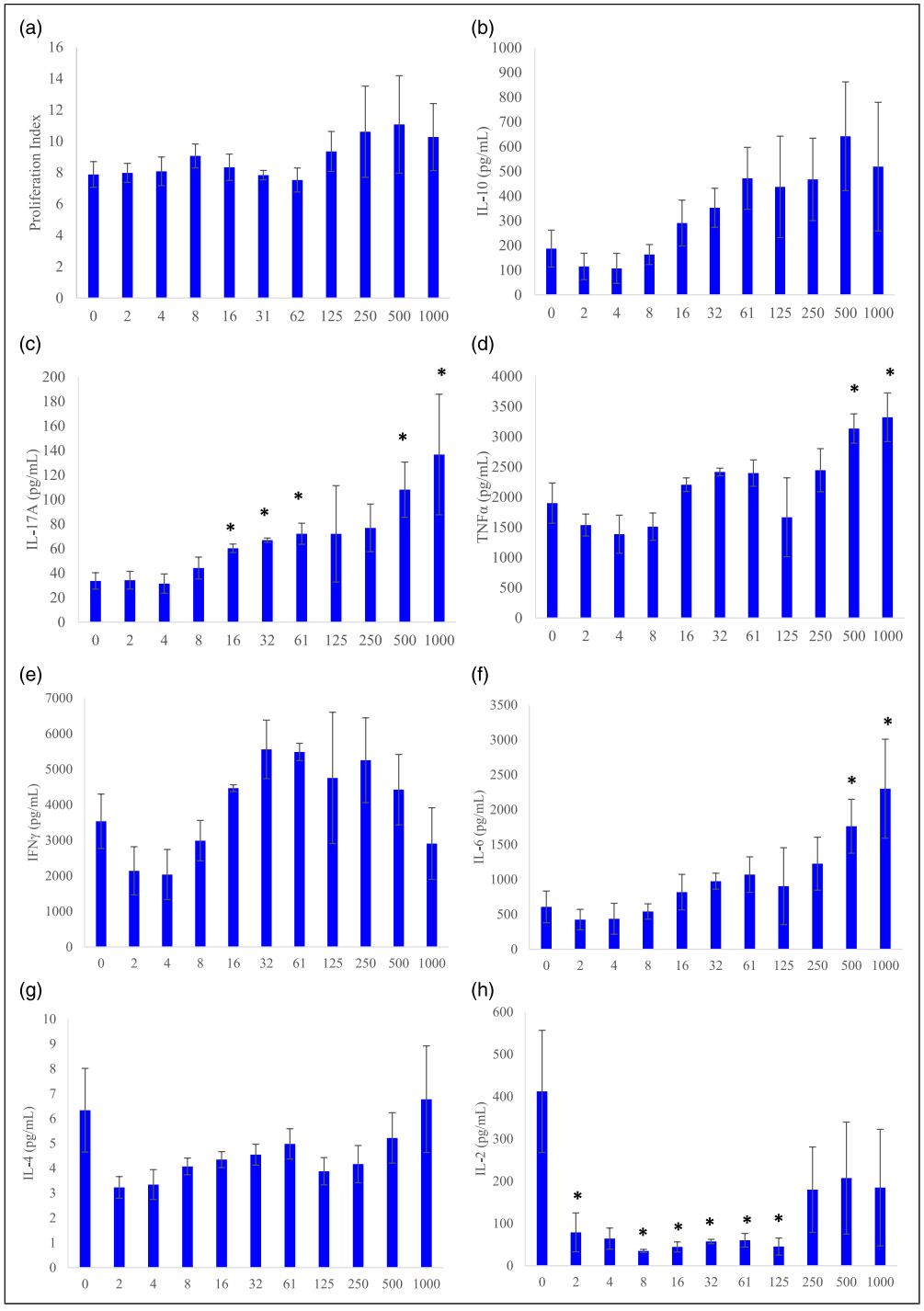

Figure 1. aGKE addition to T cell cultures *in vitro* does not affect proliferation while skewing cytokine profiles. Splenocytes were cultured with addition of ConA (3 μg/mL) and aGKE from 0 μg/mL to 1000 μg/mL, and proliferation of T cells (A), and cytokine production (B–E) were evaluated. (A) T cell proliferation was assessed after 72 h of culture spectrophotometrically by Alamar Blue reagent. The proliferation index was calculated by dividing the optical densities of ConA-stimulated by optical densities of non-stimulated T cells. Cytokines were measured by flow cytometry in the supernatants post 48 h of culture: (B) IL-10, (C) IL-17A, (D) TNF-α, (E) IFN-γ, (F) IL-6, (G) IL-4, and (H) IL-2. aGKE concentrations are shown on *x*-axes in μg/ml; Data presented as average ±SEM (n = 5); * p<0.05 compared to control group (ConA-stimulated splenocytes with addition of 0 μg aGKE/mL).

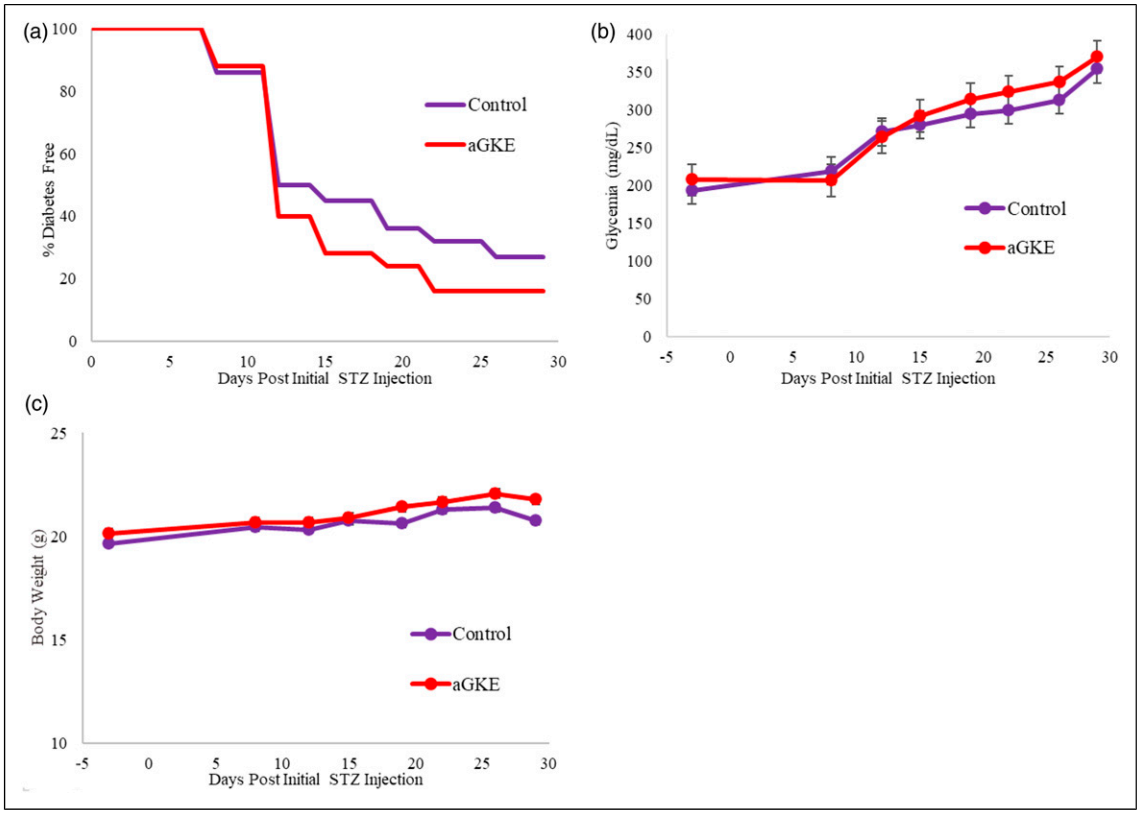

Figure 2. *In vivo* aGKE treatment does not affect T1D development in a LDSTZ mouse model. A group of 7-weeks-old male B6 mice received aGKE (100 mg/kg) in the drinking water, while control mice received only water. STZ injections (40 mg/kg) were administered at 8 weeks of age for five consecutive days intraperitoneally. Glycemia and body weight measurements started 3 days before STZ injections and continued bi-weekly from day 8 to day 30 post the initial STZ injection. A mouse was considered diabetic with two consecutive glycemia measurements of 250 mg/dL or higher. (A) Diabetes incidence presented as the percentage of diabetes-free mice; (B) Glycemia levels (mg/dL); (C) Body weights (g). Data presented as average ±SEM (n = 22–25/group).

significant reduction of IL-2 levels in aGKE-treated mice compared to controls (Figure 3(e)).

The same parameters, including the splenic cell counts, viability, T cell proliferation, immunophenotypes, and cytokine secretion, were analyzed on day 30 post-initial STZ injection (Figure 4). At that late time point, the majority of mice were diabetic. The splenic cell counts and viability were not different between the aGKE-treated and control group of mice (Figures 4(a) and (b)). The proliferation of T cells showed a trend of reduction, however, not a significant one (Figure 4(c)), while immunophenotyping revealed a significant decrease in the percentages of $CD3^+$, $CD4^+$ and $CD8^+$ T cells and an increase of B cells in mice treated by aGKE in comparison to values obtained in controls (Figure 4(d)). Whereas only IL-10 levels exhibited a significant increase, there was a trend of elevation of TNF-α and IL-6 levels, as well as a decrease of IL-2 in aGKE-treated mice.

In vivo *aGKE treatment reduces splenic T cell populations while potentiating a pro-inflammatory cytokine profile in healthy B6 mice*

STZ-induced experimental model of T1D has been used in our *in vivo* experiments that served for testing the effects of aGKE treatment on diabetes development and immune parameters. Two potentially influential factors, such as STZ and diabetic state per se, cannot be ignored while studying GKE effects on T cells. Thus, a new experiment was performed in healthy B6 male mice, which were treated in the same way with 100 mg aGKE/kg daily for the same period as their LDSTZ-exposed counterparts. After the 6 weeks of treatment, mice were sacrificed and spleens removed for the previously described analysis in LDSTZ-administered diabetic mice. Treatment with aGKE did not affect either cell counts or viability of cells isolated from spleens of treated mice

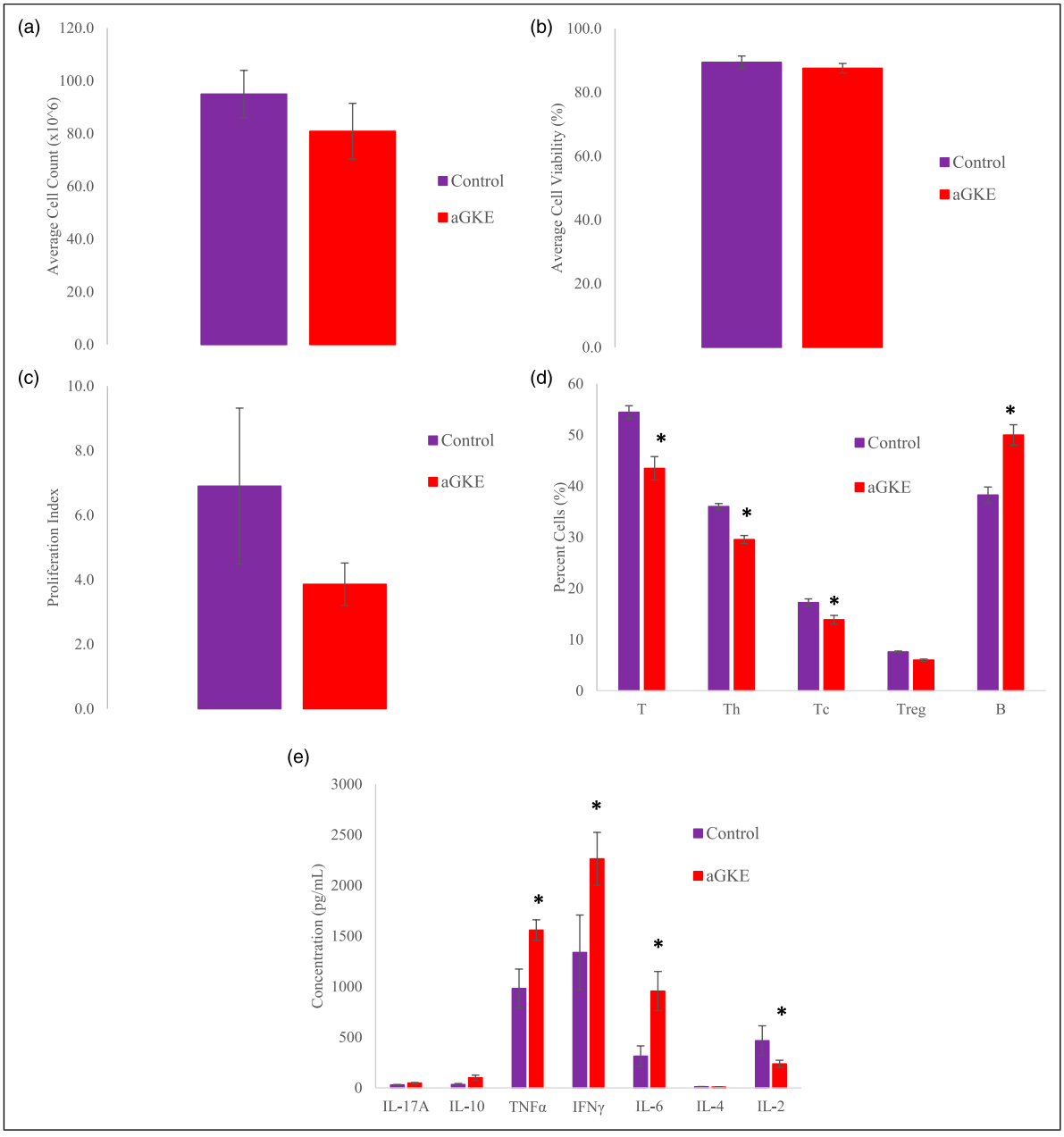

Figure 3. *In vivo* aGKE treatment affects T cell populations and cytokine production on day 11 post-LDSTZ induction of T1D in B6 mice. Total cell counts (A), viability (B), T cell proliferation (C), lymphocyte percentage (D), and cytokine production (E) were assessed *ex vivo* in the spleens of LDSTZ-injected control and aGKE-treated B6 mice euthanized on day 11 post-initial STZ injection. (A) Cell counts and (B) viability were evaluated via Trypan blue exclusion method. (C) Isolated splenocytes were cultured with the presence or absence of ConA for 72 h; proliferation was quantified by Alamar Blue colorimetric assay. The proliferation index was calculated by dividing the optical densities of Con A-stimulated by optical densities of non-stimulated T cells. (D) T cell (T), T-helper (Th), T-cytotoxic (Tc), T-regulatory (Treg), and B cells (B) were detected by flow cytometry; (E) Cytokines (IL-10, IL-17A, TNF-α, IFN-γ, IL-6, IL-4, IL-2) levels were determined by flow cytometry in the supernatants of 48-hr-cultured ConA-stimulated splenocytes. Data presented as average ±SEM (n = 6–10); * $p < 0.05$ compared to control group.

compared to controls (Figures 5(a) and (b)). A significant reduction in T cell proliferation index (Figure 5(c)), as well as in populations of $CD3^+$, $CD4^+$, and $CD8^+$ T cells (Figure 5(d)), was observed in mice treated with aGKE. Cytokine analysis (Figure 5(e)) revealed a significant increase in IFN-γ and IL-10 levels, while IL-2 was prominently reduced. A trend of increased IL-6 and TNF-α levels were observed as well.

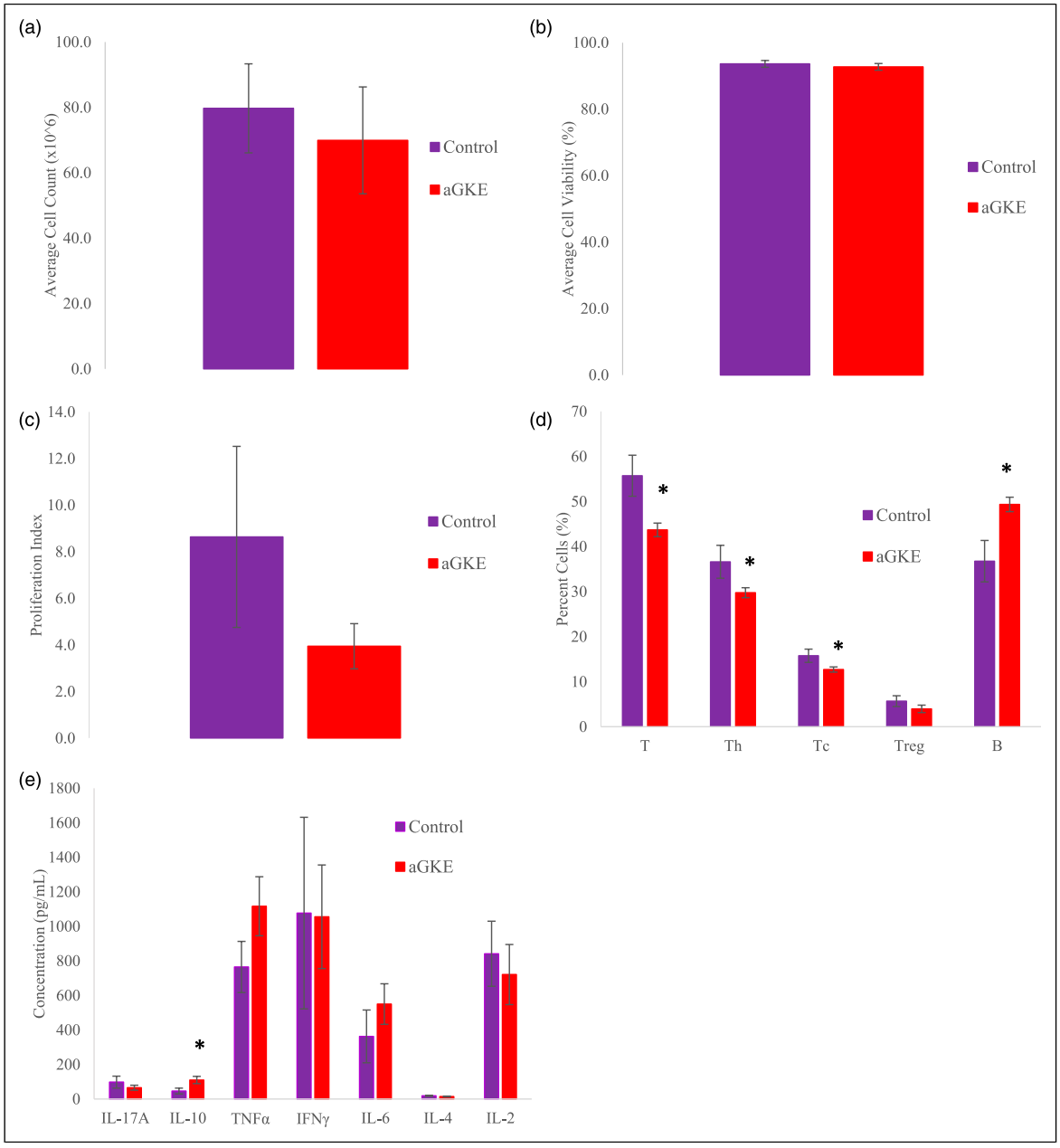

Figure 4. *In vivo* aGKE treatment affects T cell populations and cytokine production on day 30 post-LDSTZ induction of T1D in B6 mice. Total cell counts (A), viability (B), T cell proliferation (C), lymphocyte percentage (D), and cytokine production (E) were assessed *ex vivo*, as outlined in Figure 3, in the spleens of LDSTZ-injected control and aGKE-treated B6 mice euthanized on day 30 post-initial STZ injection. Data presented as average ±SEM (n = 7–11/group); * $p<0.05$ compared to control group.

Healthy B6 mice treated by aGKE for 6 weeks were followed during this experimental period for glycemia levels and body weights as well. It is found that aGKE did not affect either blood glucose levels or body weights in treated mice compared to the values obtained in control mice (data not shown).

Discussion

This study investigated the effects of aqueous GK extract on the development of autoimmune T cell-dependent T1D in a murine LDSTZ model. GKE was administered through drinking water, as the most physiologically relevant way of

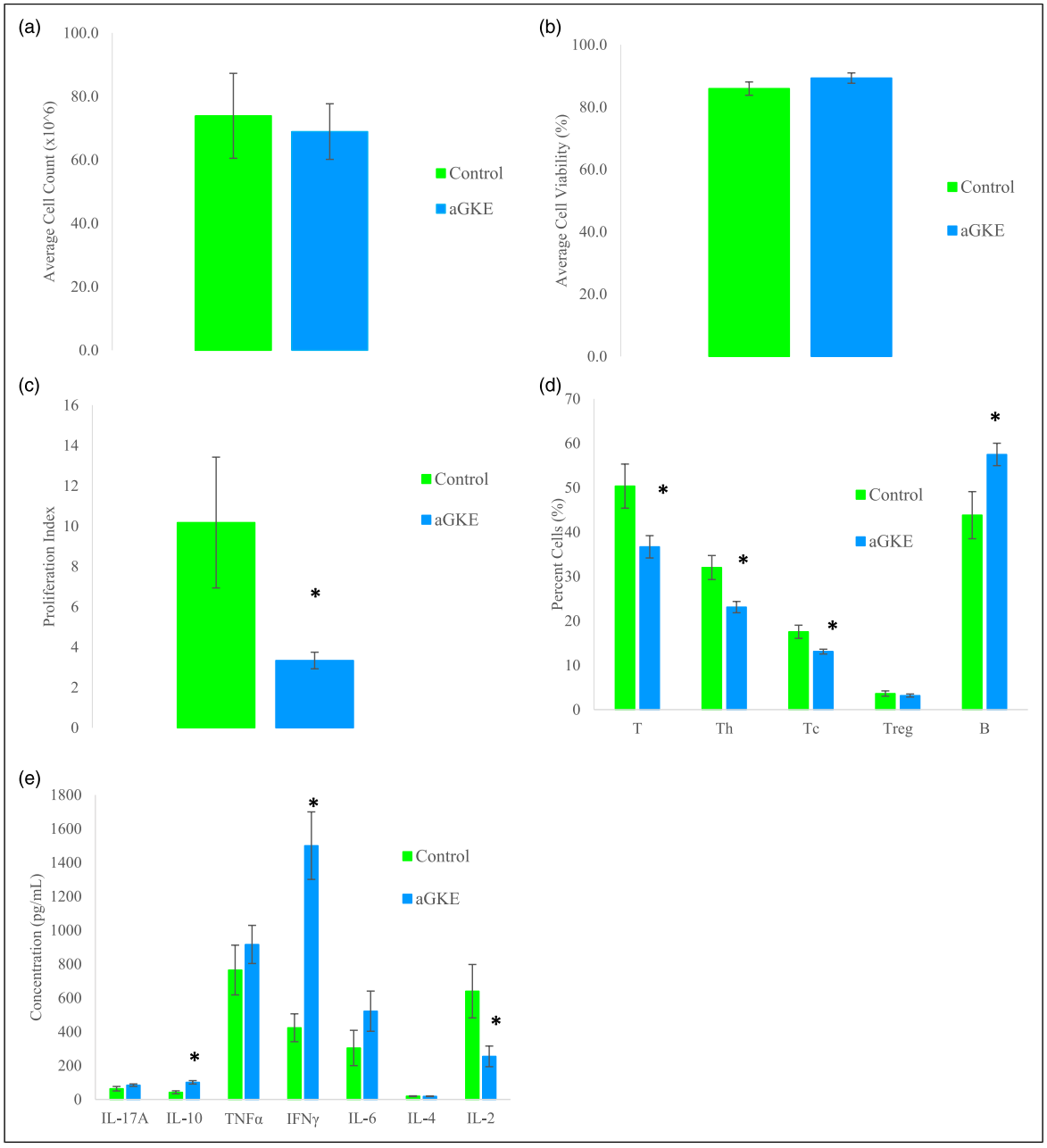

Figure 5. *In vivo* aGKE treatment affects T cell populations and cytokine production in healthy non-diabetic B6 mice. Total cell counts (A), viability (B), T cell proliferation (C), lymphocyte percentage (D), and cytokine production (E) were assessed *ex vivo*, as outlined in Figure 3, in the spleens of control and aGKE-treated B6 mice euthanized after the 6 weeks of treatment. Data presented as average ±SEM (n = 6–10/group); * p<0.05 compared to control group.

GK intake for mimicking human consumption of that seed. Results showed that GKE treatment did not affect either incidence or severity of mouse T1D, while exhibiting *in vitro* and *in vivo* immunomodulatory activity on T cells. To our knowledge, this is the first time that the effects of *G. kola* have been studied in T1D, and in the context of T cells. Whereas several studies have shown *in vitro* anti-inflammatory properties of GKE on macrophage cell lines,[17-19] T cells have never been studied in that respect. Interestingly, in contrast to the previous results obtained on innate immune cells, our initial *in vitro* experiments suggested that GKE addition to mitogen-stimulated T cells skewed their immune profile towards a pro-inflammatory type immune response. Moreover, the *in vivo* experiments, carried on in LDSTZ-induced model of T1D, as well as in healthy B6 mice, supported initially observed data *in vitro*,

since GKE treatments potentiated T_H1-type immune response of T cells, in addition to a reduced frequency of both T_H and T_C subsets of T cells.

A prominent decrease in the populations of splenic T cells, including T_H and T_C, observed in aGKE-treated mice at both time points post-induction of T1D, and confirmed in healthy mice exposed to the extract, might indicate an immunosuppressive, and thus an antidiabetic property of GKE. However, splenic T cells, isolated at an early time point during the development of T1D from aGKE-treated LDSTZ mice, showed a protuberant production of pro-inflammatory cytokines and a reduction in IL-2 levels. Thus, we speculate that T1D development was not compromised in GKE-treated mice because T cells, while being less frequent, were actually more pathogenic. In agreement with our data, a recent study, while performing flow cytometry to investigate the effect of GK phytochemical, garcinoic acid, on monocyte/macrophage population in an experimental model of inflammation-induced atherosclerosis, found a decrease of T_H cell population in $ApoE^{-/-}$ mice.[19] Furthermore, it was previously described that treatment with 250 and 500 mg kV/kg inhibited delayed-type hypersensitivity and potentiated B cell responses in rats.[20] These results actually support current findings, since a prominent increase of B cell population along with a significant reduction of $CD4^+$ and $CD8^+$ T cells have been observed in our study. Also, a leukocytosis has been observed in the blood of GK-fed catfish[21] and rats,[22] which might reflect an increase of B lymphocytes.

We hypothesized the antidiabetic properties of GKE based on previous studies implying the "anti-inflammatory" activity of GKE.[23,24] However, a vast majority of publications described antioxidative and radical scavenging properties of GKE and its active components, and postulated an anti-inflammatory potential of GK based on those findings.[11–14] Actually, just a few studies explicitly explored anti-inflammatory activities, elucidating cytokine secretion and possible molecular targets in macrophage cell lines exposed to GKEs *in vitro*.[17-19] Whereas an overall suppression of pro-inflammatory cytokines, such as TNF-α and IL-6, has been observed,[17,19] Abarikwu et al.[18] did not find a reduction of TNF-α post KV treatment of macrophages. Our *in vivo* study, supported by the data obtained in T cell cultures, showed a noticeable increase of pro-inflammatory cytokines and a reduction of IL-2 production in the spleens of both healthy and diabetic GKE-treated mice. Interestingly, Okoko et al.[17] also reported a reduction of IL-2 production in macrophage cell line induced by exposure to GK kolaflavanones.

Hypoglycemic activity of GKE has been previously shown.[8,9,25,26] KV, a complex containing flavonoids GB-1, GB-2, and kolaflavonone,[27] has been thought to serve as the main constituent responsible for the hypoglycemic effect of GKE.[8,9] Whereas the most common KV extraction method, initially described by Iwu,[27] has exploited organic solvents, the aqueous extraction has also been studied and utilized.[14,22,28,29] Since the hypoglycemic effect of aGKE exposure of LDSTZ-treated mice was not observed in our study, we further questioned the quality of the extract and the route of aGKE administration. Thus, the content of the aqueous extract was submitted to LC-MS analysis, and the presence of main active constituents of GK seed, originally shown by Iwu,[27] such as GB-1, GB-2, and kolaflavonone, has been successfully confirmed. Whereas a prominent reduction of glycemia has been described by administering an aqueous extract of GK in rats, it should be noted that extraordinarily high-doses of 450 and 900 mg GKE/kg, associated with severe side effects, were used in that study.[29] Thus, it is conceivable to assume that the most widely administered GKE dose of 100 mg/kg,[8,9,26] explored in our experiments, was actually too low for reaching the hypoglycemic effect by utilizing the aGKE. Although, it should be emphasized that this dose was clearly efficient in exhibiting effects on the immune system. In line with this observation, previous studies demonstrated the potency of 100 and 200 mg aqueous GKE/kg on different plasma enzymes and the central nervous system in mice and rats.[22,28] Our *in vivo* treatment with 100 mg GKE/kg daily did not affect splenic cell counts and viability, confirming previously published results regarding the non-toxic nature of this dose.[26,29]

The oral gavage has been mainly utilized as a method for GKE administration.[8,26,30,31] This procedure ensures the most precise dosage. However, we deliberately chose the method of GKE delivery by drinking water to mimic humans' consumption of GK seeds by chewing. Besides, exposure to GKE by drinking or eating allows the interaction with mucosal surfaces in the mouth and can lead to more efficient absorption and transport, as well as evasion of the first-pass metabolism, resulting in higher bioavailability of the compound.[4,32] Lastly, it should be noted that hypoglycemic effects of GKEs have never been studied in a murine LDSTZ-dependent autoimmune model, but rather in toxic models of T1D induced by a high-dose of STZ or alloxan.[9,10,25,30] The aGKE treatment of healthy B6 mice in our study ruled out the potential effects of LDSTZ on the extract's activity since the consistent results on glycemia, T cell population frequency, and cytokine production were obtained without the introduction of STZ.

Conclusion

Our results show that aqueous extract of GK, administered via drinking water, did not exhibit antidiabetogenic property since neither the development of LDSTZ-induced T1D in mice was delayed nor its severity reduced. However, GKE treatment prominently affected T cells, reducing the frequency of major T cell populations and skewing

cytokine production towards T_H1-type immune response, suggesting GKE efficacy in pathologies like allergies that depend on T_H2-type immune response.

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Ethics approval

Ethical approval was not sought for the present study because it is not applicable.

Animal welfare

The present study followed international, national, and institutional guidelines for humane animal treatment and complied with relevant legislation.

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