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Chokeberry (*Aronia melanocarpa*) fruit extract modulates immune response *in vivo* and *in vitro*

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ABSTRACT

Chokeberry (*Aronia melanocarpa*) is known for its anti-oxidant, anti-inflammatory and anti-diabetic properties. Since the effects of chokeberry extract on the immune response have been only sporadically assessed, our aim was to investigate chokeberry fruit water extract on the immune response *in vivo* and *in vitro*. When administered orally to healthy mice, the extract exerted immunomodulatory effects in the gut evidenced by the altered proportion of macrophages, dendritic cells and T cells. Importantly, oral consumption of the chokeberry extract resulted in blood glucose level increase in C57BL/6 mice with chemically-induced diabetes. These *in vivo* results were corroborated by observed up-regulation of nitric oxide and interelukin-1β production in macrophages and dendritic cells, up-regulated phagocytic activity of macrophages, increased T and B lymphocytes proportions and differentiation of interferon-γ-producing T cells *in vitro*. The obtained results imply that our chokeberry extract stimulates pro-inflammatory properties in immune cells of innate and adaptive immunity.

1. Introduction

Chokeberry (*Aronia melanocarpa*) contains several biologically active compounds such as phenolic acids, anthocyanins, flavonoids, and proanthocyanidins (Banjari et al., 2017; Zheng & Wang, 2003). These berries have a greater content of phenolic constituents than most other black berries (Kähkönen, Hopia, & Heinonen, 2001; Wu, Gu, Prior, & McKay, 2004). Various chokeberry extracts are commercially available and advertised as potent supplements for the treatment of diabetes, cancer, hypertension and stress. Generally, chokeberry juice, fruit or leaf extracts are considered to be anti-inflammatory, anti-oxidant, hypoglycemic, hypolipidemic, hypotensive, anti-inflammatory and antibacterial properties (Appel et al., 2015; Borowska & Brzóska, 2016; Francik et al., 2014; Handeland, Grude, Torp, & Slimestad, 2014; Thi & Hwang, 2018a). These statements only partly rely on the experimental data obtained from animal studies. For example, anti-diabetic effects were demonstrated after oral administration of a chokeberry ethanol extract to hyperglycemic rats or ICR mice (Jeon et al., 2018; Valcheva-Kuzmanova, Kuzmanov, Tancheva, & Belcheva, 2007). Also, clinical trials in volunteers with hypercholesterolemia revealed that chokeberry juice consumption reduced total cholesterol levels and mildly down-regulated blood glucose levels (Skoczyñska et al., 2007). Apart from these metabolic effects, chokeberry exhibits anti-inflammatory capacity, revealed in both *in vivo* and *in vitro* studies. Oral administration of the chokeberry extract inhibited prostaglandin E2 production in DSS-induced colitis in mice and decreased the levels of nitric oxide (NO), interleukin-6 (IL-6), and tumor necrosis factor (TNF) in *ex vivo* lipopolysaccharide (LPS)-stimulated macrophages (Kang et al., 2017). Similarly, the chokeberry extract down-regulated inflammation in LPS-induced uveitis (Ohgami et al., 2005). Versatile commercial and laboratory-made extracts also exhibited anti-inflammatory effect *in vitro*: chokeberry inhibits IL-6 and up-regulates interleukin 10 (IL-10)

Abbreviations: BSA, bovine serum albumin; ConA, concanavalin A; DC, dendritic cell; EDTA, ethylenediaminetetraacetic acid; FCS, fetal calf serum; GALT, gut-associated lymphoid tissue; IEL, intraepithelial lymphocytes; IFN-γ, interferon-γ; IL-1β, interleukin-1β; IL-17, interleukin-17; IL-6, interleukin-6; LPS, lipopolysaccharide; NO, nitric oxide; PC, peritoneal cell; PP, Peyer's patches; STZ, streptozotocin; T1D, type 1 diabetes; Th, T helper cell; Th1, type 1 T helper cell; Th17, type 17 T helper cell; TNF, tumor necrosis factor; Treg, T regulatory cell

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production in mouse splenocytes (Martin et al., 2014) and decreases NO, IL-6 and TNF production in macrophages and microglia (Lee et al., 2018; Thi & Hwang, 2018b). These anti-inflammatory effects were primarily due to particular polyphenols (cyanidin-3-arabinoside and quercetin) that are represented as a minor fraction of total polyphenols of chokeberry (Martin et al., 2014). Some of the extracts display their anti-inflammatory nature through inhibition of leukocyte transmigration from the blood into the tissue by lowering the expression of adhesive molecules on endothelial cells (Zapolska-Downar et al., 2012). Pro-inflammatory effects of chokeberry extracts have not been documented so far, except for a single result in mice with colitis, where the up-regulation of type 17 T helper cell (Th17) proportion was found within gut-associated lymphoid tissue (GALT) after the treatment with the whole chokeberry powder (Pei et al., 2019).

The aim of this study was to delineate the effects of a chokeberry extract on immune cells in healthy C57BL/6 mice and in mice with type 1 diabetes (T1D), and to evaluate whether this extract modulates immune cells *in vitro*.

2. Material and methods

2.1. Plant material

Berries were kindly provided by Conimex Trade Ltd. Chokeberries were collected in the fourth year of cultivation from the organic certificated experimental field near Šabac, Serbia, in August 2017. Conimex Trade Ltd regularly performs analyses of the complete health probity of berries to confirm the absence of contaminants. The chokeberry extract was free of viable bacteria (tested in the Brain Heart Infusion broth), and no endotoxin was detected (tested by PYROGENT™-5000 Kinetic Turbidimetric LAL Assay, Lonza Netherlands B.V., Geleen, The Netherlands). The average diameter of berries was 7.1 mm while the average weight was 1.18 g. After collection, the chokeberries were immediately dried at 40 °C for 48 h in a laboratory dryer (Instrumentaria ST 01/02, Zagreb, Croatia) and grinded by a laboratory mill at the Institute for Medicinal Plants Research, Belgrade, Serbia, which is certified with ISO 9001:2015 and HACCP for quality standards.

2.2. Extract preparation

Plant material was extracted on an orbital shaker (170 rpm) with 50% ethanol for 60 min using solid to solvent ratio 1:20 (Ćujić et al., 2016). After the extraction, ethanol was evaporated under vacuum and water extract was obtained. The water extract was frozen at −80 °C and freeze-dried (Beta 1–8 Freeze Dryer, Martin Christ, GmbH, Osteroide am Harz, Germany). Overall, from 100 g of chokeberries, 47.3 g of dried extract were obtained.

2.3. HPLC analysis

HPLC analyses were carried out on a reverse phase Lichrospher RP-18 (Agilent, Waldbronn, Germany) analytical column (250 × 4 mm i.d., 5 μm particle size) according to the previously reported method (Ćujić et al., 2016). The results were expressed as milligram per gram of dry extract. The content of total proanthocyanidin compounds in samples was determined spectrophotometrically using modified p-dimethylaminocinnamaldehyde (p-DMACA) method (Li, Tanner, & Larkin, 1996). The chokeberry extract (100 μg) was mixed with 80 μL of p-DMACA reagent, 2 mL methanol, and a drop of glycerol, and the absorbance was measured after 7 min at 640 nm. The contents of proanthocyanidins in the samples were expressed as milligrams of catechin equivalents per gram of extract (mg CE/g).

2.4. T1D induction and treatment with chokeberry extract

Male C57BL/6 mice were bred and housed in the Institute for Biological Research “Sinisa Stankovic”, under standard conditions with free access to food and tap water. The Ethic Committee of the Institute for Biological Research “Sinisa Stankovic” approved all experimental procedures (App. No 02-11/18-01-2420), which also complied with the ARRIVE guidelines and were in accordance with the Directive 2010/63/EU. T1D was induced in mice (2 months old) by intraperitoneal injection of streptozotocin (STZ) (Sigma-Aldrich, St. Louis, MO, USA) given in a dose of 40 mg/kg bw during five consecutive days. This procedure is typically used for T1D induction in C57BL/6 mice (Cetkovic-Cvrlje, Thinamany, & Bruner, 2017; Koprivica et al., 2019). The chokeberry extract was dissolved in water and administered orally every day in the doses of either 200 mg/kg bw or 50 mg/kg bw, starting from the first injection of STZ, for 7 days in total. Therefore, we formed three experimental groups: (1) a control group receiving STZ; (2) a treatment group receiving STZ and chokeberry extract (200 mg/kg bw); (3) a treatment group receiving STZ and chokeberry extract (50 mg/kg bw). Each group consisted of 7 mice. Glycemia levels were monitored on a weekly basis from blood obtained from the tail vein using a glucometer (Sensimac, IMACO GmbH, Lüdersdorf, Germany). Blood glucose level in non-fasted mice higher than 11 mmol/l was considered as hyperglycemia. Diabetes incidence was calculated as proportion of hyperglycemic mice within the total number of mice in the experimental group.

To evaluate the effect of the chokeberry extract (50 mg/kg bw) on healthy mice, we formed two experimental groups of mice: a treatment group, which received the extract orally (by gavage) for 7 days, while a control group received the same volume of water for 7 days. Each group consisted of 7 mice. On day 10 after the first oral consumption, *ex vivo* analysis of immune cells was performed.

2.5. Isolation of intraepithelial lymphocytes

Isolation of the intraepithelial lymphocytes (IEL) was performed according to the protocol described by Couter and Surana (2016). The small intestine was removed aseptically, cut into pieces (approx. 3 cm long) and cut longitudinally. After removing the intestinal content and the Peyer’s patches, the pieces were washed thoroughly in cold RPMI 1640 medium and incubated for 15 min at RT in 1 mM dithiothreitol to remove excess mucus. After centrifugation (1500 rpm, 5 min), the pellet was resuspended in RPMI medium containing 5% fetal calf serum (FCS) (both from PAA Laboratories, Pasching, Austria), 1 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (both from Sigma-Aldrich) and incubated for 1 h at 37 °C in an orbital shaker (speed 140/min). The acquired suspension was filtered through a 100 μm mesh and additionally through a 60 μm mesh and centrifuged at 1500 rpm for 5 min. The pellet was resuspended in RPMI medium containing 5% FCS, 1 mM EDTA and mixed with a Percoll gradient solution (40% final). It was then layered upon 70% Percoll and centrifuged at 1500 rpm for 20 min without rotor brakes. Intraepithelial lymphocytes (found in the layer between 40% and 70% Percoll) were collected, washed twice in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA) (AppliChem GmbH, Darmstadt, Germany), 1 mM EDTA and resuspended in RPMI 5% FCS.

2.6. Isolation of Peyer’s patches cells

Peyer’s patches (PP) were removed from the small intestine and the cells were obtained by passing the tissue through a 40 μm mesh. After centrifugation, the cells were resuspended in RPMI 5% FCS and counted, after which $5x10^5$ cells were used for flow cytometry staining (Couter & Surana, 2016).

2.7. Isolation of peritoneal macrophages

Peritoneal cells (PC) were obtained by lavage of the peritoneum with 4 mL of cold PBS. The samples were then centrifuged at 550*g* for 5 min and 5×10^6 cells were seeded in RPMI 5% FCS supplemented with 1% penicillin and streptomycin in 24-well adherent plates (Sardstedt, Numbrecht, Germany), as described by Vujicic et al. (2015). The cells were kept in an incubator at 37 °C with 5% CO_2 for 2 h, after which the wells were washed with warm PBS to remove non-adherent cells. The remaining cells were considered as macrophages and were either stimulated with LPS (Sigma-Aldrich) and interferon-γ (IFN-γ) (R &D Systems, Minneapolis, MN, USA) or kept unstimulated in the presence or absence of increasing concentrations of the chokeberry extract (between 0.08 and 50 μg/ml, depending upon the experiment). After performing a viability assay, the dose of 25 μg/ml of the chokeberry extract was chosen for application in further experiments. For flow cytometry, macrophages were detached from the adherent plates by placing the plate on ice and by washing the cells with cold PBS.

2.8. Differentiation of dendritic cells in vitro

Dendritic cells (DC) were obtained from progenitor bone marrow cells of C57BL/6 mice. The femur was isolated and flushed aseptically with RPMI 10% FCS. Cells were then centrifuged, erythrocytes lysed with red blood cells (RBC) lysis buffer (eBioscience, San Diego, CA, USA) and the cells resuspended in RPMI 20% FCS, 2 mM L-glutamine, 1 mM Na-pyruvate (PAA Laboratories). The obtained cells were cultivated with 20 ng/ml granulocyte-macrophage colony-stimulating factor (Peprotech, London, UK) for 7 days with a medium change every second day. DC differentiation was performed according to the protocol described by Djedovic et al., 2019. DC were passaged on day 7 by enzymatic digestion with acutase (ThermoFisher, Waltham, MA, USA) treatment and seeded at 5×10^5 cells/well in a 96-well plate (Sardstedt). To induce mature DC, cells were treated with 100 ng/ml LPS on day 7, in the presence or absence of different concentrations of the chokeberry extract (6–50 μg/ml). Supernatants were collected 48 h after the treatment with chokeberry extract.

2.9. Isolation of splenocytes

Spleens were removed aseptically and processed according to the protocol described by Vujicic et al., 2015. After passing through plastic mesh and centrifugation, erythrocytes were lysed with RBC lysis buffer and cells were resuspended in RPMI 5% FCS. 5×10^6 cells were seeded in RPMI 5% FCS and treated with concanavalin A (ConA – Pharmacia Fine Chemicals, Uppsala, Sweden) in the presence of the chokeberry extract (25 μg/ml). Supernatants were collected after 48 h of incubation and flow cytometry analysis was performed.

2.10. Differentiation of Th1, Th17 and Treg cells in vitro

Naïve $CD4^+CD25^-$ lymphocytes were purified from the spleens of healthy animals. Splenocytes were first incubated with biotin-conjugated anti-mouse CD25 (1:100, eBioscience) for 20 min on ice for negative selection. Cells were then resuspended in cold magnetic bead buffer (PBS 0.5% BSA, 2 mM EDTA) containing BD IMag™ Streptavidin Particles Plus - DM (1:20, BD Biosciences, Bedford, MA, USA). $CD25^-$ cells were purified by placement in a BD IMag™ Cell Separation Magnet (BD Biosciences), 3× for 8 min. After washing in PBS, $CD25^-$ cells were subjected to positive selection by incubation with biotin-conjugated anti-mouse CD4 (1:60). Purified $CD4^+CD25^-$ cells were resuspended in RPMI 10% FCS, 0.02 mM Na-pyruvate, 5 μM β-mercaptoethanol, 2 mM L-glutamine, 25 mM HEPES (PAA Laboratories) and antibiotics. $CD4^+CD25^-$ cells (5×10^5) were seeded in U-bottom 96-well plates and received stimuli from plate-bound anti-CD3 (1 μg/ml) and soluble anti-CD28 antibodies (1 μg/ml) (both from eBioscience). For type 1 T helper (Th1) differentiation the cells were additionally stimulated with interleukin-12 (IL-12) (20 ng/ml, R&D Systems) and anti-interleukin-4 (IL-4) antibody (10 ng/ml, eBioscience), for Th17 differentiation the cells were stimulated with transforming growth factor-β (TGF-β) (10 ng/ml) and IL-6 (10 ng/ml) (both from R&D Systems), while for T regulatory (Treg) differentiation the cells were stimulated with TGF-β (2 ng/ml) and interleukin-2 (IL-2) (10 ng/ml, R&D Systems). The chokeberry extract was added 24 h after starting the culture, and 3 days later the presence of T cell subsets was analyzed by flow cytometry.

2.11. Rat insulinoma m5F (RINm5F) cell culture

RINm5F cells were a kind gift from Dr Melita Vidakovic, Institute for Biological Research “Sinisa Stankovic”, and were purchased from ATCC® (ATCC® CRL-11605™). The cells (4.5×10^4/well) were seeded in 96-well plates (Sardstedt) in RPMI 10% FCS, 2 mM L-glutamine, 50 μM β-mercaptoethanol and antibiotics. They were cultured in the presence or absence of a pro-inflammatory cytokine mixture – IL-1β, TNF and IFN-γ (all from R&D Systems), 10 ng/ml each, and treated with increasing concentrations of the chokeberry extract (100–400 μg/ml). After 24 h of incubation, a cell viability assay was performed.

2.12. Griess test

Culture supernatants from peritoneal macrophages and splenocytes were used for measuring nitrite accumulation as an indicator of NO production. Equal volumes of supernatants and Griess reagent were mixed and after 10 min the mixture color intensity was detected by LKB microplate reader (LKB Instruments, Vienna, Austria) at 540/670 nm (for impurity corrections).

2.13. Viability assays

The viability of DC, macrophages and RIN5mF in cell cultures was tested by MTT and crystal violet assays. After the collection of supernatants, cells were treated with 0.5 mg/ml 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution (Sigma-Aldrich) for 30 min at 37 °C in a humidified incubator. After the addition of dimethyl sulfoxide, the developed color intensity was measured by a microplate reader at 540/670 nm. Plates were then washed and crystal violet solution was added. After 30 min at RT, the solution was thoroughly washed with running water and 50 μL of 33% acetic acid per well was added. Again, the absorbance was measured by a microplate reader at 540/670 nm.

2.14. Flow cytometry

Viable cells were used for detection of surface molecules. Surface staining was performed in PBS 1% BSA by adding the following antibodies: anti-mouse CD4 PerCP-Cyanine5.5 (rat IgG2a,κ), CD4-FITC (rat IgG2b,κ), CD8-PE (rat IgG2a,κ), B220-FITC (rat IgG2a,κ), CD11c-PE-Cy5 (Armenian hamster IgG), F4/80-FITC (rat IgG2a,κ), CD25-PE (rat IgG1,λ), CD206-PE (rat IgG2b,κ), MHC II-FITC (rat IgG2b,κ), CD80-PE-Cy5 (Armenian hamster IgG), CD86-PE-Cy5 (rat IgG2a,κ), CD19-PE-Cy5 (rat IgG2a,κ) and CD40-PE (Armenian hamster IgM,κ) (all from ThermoFisher Scientific). The staining was performed for 45 min at 4 °C. Intranuclear FoxP3 was detected by Mouse Regulatory T cell Staining Kit according to the manufacturer’s instructions (eBioscience). Similar procedure was used for Ki67 detection. For intracellular cytokine staining, cells were stimulated with Cell Stimulation Cocktail (plus protein transport inhibitors) (1:500, eBioscience) for 4 h. Cells were then fixed in 2% paraformaldehyde, permeabilized and stained with the following antibodies: anti-mouse IFN-γ-PE (rat IgG1,κ) (eBioscience) or IL-17-PE (rat IgG1,κ) (BD Biosciences). Isotype-matched controls were included in all experiments (eBioscience). Cells were acquired on Partec CyFlow Space and analyzed by FlowMax software (Partec, Görlitz,

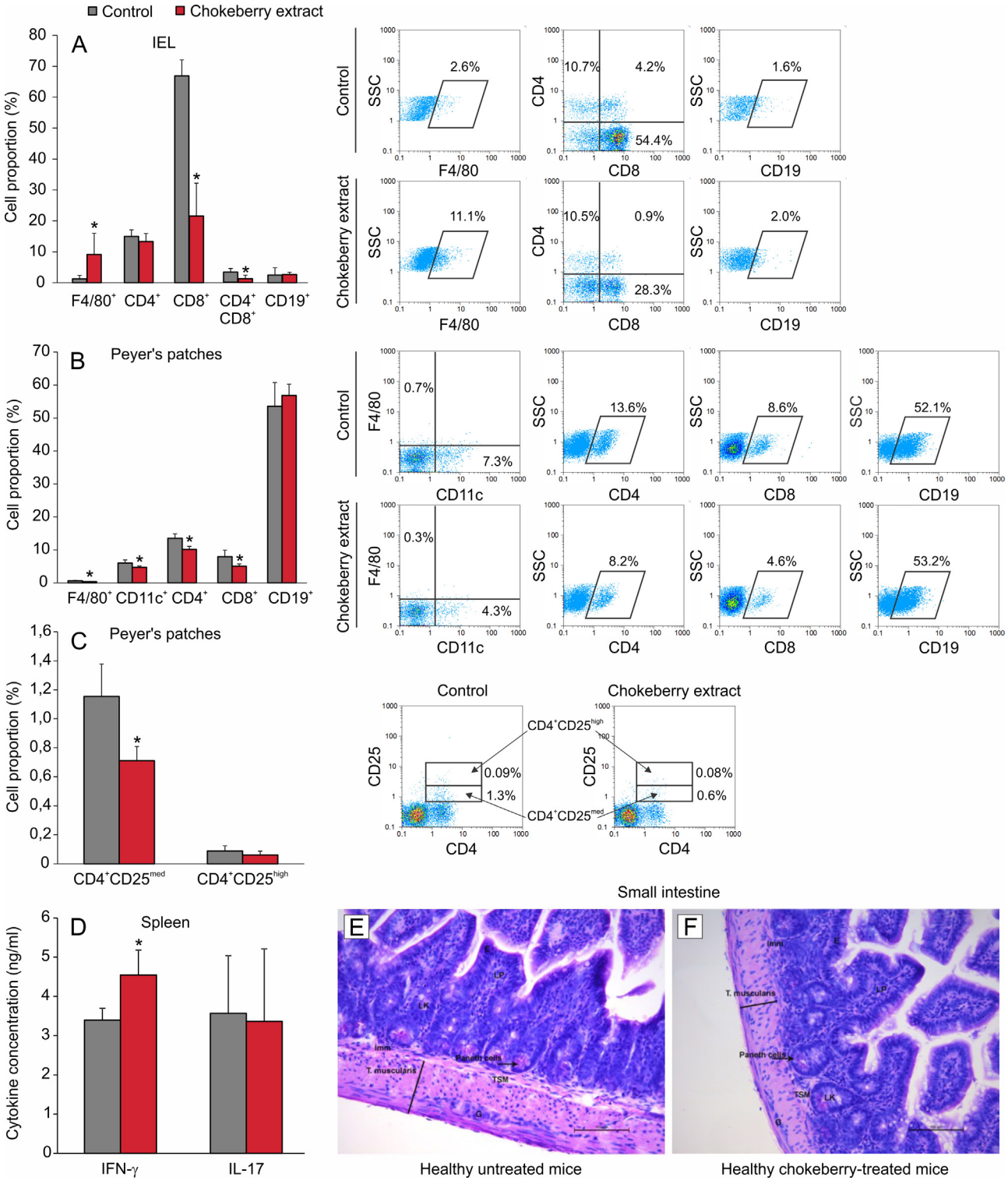

Fig. 1. The effect of the chokeberry extract on the immune response in healthy C57BL/6 mice. (A) The proportion of F4/80$^+$ macrophages, CD4$^+$ T helper lymphocytes, CD8$^+$ cytotoxic lymphocytes, CD4$^+$CD8$^+$ memory cells and CD19$^+$ B lymphocytes in the compartment of gut intraepithelial lymphocytes (IEL). (B) The proportion of F4/80$^+$ macrophages, CD11c$^+$ dendritic cells, CD4$^+$ T helper lymphocytes, CD8$^+$ cytotoxic lymphocytes and CD19$^+$ B lymphocytes in Peyer's patches. (C) The proportion of activated T helper (CD4$^+$CD25^{med}) or T regulatory (Treg – CD4$^+$CD25^{high}) cells. Representative dot plots are shown on the right hand side. (D) Production of cytokines from splenocytes *ex vivo* after 24 h of cultivation. (E and F) Representative histological sections of the small intestine. $p < 0.05$ represents the significant change between populations or cytokine production of cells from chokeberry-treated mice compared to control mice.

Table 1
The absolute number of immune cell subsets in the intraepithelial lymphocytes (IEL) and Peyers's patches (PP) of healthy C57BL/6 mice treated with chokeberry extract.

($\times 10^6$ cells)	Control (IEL)	Chokeberry (IEL)	Control (PP)	Chokeberry (PP)
$F4/80^+$	0.092 ± 0.044	0.268 ± 0.105*	0.005 ± 0.002	0.006 ± 0.001
$CD11c^+$	NM	NM	0.057 ± 0.031	0.072 ± 0.012
$CD4^+$	2.0 ± 1.6	1.2 ± 0.4	0.123 ± 0.049	0.157 ± 0.036
$CD8^+$	7.9 ± 4.7	2.2 ± 1.6*	0.073 ± 0.033	0.076 ± 0.012
$CD19^+$	0.18 ± 0.10	0.12 ± 0.12	0.484 ± 0.192	0.868 ± 0.110*

NM – not measured.
* $p < 0.05$, statistical significance between the absolute numbers of IEL and PP of chokeberry extract-treated vs untreated (control) C57BL/6 mice.

Germany). The cells were first gated on live cells (empirically determined) and then further gated appropriately to the required analysis.

2.15. Phagocytosis assay

For the detection of phagocytosis, macrophages were plated in 24-well plates at 1×10^5/well and incubated at 37 °C for 1 h. After washing with PBS, cells were incubated at 37 °C for in the presence or absence of the chokeberry extract (25 μg/ml). After 24 h, PE-labeled latex beads (amine-modified polystyrene particles around 1 μm in diameter in the aqueous suspension, Sigma-Aldrich) were pre-opsonized in PBS 50% FCS. Macrophages were incubated with the pre-opsonized beads (10 beads per cell) for 1 h, after which the cells were analyzed with cytofluorimetry (Partec CyFlow Space).

2.16. ELISAa

Cell supernatants were used for the determination of cytokine concentration by sandwich ELISA using MaxiSorp plates (Nunck, Rochild, Denmark) and anti-mouse paired antibodies according to the manufacturer's instructions. Samples were analyzed in duplicate for murine TNF, IL-1β, IL-17, IFN-γ and IL-12 (eBioscience), TGF-β (R&D Systems), IL-6 (Abcam, Cambridge, MA, USA) and the absorbance was measured by a microplate reader at 450/570 nm. A standard curve was created using known concentrations of appropriate recombinant cytokines and was used to calculate the sample cytokine concentrations.

2.17. Histological examination of intestinal sections

Samples of the small intestine were fixed in 10% neutral buffered formalin, processed to paraplast and cut on a microtome at 5 μm thick sections. Tissue sections were stained with hematoxylin-eosin (Biooptica, Milan, Italy). Morphometric measurements were performed on photomicrographs acquired with digital camera Leica DFC295 (Leica, Heerbrugg, Switzerland) connected to a light microscope Leica DM4000 B LED (Leica, Wetzlar, Germany). All elements of the small intestine were measured (in μm) using open-source software Fiji (Schindelin et al., 2012). Measurements were performed at the magnification ×200, on 3 tissue sections for each sample. For each parameter 3 measurements per section were done.

2.18. Statistical analysis

Data are presented as mean ± SD. The significance of differences between groups was determined by a two-tailed Student's *t*-test. Differences are regarded as statistically significant if $p < 0.05$. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. The effect of chokeberry extract oral consumption on the immune cells in the gut-associated lymphoid tissue (GALT) and spleen of healthy C57BL/6 mice

To determine whether the orally-delivered chokeberry extract affected immune cells in GALT, IEL and PP cells were analyzed. The chokeberry extract was administered for 7 days and *ex vivo* analysis was performed at day 10 post-treatment. The results indicate that immune cells in the GALT of healthy C57BL/6 mice were markedly affected by oral consumption of the chokeberry extract. The absolute number of IEL was mildly decreased in chokeberry-treated mice (control *vs*. chokeberry-treated $(11.7 \pm 8.7) \times 10^6$ *vs* $(5.9 \pm 4.7) \times 10^6$, respectively, $p = 0.299$), but the number of cells per PP was significantly higher after the administration of chokeberry extract (control *vs* chokeberry-treated $(0.9 \pm 0.4) \times 10^6$ *vs* $(1.5 \pm 0.3) \times 10^6$, respectively, $p = 0.028$). The proportion of $F4/80^+$ macrophages was significantly up-regulated, while $CD4^+CD8^+$ and $CD8^+$ lymphocytes were down-regulated in IEL of chokeberry-treated mice (Fig. 1A). Proportions of $CD4^+$ T lymphocytes and $CD19^+$ B lymphocytes were comparable between groups (Fig. 1A). Similar results were obtained when the absolute number of cells was calculated (Table 1). Cell proportions in PP had a slightly different distribution pattern compared to IEL. Namely, PP from chokeberry-treated mice had a lower proportion of $F4/80^+$ macrophages, $CD11c^+$ dendritic cells, $CD4^+$ T helper lymphocytes and $CD8^+$ cytotoxic lymphocytes (Fig. 1B). Again, when comparing absolute numbers, the only significant change was detected in the pool of B cells ($CD19^+$) in favor of higher numbers of B cells after chokeberry extract treatment (Table 1). Among T helper cells, a lower proportion of activated inflammatory $CD4^+CD25^{med}$ T cells was observed, while the proportion of suppressive T regulatory (Treg) cells was similar between the control and treated group of mice (Fig. 1C).

In order to clarify whether the observed alterations in immune cell composition in the GALT of mice treated with the chokeberry extract reflect its anti- or pro-inflammatory effects *in vivo,* we examined the production of IFN-γ and IL-17 in *ex vivo* cultures of splenocytes. Oral consumption of the chokeberry extract significantly stimulated the production of IFN-γ, while IL-17 secretion remained unaltered (Fig. 1D). These results imply that in addition to affecting the GALT, chokeberry affected the systemic immune response and provoked IFN-γ production in splenocytes.

We next evaluated the morphological characteristics of the luminal side of the intestinum and no differences were found in the height of the three principal layers of intestinal wall (mucosa, submucosa, muscularis), the elements of mucosa (the height of intestinal villi, the depth of Lieberkühn crypts, the height of surface epithelium, the width of muscularis mucosa) and the width of the circular and longitudinal layer within muscularis (Fig. 1E, F and Table 2).

Table 2
Morphometric measurements of elements of small intestine (μm).

	Mucosal height	Villus height	Depth of crypts	Surface epithelium height	Width of muscularis mucosa	Submucosal width	Muscularis width	Circular layer width	Longitudinal layer width
Control	314.6 ± 38.3	203.7 ± 44.7	106.8 ± 28	14.1 ± 3.2	7.5 ± 2.3	11.7 ± 3.6	73.7 ± 31.6	45.2 ± 13.8	16.9 ± 9.7
Chokeberry extract	286.7 ± 46.4	191.7 ± 41.1	89.4 ± 20.4	17.5 ± 3.9	7.5 ± 3.8	14.6 ± 6.2	63.5 ± 16.1	42.9 ± 12.6	15.6 ± 7.1

3.2. The effects of oral consumption of the chokeberry extract on T1D in mice

Since our results indicated that the chokeberry extract had a profound impact on immune cells *in vivo*, we next investigated its effects in the model of T1D induced by multiple low doses of STZ in C57BL/6 mice. The chokeberry extract was orally consumed starting from the first injection of STZ, once a day for 7 days in total (Fig. 2A). A higher concentration of chokeberry extract (200 mg/kg bw) was shown to be lethal in 2 out of 7 mice on day 7 after T1D induction. This group of mice exhibited significant weight loss by day 7 (Fig. 2B) and hyperglycemia on day 14 after T1D induction (Fig. 2C). We then investigated the effect of a lower dose of chokeberry extract (50 mg/kg bw). Similarly, oral administration of the extract resulted in higher blood glucose levels compared to untreated diabetic mice (Fig. 2E and F), while no change in the body weight was observed (Fig. 2D). In contrast to the diabetic mice, chokeberry administration to healthy C57BL/6 mice did not provoke the increase of blood glucose levels compared to untreated mice at day 7 post-treatment (8.5 ± 1.7 *vs* 7.3 ± 1 mmol/L, respectively). The observed elevation of glycemia in diabetic animals was probably not a result of a direct harmful effect of the chokeberry extract on pancreatic β-cells, since its application *in vitro* on RIN5mF cells (insulinoma cell line) caused significant protection from the detrimental influence of pro-inflammatory cytokines (Fig. 2G). Since the chokeberry extract increased glycemia, the next step was to investigate the effect of chokeberry on the cells of the immune system that are involved in the inflammatory/autoimmune response during T1D.

3.3. The influence of the chokeberry extract on antigen-presenting cells in vitro

In order to further delineate the effect of chokeberry on immune cells, we performed *in vitro* studies. First, we examined the effect of the chokeberry extract on innate immune cells. DC were exposed to the maturation signal (LPS) in the presence or absence of various concentrations of the chokeberry extract. The viability of DC did not decrease after 48 h of incubation with the extract (Fig. 3A and B), while the presence of the chokeberry extract boosted LPS-driven production of NO (Fig. 3C). Since chokeberry slightly increased the number of DC (measured by crystal violet), when NO production was normalized to the number of cells, the observed increase in NO was annulled. The production of pro-inflammatory cytokines IL-β and IL-6 remained unaltered in DC cultures exposed to the chokeberry extract, while the production of TNF was increased (Fig. 3D).

The chokeberry extract exerted similar effects on *in vitro* cultured macrophages. The viability of macrophages was not affected by the chokeberry extract in any of the tested concentrations (Fig. 4A). The chokeberry extract increased NO production by macrophages (Fig. 4B) and increased the proportion of macrophages expressing the co-stimulatory molecule CD86 (Fig. 4C), while it increased the proportion of both pro-inflammatory M1 macrophages and anti-inflammatory M2 macrophages (Fig. 4D). The ability of macrophages to phagocytize particles *in vitro* was significantly increased after the chokeberry treatment (Fig. 4E). The production of pro-inflammatory IL-6 and IL-12 was increased in macrophage cultures after chokeberry extract treatment, while IL-1β and TNF production remained unchanged (Fig. 4F). These results demonstrate the stimulatory effect of chokeberry extract on innate immunity cells *in vitro*.

3.4. The effect of the chokeberry extract on adaptive immune cells in vitro

To determine the effect of the chokeberry extract on cells of acquired immunity, a suspension of splenocytes was subjected to chokeberry extract treatment in the presence of ConA. After 48 h of incubation, an MTT test revealed that chokeberry extract increased the

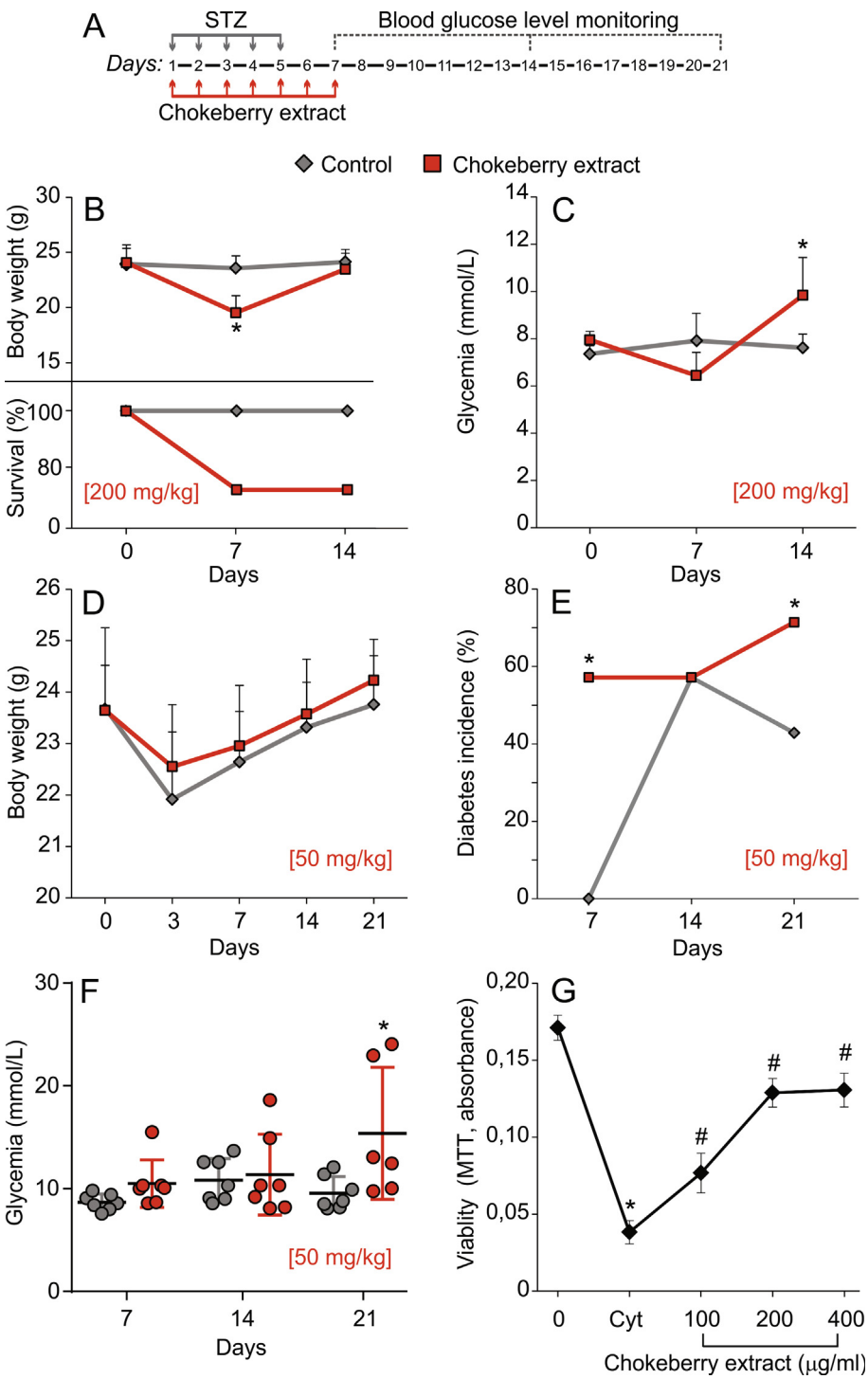

Fig. 2. The effect of the chokeberry extract on T1D pathogenesis. (A) Schematic overview of the T1D induction and chokeberry treatment of C57BL/6 mice. (B) Body weight and survival and (C) glycemia of mice treated with chokeberry extract (200 mg/kg bw). (D) Body weight, (E) diabetes incidence and (F) glycemia of mice treated with chokeberry extract (50 mg/kg bw). (G) The viability of RINm5F treated with cytokines (IL-1β + TNF + IFN-γ) in the presence of the chokeberry extract. Results were obtained from 7 mice per group. $p < 0.05$ represents the significant change between chokeberry-treated mice compared to control mice or chokeberry + cytokine-treated RIN5mF cells *vs* cytokine-treated cells.

number of viable cells (ConA-treated *vs.* chokeberry extract + ConA-treated 0.0800 ± 0.0017 *vs* 0.0917 ± 0.0051, respectively, $p = 0.001$) as well as the proportion of $CD4^+$, $CD8^+$ and $B220^+$ B lymphocytes (Fig. 5A). The production of IL-17, IFN-γ and TGF-β did not change after chokeberry extract treatment (Fig. 5B).

We further examined whether the chokeberry extract impacts *in vitro* induced differentiation of T helper cells. To this end, we cultured naïve $CD4^+CD25^-$ cells in conditions that favor differentiation of Th1, Th17 or Treg cells in the presence of the chokeberry extract. The extract promoted Th1 cell differentiation and suppressed Treg cell differentiation, while no effect on Th17 cells was observed after 5 days of cultivation (Fig. 5C). The expression of signature cytokines IFN-γ (Th1) and IL-17 (Th17) was not altered in the presence of chokeberry extract (Fig. 5D). These results suggest that the chokeberry extract favored the differentiation of pro-inflammatory Th1 cells.

3.5. HPLC analysis of the chokeberry extract

In order to compare the composition of our chokeberry extract to others, phenolic constituents were quantified using the HPLC method. Cyanidin-3-galactoside was the main anthocyanin compound (0.34 mg/g), followed by cyanidin-3-arabinoside (0.16 mg/g) and cyanidin-3-glucoside (0.06 mg/g). Quercetin derivatives were the major flavonoids in the chokeberry, with quercetin-3-glucoside, quercetin-3-galactoside

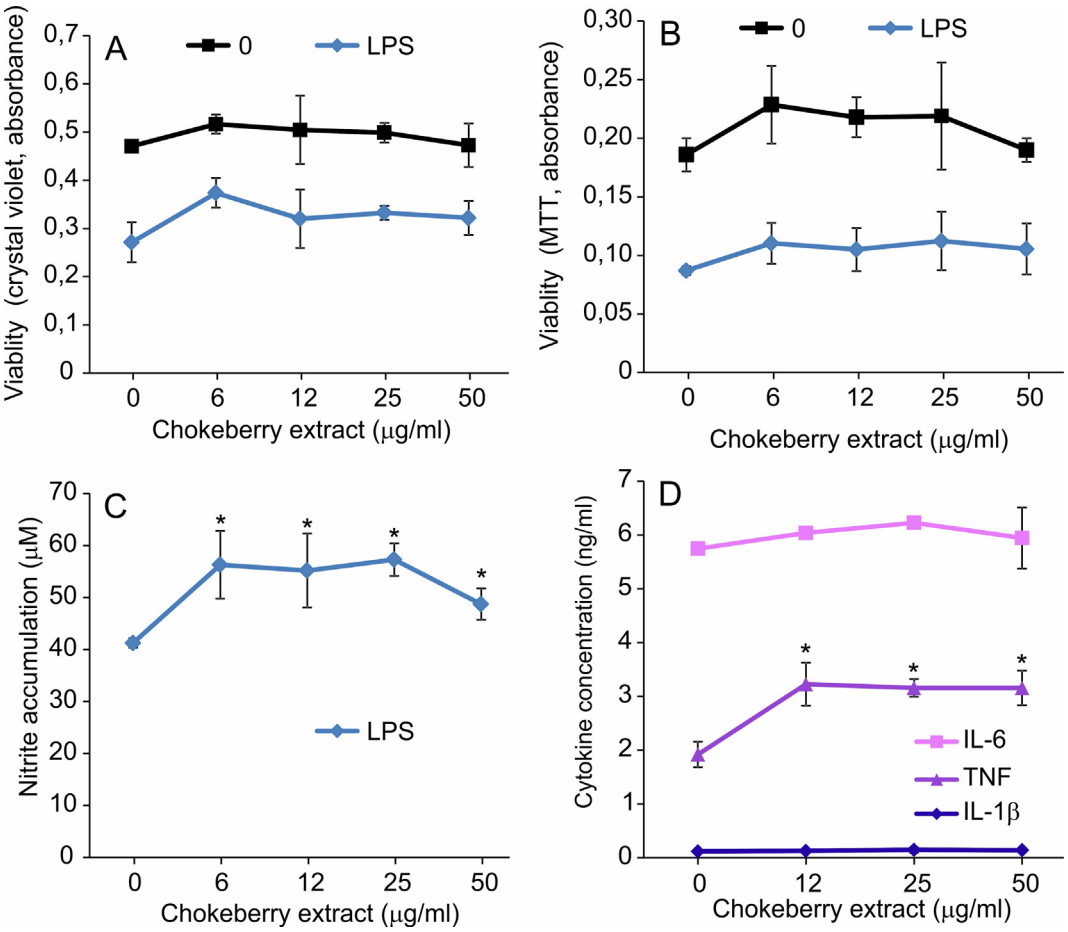

Fig. 3. The effect of the chokeberry extract on DC *in vitro*. Viability measured by (A) crystal violet or (B) MTT assay and (C) NO production of DC stimulated with LPS in the presence or absence of increasing concentrations of the chokeberry extract. (D) Production of cytokines from DC after 24 h of cultivation. $p < 0.05$ represents the significant change between populations or cytokine production of chokeberry-treated cells compared to adequate controls.

and quercetin-3-rutinoside found in amounts of 0.15 mg/g, 0.31 mg/g, and 0.28 mg/g, respectively. Chlorogenic acid was the main phenolic acid, and its content was 3.53 mg/g. The amount of total proanthocyanidins was 36.5 mg CE/g. This phenolic pattern is in agreement with previously reported data (Kulling & Rawel, 2008).

4. Discussion

This study points to the pro-inflammatory properties of the chokeberry extract: it exacerbated T1D clinical signs in C57BL/6 mice, triggered a significant perturbation of immune cell composition within the GALT and stimulated pro-inflammatory features of innate and adaptive immune cells *in vitro*.

Chokeberry extracts have already been used in animal models of various inflammatory conditions. The applied doses have ranged from 10 to 2680 mg/kg bw (Kang et al., 2017; Valcheva-Kuzmanova, Kuzmanov, Kuzmanova, & Tzaneva, 2018) and each of them was well tolerated and exerted biological effects. We have first chosen the dose of 200 mg/kg bw according to the study by Lee et al. (2016) that was performed on ICR mice. However, the dose of 200 mg/kg bw of our chokeberry extract showed lethal effects in the diabetes model in C57BL/6 mice. The lower dose of the chokeberry extract (selected according to our study performed on spontaneously hypertensive rats) (Ćujić et al., 2018) had no lethal effects, but it did increase blood glucose levels in diabetic C57BL/6 mice. This finding is in contrast to the study which demonstrated beneficial effects of a chokeberry extract on hyperglycemia in ICD mice (Jeon et al., 2018). In a human intervention study, a daily intake of 200 mL of chokeberry juice over a period of 3 months was effective in lowering fasting glucose levels in patients with non-insulin dependent diabetes (Simeonov et al., 2002). Although chokeberry fruit contains high amounts of sugar, with sorbitol being the most abundant, the results of Simeonov et al. suggest that this sugar content does not raise blood glycemia levels. Therefore, the increased glycemia in mice with T1D could be attributed to the chokeberry-mediated influence on immune cells rather than the direct contribution of the sugar content to the overall blood glucose level. Additionally, the aggravation of T1D by the chokeberry extract was most probably not prompted by its interference with pancreatic β-cells. This is corroborated by the finding that *in vitro* application of the chokeberry extract on RIN5mF β-cell line protected the cells from the cytotoxic influence of pro-inflammatory cytokines. Therefore, our next aim was to investigate the effect of chokeberry extract on immune cells *in vivo* and *in vitro*.

Generally, chokeberry extracts contain mixtures of biologically-active substances that inhibit inflammation. Polyphenol compounds, more precisely anthocyanins, are responsible for the anti-inflammatory properties of berries (Joseph, Edirisinghe, & Burton-Freeman, 2014; Martin et al., 2014). According to literature, the main anthocyanin compounds in chokeberry extracts - cyanidin and its glucosides show an extremely potent anti-inflammatory activity (Jung, Kwak, & Hwang, 2014; Wang et al., 1999). In addition to specific constituents that show anti-inflammatory effects, the application of a whole berry extract was highly operative in preventing inflammation as well. For example, oral administration of a chokeberry extract inhibited prostaglandin E2 production in DSS-induced colitis (Kang et al., 2017), down-regulated inflammatory markers in LPS-induced uveitis (Ohgami et al., 2005),

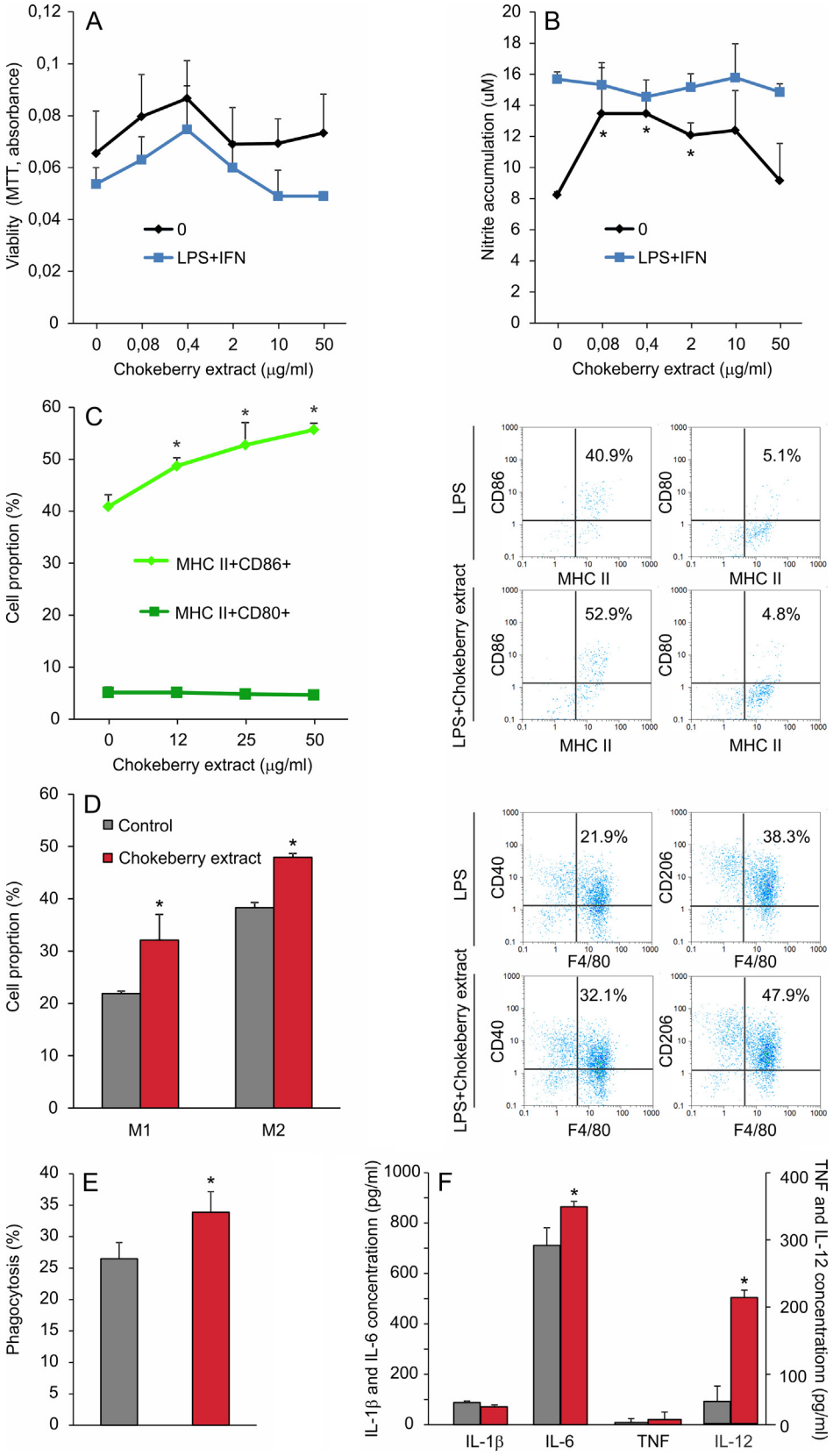

Fig. 4. The effect of the chokeberry extract on macrophages *in vitro*. (A) Viability and (B) NO production of peritoneal macrophages stimulated with IFN-γ + LPS in the presence or absence of increasing concentrations of the chokeberry extract. (C) The proportion of MHC II^+ macrophages expressing CD80 or CD86 co-stimulatory molecules after the treatment with the chokeberry extract. (D) The proportion of M1 ($F4/80^+CD40^+$) and M2 ($F4/80^+CD206^+$) macrophages after the treatment with the chokeberry extract (25 μg/ml). Representative dot plots of 25 μg/ml chokeberry treatments are shown on the right hand side. (E) The proportion of macrophages that phagocytized PE-labeled latex beads, analyzed by flow cytometry after 24 h of cultivation. (F) Production of cytokines from macrophages after 24 h of cultivation. $p < 0.05$ represents the significant change between populations or cytokine production of chokeberry-treated cells compared to adequate controls.

and decreased the levels of NO, IL-6, and TNF-α in *ex vivo* LPS-stimulated macrophages (Kang et al., 2017). Our results suggested the opposite effects: the chokeberry extract stimulates the pro-inflammatory functions of both innate and adaptive cells of the immune response *in vitro*. As we have excluded the possible contamination of the extract with either live bacteria or endotoxin (LPS) that could potentially activate immune cells, the discrepancy in the effect between this specific chokeberry extract and other ones can be only attributed to different extraction procedures and subsequent difference in extract composition. Although the phenolic content is similar to some of the chokeberry

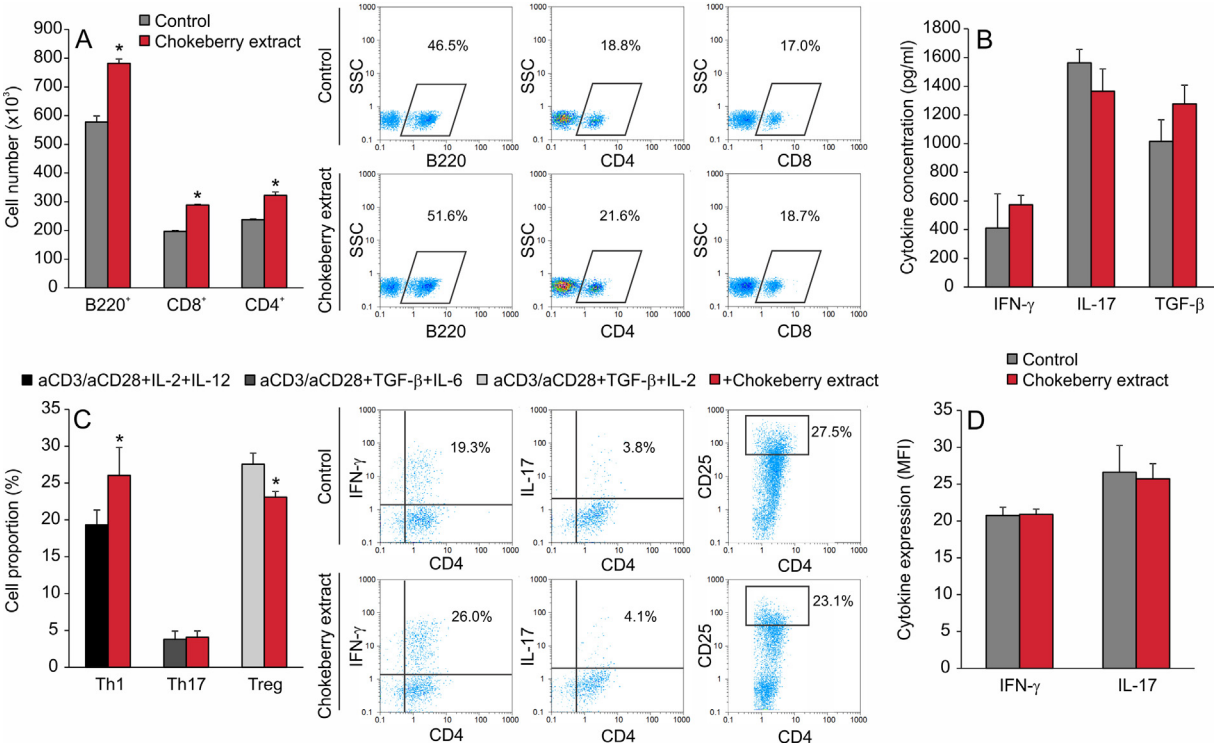

Fig. 5. The effect of the chokeberry extract on lymphocytes *in vitro*. Splenocytes were treated *in vitro* with the chokeberry extract (25 μg/ml) and cell populations were analyzed 24 h after the addition of chokeberry (A) and cytokine production was measured in the presence of ConA (B). (C) Purified $CD4^+CD25^-$ lymphocytes were stimulated either toward Th1 cell differentiation (anti-CD3 + anti-CD28 antibodies + IL-2 + IL-12) or Th17 (anti-CD3 + anti-CD28 antibodies + TGF-β + IL-6) or Treg (anti-CD3 + anti-CD28 antibodies + IL-2 + TGF-β) for 5 days. The chokeberry extract was added 24 h after the initiation of differentiation. Representative dot plots are shown on the right hand side. (D) Cytokine expression within Th1 and Th17 lymphocytes measured as mean fluorescence intensity (MFI). $p < 0.05$ represents the significant change between populations of chokeberry-treated cells compared to adequate controls.

extracts described by Kulling and Rawel (2008), the other constituents such as organic acids, sugars, dietary fiber, minerals, vitamins and proteins could be different. Therefore, the ratio between specific bioactive compounds can result in different bioactivity. Also, the content of the extract is highly influenced by localization, culture conditions, depends on the variety of species, season and harvest date (Sidor & Gramza-Michałowska, 2019). Moreover, in our study the chokeberry extract dose of 200 mg/kg bw exerted lethal effects, which is in contrast to reported studies. This finding further implies a different composition of our chokeberry extract.

DC and macrophages were the main representatives of innate immunity affected by the chokeberry extract. NO secretion from macrophages was increased after chokeberry extract administration. NO is a pro-inflammatory molecule that participates in the elimination of invading microbes and could act in an anti-proliferative manner regarding T cells (Fonseca et al., 2003). The production of NO in DC seemed to be higher after the chokeberry treatment, but when normalized to the number of viable DC, there was no statistical significance. The administration of LPS decreased the viability of DC, while the chokeberry extract mildly prevented LPS-induced cell death. Overall, it can be concluded that the chokeberry extract exerted a biological effect on DC *in vitro*, either through the preservation of their viability or NO induction. Along with NO, other mediators such as TNF and IL-6 produced by phagocytes were stimulated by the chokeberry extract. To support the increased activation of macrophages, their phagocytic ability was also enhanced in the presence of the chokeberry extract. Although all data point to the stimulation of M1 pro-inflammatory macrophages by the extract, the number of M2 anti-inflammatory macrophages was elevated as well, suggesting an overall stimulatory effect of chokeberry on macrophage proliferation. The effect of the chokeberry extract on the representatives of adaptive immunity, Th1, Th17 or Treg cells, was again in favor of inflammation. Namely, chokeberry specifically stimulated Th1 and inhibited Treg cells. Th1 cell differentiation is a process dependent on IL-12 (Zhang, Zhang, Gu, & Sun, 2014). The chokeberry extract stimulated the production of this cytokine from phagocytes, and this might be one of the reasons behind stimulated Th1 differentiation. In concordance with the generally pro-inflammatory effects, Treg cell differentiation was partially blocked after the treatment with the chokeberry extract. Treg cells are important for the maintenance of immune system homeostasis, as they inhibit effector T cell proliferation. Therefore, the observed negative effect on Treg cells may have been another reason for the stimulated Th1 differentiation. Interestingly, although the chokeberry extract stimulated IL-6 production in macrophages, the differentiation of Th17 cells remained unaltered. Th17 cells require the presence of both IL-6 and TGF-β. Therefore, the reason for this effect might be found in the unchanged TGF-β production.

When given orally, the chokeberry treatment enhanced the number of cells within the PP in healthy mice, either by stimulating their *in situ* proliferation or by attracting them from other lymphoid tissues. Furthermore, the number of macrophages was increased in the GALT, as well as *ex vivo* splenic production of IFN-γ. However, we found a reduction in effector $CD4^+CD25^{med}$ cell proportion in the GALT as well as the proportion of highly activated $CD4^+CD8^+$ memory cells (Pahar, Lackner, & Veazey, 2006). These results suggest that oral consumption of chokeberry extract in healthy mice affects the proportions of immune cells in GALT.

To date there are very little data on pro-inflammatory effects of plant extracts in general (Yahfoufi, Alsadi, Jambi, & Matar, 2018). Similarly to the chokeberry extract, the water extract of litchi (Asian cherry) stimulates pro-inflammatory mediators IL-1β, inducible NO synthase and cyclooxygenase-2 in a macrophage cell line (Wang, Hu, Yan, Ma, & Deng, 2016). Specific proteins were identified in litchi as the bioactive compounds that caused the observed effects. Therefore,

the observed chokeberry extract effect can be attributed to proteins. Furthermore, citrus-derived pectins may cause an increased production of IL-17, IFN-γ and TNF from mouse splenocytes *in vivo* (Merheb, Abdel-Massih, & Karam, 2019). Pectins are also highly abundant in chokeberry extracts, with pectin content similar to those of apples or pears, fruits that have high pectin content (Nawirska & Kwaśniewska, 2005). Therefore, the pro-inflammatory nature of the chokeberry extract may be related to its pectin levels. Of note, herbal extracts that show anti-cancer properties may mediate their effects through the stimulation of the immune response. For example, extracts from *Astragalus* species stimulate T cell activation and the production of pro-inflammatory cytokines *in vivo* and *in vitro*. These effects are attributed to astragalosides (Qi, Gao, Hou, & Wan, 2017). It is still uncertain which constituents of our chokeberry extract causes the observed pro-inflammatory effects, but it can be speculated that the specific ratio between organic compounds, vitamins and minerals present in the chokeberry extract (Kulling & Rawel, 2008; Nawirska & Kwaśniewska, 2005) could provoke such an effect, which warrants further investigation. One of the possible reasons for the observed pro-inflammatory effects of our chokeberry extract could be the low content of polyphenols. For example, in contrast to an extract used in the treatment of uveitis where the extract contained 16.9% of polyphenols (Ohgami et al., 2005), our chokeberry extract contains only 4%.

The observed feature of the chokeberry extract used in our study could be utilized in the fight against infections or cancer, in which an enhanced pro-inflammatory response is beneficial. The results of this study point to the necessity of thorough investigation of plant extracts before their commercialization.

5. Ethics statements

Ethic Committee of the Institute for Biological Research “Sinisa Stankovic” approved all experimental procedures with C57BL/6 mice (App. No 02-11/18 - 01-2420) and they comply with the ARRIVE guidelines and were in accordance with the Directive 2010/63/EU.

CRediT authorship contribution statement

Dragica Gajic: Data curation, Investigation, Methodology, Formal analysis, Writing - review & editing. **Tamara Saksida:** Data curation, Investigation, Methodology, Formal analysis, Writing - review & editing. **Ivan Koprivica:** Investigation, Methodology, Writing - review & editing. **Milica Vujicic:** Investigation, Methodology, Writing - review & editing. **Sanja Despotovic:** Visualization, Writing - review & editing. **Katarina Savikin:** Investigation, Methodology, Writing - review & editing. **Teodora Jankovic:** Investigation, Methodology, Writing - review & editing. **Ivana Stojanovic:** Conceptualization, Data curation, Formal analysis, Supervision, Investigation, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

None.

Acknowledgments

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.jff.2020.103836.

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