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Jenna Nelson
St. Cloud State University

Logan Olson
St. Cloud State University

Amira Zaher
St. Cloud State University

Jace Engelmann
St. Cloud State University

Marina Cetkovic-Cvrlje
St Cloud State University

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Jenna Nelson[1,2], Logan Olson[1,2], Amira Zaher[1,2], Jace Engelmann[1,2],
and Marina Cetkovic-Cvrlje[1,2]

[1]*Department of Biology, College of Science and Engineering, St. Cloud State University, St. Cloud, MN, USA*
[2]*Immunology Laboratory, St. Cloud State University, St. Cloud, MN, USA*

Abstract

Type 1 diabetes (T1D) is a chronic inflammatory autoimmune disease in which T cells destroy insulin-producing β cells in the pancreas, leading to hyperglycemia. Some T cells directly kill β cells, such as T-cytotoxic, or indirectly such as T-helper, while others, like regulatory T cells, actually protect them. A recent study showed that sodium bicarbonate (SB) exhibited anti-inflammatory activity by affecting immune cells other than T cells, implying its potential for the treatment of autoimmune diseases. Since SB has never been tested in an experimental mouse model for autoimmunity, we studied the effects of SB treatment on the development and severity of T1D, as well as on T cell subsets and T cell function. It was hypothesized that SB administration (200 mM, administered via drinking water) would decrease the incidence and severity of streptozotocin-induced T1D in 8-week-old C57BL/6 mice by its action on T cells. Glucose and body weight measurements were taken biweekly until mice were sacrificed four weeks later, and their spleens obtained for analysis of cell counts, viability, T cell proliferation, and quantification of T cell subsets by flow cytometry. There were no differences in splenic lymphocyte counts and viability between SB-treated and control mice. Although results showed that SB significantly decreased glucose levels and delayed diabetes development, it does not seem to affect the frequency of T cell populations nor their proliferation capacity. Our results suggest beneficial effects of SB in the prevention of mouse autoimmune T1D and highlight the need for further studies on its mechanism of action.

Introduction

Type 1 diabetes (T1D) is an autoimmune disease that occurs when inflammatory T cells attack insulin-producing β cells in the pancreas, which produces a halt in the body's production of insulin, leading to increased blood glucose levels (hyperglycemia). T1D is the second most common autoimmune disease among children (Jamshidi et al., 2019). The onset of this disease typically occurs during childhood and generates many complications throughout the life of a patient. Early symptoms include polydipsia (increased thirst), polyphagia (increased appetite), polyuria (increased urination), weight loss, and fatigue (Kahanovitz, Sluss, & Russell, 2017). If untreated, ketoacidosis is likely to occur and more serious

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symptoms can follow, including cerebral edema, mental confusion, unconsciousness, coma, and death (Kahanovitz et al., 2017).

The incidence of T1D increased at varying rates throughout the 20th century and the same trends are being discerned now (Egro, 2013). Egro (2013) stated several hypotheses affirming that an increase in T1D incidence could be an implication of increased hygiene over the past decades, viral infections, vitamin D deficiency, and even a lack of breastfeeding. The only definitive answer that scientists have come up with on T1D etiopathogenesis is that it is a matter of both genetics and environment (Knip & Simell, 2012). Without knowing the exact cause of disease, the efforts that have been made toward prevention of disease have been futile. Since T1D depends on the over-reactivity of T cells, many preventative immunosuppressive approaches are aimed at the destruction of these immune cells (Chatenoud, Warncke, & Ziegler, 2012). However, these efforts did not provide expected results. Often times the treatments had an overwhelming effect on the immune system, eliminating the cells necessary to fight infections and tumors (Chatenoud et al., 2012). The only current treatment, once T1D is diagnosed, is insulin injections, leaving a great need for investigating alternative intervention methods for T1D.

Sodium bicarbonate (SB) has been used for decades to treat minor health issues, including dyspepsia and reflux. It is also implemented in the treatment of serious health complications, including metabolic acidosis which may occur in severe renal disease, uncontrolled diabetes, circulatory insufficiency due to shock or severe dehydration, extracorporeal circulation of blood, cardiac arrest and severe primary lactic acidosis (NCBI, n.d.). In a recent study, SB was shown to have *in vitro* anti-inflammatory properties, through its action on macrophages (Ray et al., 2018). One can assume that SB's anti-inflammatory effects on macrophages might also be observed on T cells as well. The same study speculated about SB's potential in prevention of autoimmune diseases, however it had never been tested *in vivo* in an experimental model of autoimmune disease. With T1D being a T cell-mediated autoimmune disease, and there being a lack of knowledge on the effects of SB on T cells, further research in this area is imperative.

When studying T cells in T1D, several types of T cells must be considered, as they are crucial in the disease's mechanism of action. Cytotoxic T cells (T_C), helper T cells (T_H1, T_H2, T_H17), and regulatory T cells (T_{reg}), each of which can be characterized by their particular cell markers (immunophenotypes) and the cytokines they release, and can be described as protective or destructive during disease development (Cetkovic-Cvrlje, Thinamany, & Bruner., 2017). Contradictory to their names, T_H1, T_H2, and T_H17 are not all helpful, nor do they play the same roles in T1D. T_H1 release the cytokines interleukin (IL)-2 and interferon (IFN)-γ, which are considered to be pathogenic, while T_H2 release protective cytokines, IL-4 and IL-10 (Kuiper, Moran, Cetkovic-Cvrlje., 2016). T_H17 release IL-17, which is also considered destructive (Kuiper et al., 2016). T_{reg} do their implied job in regulating the immune system and are said to have protective qualities during disease development (Kuiper et al., 2016). C57BL/6J (BL6) mice injected by chemical streptozotocin (STZ, Sigma-Aldrich) and NOD/LtJ (NOD) are both acceptable model organisms for T1D research. NOD mice spontaneously generate T1D, while BL6 mice must be chemically-induced, using 5 low-doses of STZ. Both models exhibit immunological changes during the development of T1D, comparable to humans (Cetkovic-Cvrlje et al., 2017).

The aim of this study was to illuminate antidiabetic properties of SB using a STZ-induced T1D mouse model. Since T1D is a T cell-mediated disease, the effects of SB treatment on T cells were closely examined. It was hypothesized that SB administration would decrease the incidence and severity of STZ-induced T1D by its action on T cells in a BL6 mouse model.

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Methods

Mice. BL6 breeding pairs were purchased from Jackson Laboratory in Bar Harbor, ME for continued breeding at St. Cloud State University. Mice were housed in NexGen Lo-Profile caging systems in temperature- and relative humidity- controlled rooms with a 12-hour light/dark cycle. All mice had constant access to casein-based phytosterol-gen-free food and filtered water. St. Cloud State University Institutional Animal Care and Use Committee approved all protocols/procedures before experimentation began (protocol 5-113).

Sodium Bicarbonate Preparation. 200 mM concentration of SB (Sigma-Aldrich) drinking water was prepared by mixing SB powder with deionized water (Ray et al., 2018). The solution was then autoclaved along with non-treated, deionized (control) water. The water solutions were distributed to the appropriate cages via glass bottles with rubber sips when the mice were 7 weeks old. For the remainder of the experiment, the water bottles and sips were changed weekly.

Type 1 Diabetes Induction by Streptozotocin Injections. On 5 consecutive days, STZ was injected at 40 mg/kg/day, intraperitoneally (i.p.), to control and SB-treated 8-week-old, male mice (Cetkovic-Cvrlje et al., 2017). Prior to injections each day, STZ was dissolved in 0.05 M citrate buffer (pH 4.5, Sigma-Aldrich), vortexed, and within 30 minutes i.p. injected at 6.52 µl/g (ter Veld et al., 2008).

Blood Glucose and Body Weight Measurements. At pre-decided time points, a lateral tail vein puncture was performed in a mouse to produce a small drop of blood that was then placed onto an Accu-Chek Aviva blood glucose meter strip to determine blood glucose levels. Glucose testing, along with body weight (g) measuring, was performed biweekly, starting 8 days after the initial STZ injection, until the experiment end. A mouse was considered diabetic after two consecutive readings of ≥ 250 mg glucose/dL (Cetkovic-Cvrlje et al., 2017).

Euthanasia. At 12 weeks of age, mice were euthanized via CO_2 asphyxiation. The mice were placed into an empty, clean 10-gallon plastic box. A stainless-steel lid was placed on top of the box, which is attached to the CO_2 tank by flexible polyvinyl tubing. The CO_2 tank was opened, allowing CO_2 (at ≥ 200 psi) to fill the tank. The mice remained in the tank for at least 10 minutes; during this time, their heartbeats stopped, and the mice were considered deceased.

Single Cell Suspension. Spleens were harvested from euthanized 12-week-old SB-treated and control mice and were smashed using a 10 cc syringe plunger and a 70 mm nylon mesh strainer with the addition of phosphate-buffered salt solution (PBS, Sigma-Aldrich). This process produced single (spleen) cell suspensions (SCS). The SCSs were put into a centrifuge and spun for 5 minutes at 1200 rpm 4°C. The supernatants decanted, and the pellets were resuspended in 0.75 ml of ACK lysing buffer (Lonza-BioWhittaker) which was used to lyse the erythrocytes. The SCSs were washed three more times using PBS. A small portion of each SCS was mixed with Trypan blue (Lonza BioWhittaker), which stains dead cells blue. 20 µl of the mixtures were inserted into a hemocytometer for cell counting with a light microscope. Alive and dead cells were counted separately; this allowed for conclusions to be drawn, not only about how many cells there were in each sample, but also what percentage of those cells were living. This procedure has been described in more detail in the Cetkovic-Cvrlje et al. (1997) manuscript.

T-cell Proliferation. Proliferation assays were performed according to Cetkovic-Cvrlje et al. (2016). To summarize, splenocytes from SB-treated and control mice were suspended in complete media (CM), which contained RPMI-1640 medium, 1 U penicillin/ml, 100 µg streptomycin/ml and 10% fetal calf serum (FCS) (all purchased from Sigma-Aldrich). The CM-suspended cells were cultured in triplicates in a 96-well-plate with and without the addition of 3 µg/ml Concanavalin A (ConA, Sigma-Aldrich) for 72 hours at 37°C and 5% CO_2. ConA is T cell mitogen, which means it is used to induce proliferation (mitosis) of T cells (Dwyer & Johnson, 1981). After 72 hours, 10 µl of Alamar blue (Invitrogen), which is used to measure the reducing capacity of cells, was added to each well, and the plate was incubated for an additional 4-6 hours. Optical densities were then measured, at 570nm, with an ELISA plate reader (GeneMate).

Immunophenotyping. Splenocytes were analyzed in order to detect particular types of immune cells (immunophenotypes) by flow cytometry (Cetkovic-Cvrlje et al., 2016). Aliquots of 10^6 splenocytes were obtained from the SCS of each mouse. Aliquots were spun down via centrifugation, the supernatants were decanted, and the pellets were resuspended in FACS buffer (0.1%NaN3, 1% FCS in PBS). Each sample was stained with a particular antibody mixture, incubated in the dark at 4°C for 45 minutes, washed, and analyzed using a FACSCalibur (BD Biosciences) flow cytometer. The antibodies against particular cell markers were purchased from BD Biosciences; these immune cell markers and their associated fluorochromes can be found in Table 1. A minimum of 10,000 cells were acquired for each analysis. Immunophenotype analysis was performed using CellQuest Prosoftware (BD Biosciences).

Table 1. Cell types, cell markers, and antibody-associated fluorochromes, used for the flow cytometry. The fluorochrome column displays abbreviations of the following fluorochromes: peridinin chlorophyll-a protein (PerCP), fluorescein isothio-cyanate (FITC), allophycocyanin (APC), and phycoerythrin (PE).

Cell Type	Cell Marker	Fluorochrome
T cell (all types)	CD3	PE
T-cytotoxic	CD8	FITC
T-helper	CD4	PerCP
T-regulatory	CD4 & CD25	APC
B cell	CD45RB220	APC
Natural Killer Cell	CD335	FITC
Macrophage	CD11b	PerCP

Statistical Analysis. For each of the statistical analyses, a p-value of < 0.05 was considered significant. Differences in diabetes incidence among experimental groups were determined using a life-table analysis, specifically looking at the log-rank test, using JMP Pro 14 statistical analysis software. For blood glucose levels and body weight data, Microsoft Excel was used to run a one-way analysis of variance with repeated measures, otherwise known as an ANOVA test. For immunophenotyping, T cell proliferation, cell counts, and cell viability a Student t test was performed using Microsoft Excel.

Experimental Timeline. The mice were divided into two groups, control and treatment, and treatment began at 7 weeks of age (Day -7). Initial blood glucose and body weight measurements were also taken at that time. At 8 weeks of age (Day 0), STZ injections began. Between 9 and 12 weeks of age (Day 7 to

Day 28), blood glucose and body weight measurements were taken biweekly. At 12 weeks of age (Day 30), all T cell-related experimental analyses were performed (SCS preparation, cell counting, the initiation of the T cell proliferation assay, and immunophenotyping).

Results

Effect of Sodium Bicarbonate on the Development and Severity of T1D. To test the effect of SB treatment on the development and severity of T1D in a STZ-induced BL6 mouse model of disease, mice were treated with 200 mM SB from 7 to 12 weeks of age. One week after the initiation of treatment, each mouse, control and SB-treatment, received a daily low-dose of STZ for a period of 5 days to induce T1D. Prior to the induction to T1D (Day -7) there were no differences in glucose levels observed between the control and treatment groups (Figure 2). At 9 weeks of age, biweekly glucose measurements were taken until the end of the experiment (Day 7 to Day 28). As illustrated in Figure 1, 200 mM SB treatment significantly ($p = 0.0444$) decelerated T1D diabetes development and decreased diabetes incidence compared to control group. On Day 14, 47.6% of SB-treated mice were diabetes-free while only 14.3% of control mice were diabetes-free; at the end of experimental period, 33.3% of SB-treated mice were diabetes-free, while 9.5% of control mice were diabetes-free (Figure 1).

Throughout the aforementioned time period, on average, the SB-treated group had lower blood glucose levels than the control group, though no statistical significance was reached ($p = 0.1819$) (Figure 2).
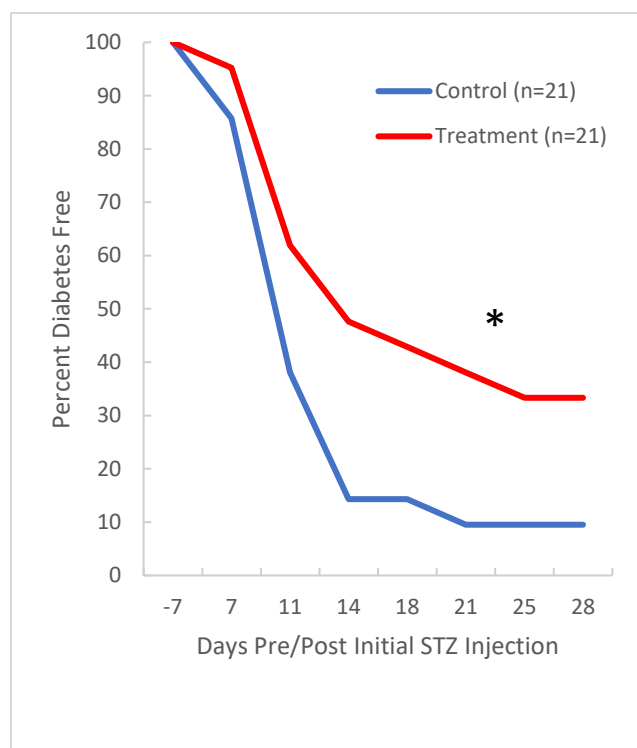

Figure 1. Incidence of diabetes in STZ-administered mice treated by 200 mM SB vs control group. Statistical significance was observed for incidence of diabetes in treated vs control mice; $p = 0.0444$.
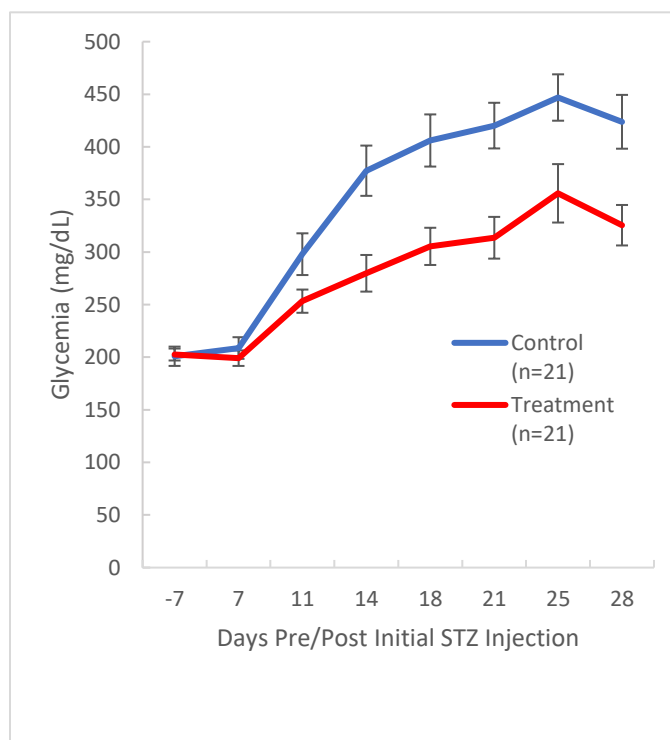
Figure 2. Average blood glucose levels (mg/dL) +/- standard error of the mean (SEM) for 200 mM treatment vs control group of mice. No statistical significance was observed ($p > 0.05$).

Sodium Bicarbonate and Immunotoxicity. The main focus of this project was to test SB's potential in decreasing the incidence and severity of T1D, but it was also vital to observe whether a 200 mM dose of SB exhibited toxic effects. Thus, certain parameters were monitored *in vivo* and *ex vivo*. *In vivo*, an initial body weight measurement was collected for each mouse at 7 weeks of age (Day -7). Following 5 low doses of STZ given to induce T1D, body weight measurements were recorded biweekly (Day 7 to Day 28) until the mice reached 12 weeks of age. During this time, there was no significant difference found between the 200 mM SB-treated group and the control group ($p = 0.2706$) (data not shown). A Trypan blue exclusion test was performed *ex vivo* after the preparation of the SCS. A small portion of the SCS was mixed with Trypan blue and splenocytes were counted. When comparing 200 mM SB-treated and control mice, there were no significant differences found in the context of total cell counts ($p = 0.5288$) (Figure 3), nor in the cell viability ($p = 0.3681$) (Figure 4).
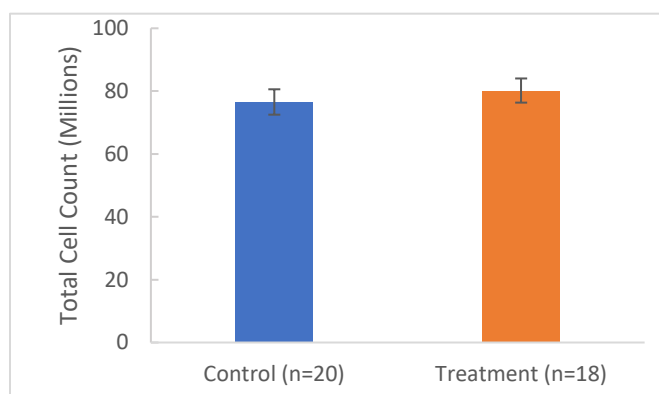

Figure 3. Splenic cell counts (millions) +/- standard error of the mean (SEM) for treatment vs control group of mice. No statistical significance was observed ($p > 0.05$).
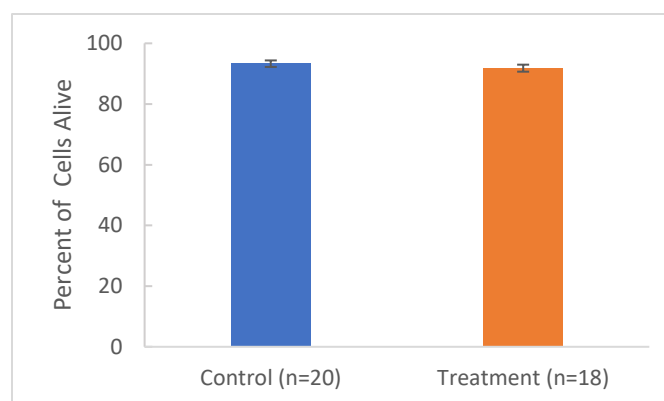
Figure 4. Splenic cell viability +/- standard error of the mean (SEM) for treatment vs control group of mice. No statistical significance was observed ($p > 0.05$).

Effect of Sodium Bicarbonate on T cell Function. If SB had the potential to decrease the incidence and severity of T1D, which is a T cell mediated autoimmune disease, one would expect to observe SB-mediated effects on T cell function. Thus, a T cell proliferation assay was performed. A portion of each SCS was plated with and without the addition of ConA and was stained with Alamar blue after the 3-day-culture period and was analyzed via spectrophotometry. When comparing T cell proliferation levels, in stimulated and non-stimulated conditions, there was no significant difference for non-stimulated ($p = 0.3614$), as well as stimulated conditions ($p = 0.6287$) between the 200 mM SB-treated group and the control group (Figure 5).
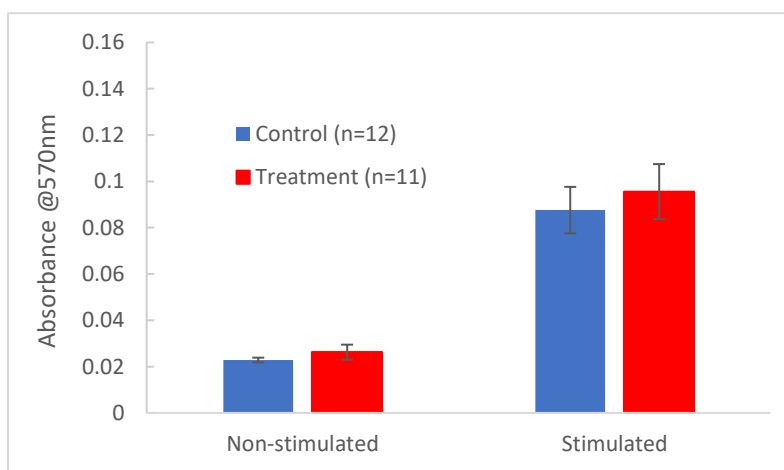

Figure 5. Proliferation levels +/- standard error of the mean (SEM) for 200 mM treatment vs control group of mice. No statistical significance was observed ($p > 0.05$).

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Effect of 200 mM Sodium Bicarbonate on Immune Cell Subpopulations. To shed light on SB's mechanism of action in the context of immune cells, quantification of immune cell populations and subpopulations was performed. Thus, mouse splenocyte samples were stained with fluorochrome-labeled antibodies and a flow cytometer was used to quantify the immune cell types/subtypes. The percent of T_C, T_H, and T_{reg} cells was slightly lower in the 200 mM SB-treated mice compared to the control, while there was a trend of slight increase in the percent of B cells, natural killer (NK) cells, and macrophages in the SB-treated group. However, comparing the SB-treated against the control group, across all immune parameters, no significant differences were observed (Figure 6). The p-values for the 200 mM SB-treated group versus the control group are as follows: CD3+ (p = 0.4697), CD4+ (p = 0.3958), CD8+ (p = 0.4718), Treg (p = β0.4739), B cell (p = 0.4218), NK cells (p = 0.1696), and macrophages (p = 0.5460). The cell types that coincide with these cell markers can be found in Table 1.
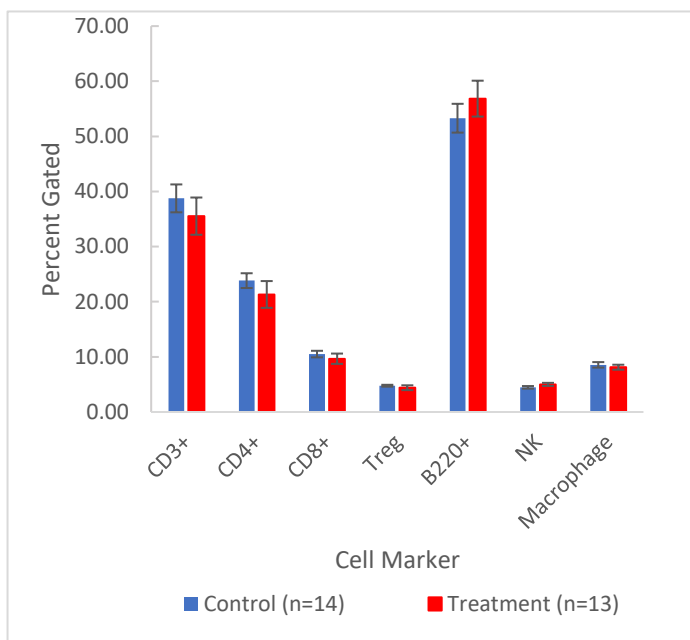

Figure 6. Spleen cell immunophenotypes +/- standard error of the mean (SEM) for 200 mM treatment vs control group of mice. No statistical significance was observed (p > 0.05).

Conclusions

This study showed for the first-time effects of SB treatment in an *in vivo* T1D mouse model. The results imply that SB has the potential to reduce the incidence and severity of the disease, as SB treatment significantly delayed the onset of disease, while simultaneously lowering glycemia levels. SB did not show toxic effects; this was confirmed with no changes in the body weights during the treatment period, as well as with intact splenic cell counts and viability. Interestingly, SB did not affect T cell function nor the frequency of T cell populations/subpopulations. Thus, further research into SB's mechanism of action, in the context of T cells, needs to be performed. Cytokine secretion will be studied to elucidate how SB affects particular T cell subpopulations regardless of not affecting their numbers. Overall, whereas these results are not conclusive about the mechanism of action of SB in T1D, they serve as a promising first step towards retardation of T1D onset and potential preventative efforts for autoimmunity in general.

Acknowledgments

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