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Chokeberry (*Aronia melanocarpa*) fruit extract abrogates melanoma progression through boosting up IFN-γ-producing cells

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ABSTRACT

Chokeberry has exhibited cardioprotective, anti-bacterial, immunomodulating and anti-cancer properties. Chokeberry extract (CE) was tested in the model of melanoma induced by B16 cells inoculation in C57BL/6 mice.

CE treatment that began 7 days before inoculation and continued through the observation period, delayed melanoma appearance and increased infiltration of immune cells in the tumor microenvironment (TME). Levels of TNF, perforin, granzyme B and IL-1β did not differ between the CE-treated and control animals, but the TME of CE-treated mice contained more IFN-γ-producing cells and a lesser frequency of CCR5-expressing MDSC. *In vitro*, CE displayed no direct cytotoxicity to B16 cells. However, splenocytes isolated from CE-treated animals exerted strong cytotoxic effect on B16 cells *in vitro*. Neutralization of IFN-γ diminished the observed B16 death, suggesting that this effect was mediated mainly by splenocyte-derived IFN-γ.

In conclusion, pre-treatment with CE stimulated the anti-tumor immune response by enhancing IFN-γ-producing cells to act against melanoma.

1. Introduction

Melanoma, a malignant neoplasm that arises from melanocytes, is a very aggressive disease with high mortality rates world-wide and a rising incidence (Karimkhani et al., 2017). Unfortunately, metastatic melanoma is often characterized by unresponsiveness to conventional therapies, radiotherapy and chemotherapy (Singh & Salama, 2016), while early-stage melanoma can be surgically removed (Prado, Svoboda, & Rigel, 2019). The immune response against melanoma is at first mediated by fast, non-specific action of innate immune cells, crucial for early stages of tumor development. Dendritic cells (DC), macrophages (Mφ), NK and NKT cells within the tumor microenvironment (TME) display direct cytotoxic action towards melanoma cells or activate adaptive immune cells to eliminate the tumor, either by producing pro-inflammatory cytokines or by antigen presentation. As a part of the anti-tumor response, effector CD4 and CD8 T cells produce interferon-γ (IFN-γ) and tumor necrosis factor (TNF). Other mediators derived from CD8 T and NK cells include perforin and granzyme B, molecules that form pores and induce apoptosis in tumor cells, respectively. In an efficient anti-tumor immune response, the presence of suppressor cells, regulatory T (Treg) cells and myeloid-derived suppressor cells (MDSC), is down-regulated, as well as the production of immunosuppressive mediators (*e.g.* interleukin-10) (Marzagalli, Ebelt, & Manuel, 2019). The usage of monoclonal antibodies to block the inhibitory signal through Cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) and Programmed cell death protein 1 (PD-1) has been shown to be an efficient anti-tumor therapy which acts by stimulating the activation and expansion of effector T (Teff) cells. These immune checkpoint inhibitors, CTLA-4 and PD-1, are expressed not only on activated Teff cells, but also on Treg cells. The expression of CTLA-4 on Treg cells is upregulated in the TME (Kwiecien, Stelmaszczyk-Emmel, Polubiec-Kownacka, Dziedzic, & Domagala-Kulawik, 2017) and correlates with poor prognosis (Paulsen et al., 2017). The checkpoint inhibitors are effective against inflamed tumors, in which many pro-inflammatory cytokines are detected (Herbst

Abbreviations: CE, chokeberry extract; CTLA-4, cytotoxic T-lymphocyte-associated protein 4; DC, dendritic cell; IFN-γ, interferon gamma; MDSC, myeloid-derived suppressor cell; MLN, mesenteric lymph node; Mφ, macrophage; PD-1, programmed cell death protein 1; PI, propidium iodide; SPC, splenocytes; Teff, effector T cell; TME, tumor microenvironment; TNF, tumor necrosis factor; Treg, regulatory T cell.

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et al., 2014); while there is resistance to immune checkpoint blockade in melanoma patients (Yan et al., 2019). In that sense, significant efforts are being made to identify new treatments that activate or enhance anti-tumor immune responses.

Chokeberry (*Aronia melanocarpa*), a fruit rich in phenolic compounds, may have numerous health benefits, including cardioprotective (Ćujić et al., 2018), anti-bacterial (Gajić et al., 2020), immunomodulating (Ali et al., 2021; Gajic et al., 2020) and anti-cancer properties (Gill et al., 2021). It was shown that chokeberry juice is cytotoxic for embryonal carcinoma stem cells (Sharif et al., 2013) and acute lymphoblastic leukemia Jurkat cells (Sharif et al., 2012). Also, polyphenols from *Aronia melanocarpa* are cytotoxic for pancreatic cell line AsPC-1 (Thani, Keshavarz, Lwaleed, Cooper, & Rooprai, 2014). Anthocyanins from chokeberry extract (CE) decreased the proliferation of melanoma murine cell line B16-F10 in a dose dependent manner (Diaconeasa et al., 2017). These experiments explored the direct contact of tumor cells with *Aronia melanocarpa* juice or extract and did not examine the effects of their oral administration, which resembles the human route of *Aronia* utilization and is thus more adequate. The CE was produced and characterized as part of our previous study (Gajic et al., 2020) and we have shown its immunomodulating properties when the CE is applied via oral gavage. Oral consumption of the CE altered the proportion of Mφ, DC and T cells in the gut and significantly stimulated the production of IFN-γ in splenocytes. To the best of our knowledge, no *Aronia melanocarpa* extract has ever been tested in models of melanoma, although it shows anti-proliferative potential towards a melanoma cell line (Diaconeasa et al., 2017). In this study, we used murine B16 melanoma cells to induce tumors in C57BL/6 mice and investigated the effects of oral consumption of CE on the tumor development.

2. Material and methods

2.1. Plant material and reagents

The CE was produced and characterized as part of our previous study (Gajic et al., 2020). In short, chokeberry collected from a certificated organic cultivation field was immediately dried at 40℃ for 48 h and ground in a laboratory mill. The obtained material was extracted with 50 % ethanol and after vacuum evaporation, the water residue was freeze-dried. The CE had no presence of endotoxin or viable bacteria. The characterization of CE was performed by HPLC analysis (Gajic et al., 2020). Briefly, the main anthocyanin compound is cyanidin-3-galactoside (0.34 mg/g), the main phenolic acid is chlorogenic acid (3.53 mg/g) and the total amount of proanthocyanidins is 36.5 mg CE/g.

Unless stated otherwise, all the chemicals were from Sigma Aldrich (St. Louis, MO, USA).

2.2. Animals and experimental design

The murine melanoma B16 cell line was obtained from Dr. Danijela Maksimović-Ivanić, Institute for Biological Research "Siniša Stanković" – National Institute of Republic of Serbia, University of Belgrade (IBISS), and grown in RPMI 1640 supplemented with 10 % fetal calf serum (FCS), 1 % penicillin and streptomycin (all from PAA Laboratories, Pasching, Austria), 2 mM L-glutamine and 25 mM HEPES (culture medium) at 37C in a humidified atmosphere with 5 % CO_2. The density of tumor cells for seeding was 4×10^3 cells/well in 96-well plates and 2×10^4 cells/well in 24-well plates.

Female 8–12 weeks old C57BL/6 mice were used for all experiments in accordance with the 3R principles. Animals were bred and maintained at the Animal Facility of IBISS with unlimited access to tap water and standard chow. The study was conducted according to the guidelines of the Directive 2010/63/EU on the protection of animals used for scientific purposes, and it was approved by the Veterinary Administration, Ministry of Agriculture, Forestry and Water Management, Republic of Serbia (N° 323–07-02501/2020–05).

C57BL/6 mice were treated with the CE, at the dose of 50 mg/kg body weight or with vehicle (potable water) by oral gavage, daily, starting 7 days before the melanoma cells inoculation. The treatment was continued until the end of experiment. Tumors were induced by subcutaneous administration of 2.5×10^5 B16 cells in the dorsal right lumbosacral region of mice. Tumor dimensions were measured daily and the volume was calculated with the following formula: length × width^2 × 0.52. *Ex vivo* analyses were performed on the 13th day from the B16 cells inoculation, as described previously (Paul, Chhatar, Mishra, & Lal, 2019).

2.3. Isolation of cells from tissues and cell cultures

Spleens and mesenteric lymph nodes (MLN) were aseptically removed, passed through a cell strainer (pores of 70 μm), centrifuged at 550g for 5 min and resuspended in the culture medium. For obtaining splenocytes (SPC), erythrocytes were lysed with RBC Lysis Buffer (eBioscience, San Diego, CA, USA) and then resuspended in the culture medium for further analyses. For the SPC culture, 5×10^6 cells were seeded in 24-well plates for 24 h. The supernatants were collected after centrifugation and used for the determination of cytokines with ELISAs and for the IFN-γ neutralization experiment.

For obtaining of tumor-infiltrating immune cells, the tumors were harvested and passed through a cell strainer (pores of 70 μm). After centrifugation (550g, 5 min), pellets were resuspended in the culture medium and overlaid on density gradient media (HistoPaque-1083). The samples were centrifuged at 550g for 20 min at RT. The cells between the layers were collected, washed twice and resuspended in the culture media.

2.4. Viability assay

After treating B16 cells with different CE concentrations, the supernatants were discarded and cells were exposed to 0.5 mg/ml 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) solution for 30 min at 37C in a humidified incubator. Cells were then resuspended in dimethyl sulfoxide and color intensity was measured on a microplate reader (LKB Instruments, Vienna, Austria) at 540 and 670 nm (for impurity corrections).

2.5. Detection of in vitro apoptosis by flow cytometry

2×10^4 B16 cells/well were incubated in a 24-well plate in the presence or absence of CE (200 μg/ml and 400 μg/ml) for 24 h. Cells were stained with Annexin V-FITC (Ann, BD Pharmingen, San Diego, CA, USA) and propidium iodide (PI) for detection of apoptosis with flow cytometry according to the manufacturer's instructions.

2.6. Cytotoxicity (LDH) assay

For the cytotoxicity assay, 4×10^3 B16 cells were seeded in a 96-well plate. Splenocytes obtained from tumor-bearing animals treated with vehicle or CE were mixed in 20:1 or 10:1 ratios with B16 cells, according to Isvoranu et al. (Isvoranu et al., 2019). Supernatants, containing lactate dehydrogenase (LDH) released upon cell lysis, were collected. LDH assay was performed according to the manufacturer's instructions, using Triton X-100-treated B16 cells as a positive control and non-treated B16 cells as a negative control. Absorbance was measured at 492 and 592 nm and the ratio of necrotic cells was calculated using the following equation: ((SPC + B16 cells absorbance - non-treated B16 cells absorbance) / (Triton X-100-treated B16 cells absorbance - non-treated B16 cells absorbance)) × 100. The obtained proportion of necrotic cells is shown as cytotoxicity of effector cells. Neutralization of IFN-γ was performed with neutralizing anti-IFN-γ antibody (Thermo Fisher Scientific, Waltham, MA, USA) during 48 h of co-cultivation of B16 and SPC supernatants from CE-treated animals. Goat anti-mouse IgG served as

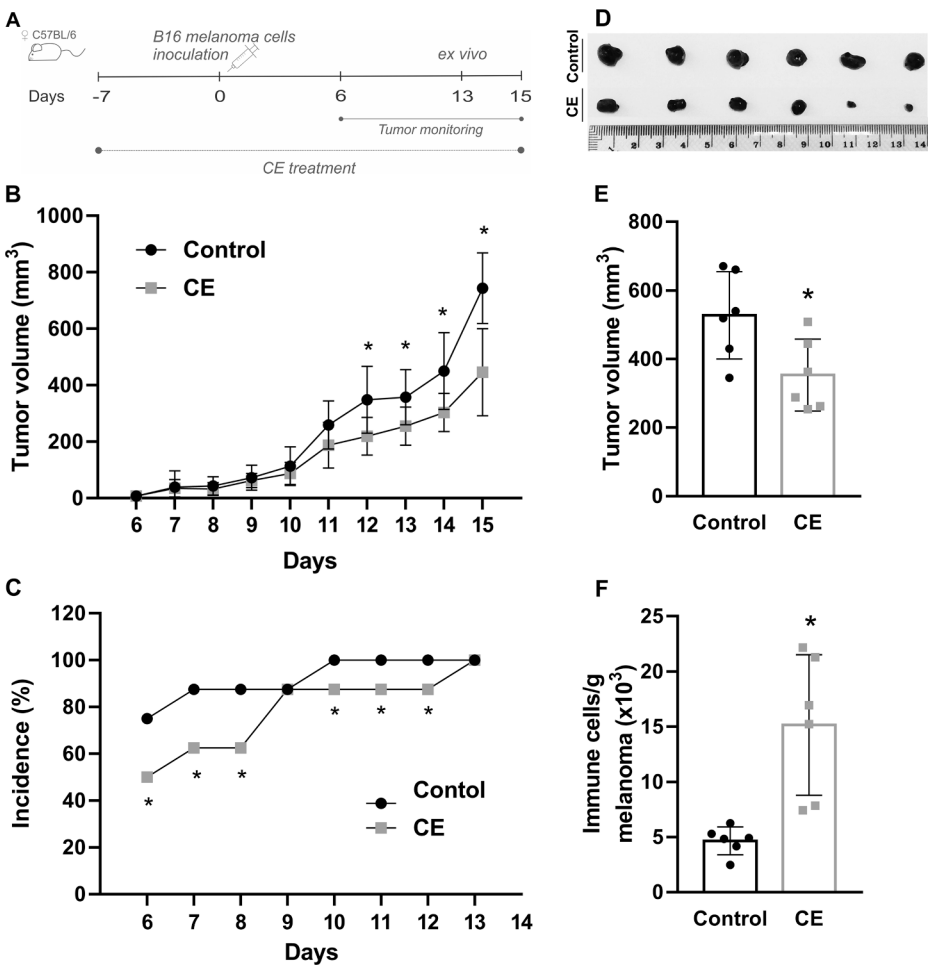

Fig. 1. Tumor progression is slowed down by oral CE treatment. C57BL/6 mice were treated with CE (50 mg/kg body weight) or vehicle (potable water, control) by oral gavage starting 7 days before the melanoma cells inoculation. 2.5×10^5 B16 cells were injected subcutaneously in syngeneic C57BL/6 mice (A) and the treatment was continued until the end of the experiment. Tumor volume calculated according to the formula: length $\times$ width^2 $\times$ 0.52 (B), the incidence of melanoma (C), representative image of excised tumors (D), volume of excised tumors (E) and the number of tumor-infiltrating immune cells per g of tumor after tumor excision and processing to cellular suspension (F) on the day of *ex vivo* analyses (13th day). The results are shown as mean $\pm$ SD, n = 6. * $p < 0.05$ represents significant difference versus the control group.

the isotype control. The viability of B16 cells was assessed with LDH assay, as described above.

2.7. Detection of immune cells by flow cytometry

For the detection of specific intracellular or extracellular markers 1×10^6 or 0.5×10^6 were stained. The following anti-mouse antibodies and isotype controls were used: CD4-FITC (rat IgG2b, κ), CD8-PE (rat IgG2a, κ), CD8-APC (rat IgG2a, κ), CD3e-PE (Armenian hamster IgG), CD3e-PE-Cy5.5 (Armenian hamster IgG), CD25-PE-Cy5.5 (rat IgG1, λ), NK1.1-FITC (mouse IgG2a, κ), NK1.1-PE-Cy7 (mouse IgG2a, κ), CD11b-FITC (rat IgG2b, κ), CD11c-PE (Armenian hamster IgG), CD40-PE-Cy5 (Armenian hamster IgM, κ), CD206-PE (rat IgG2b,κ), F4/80-FITC (rat IgG2a, κ), MHCII-FITC (rat IgG2b, κ), CD86-PE-Cy5 (rat IgG2a, κ), Ly-6G/Ly-6C- PE-Cy5.5 (rat IgG2b, κ), KLRG1-PE-eFluor® 610 (Syrian hamster IgG), PD-1-FITC (Armenian hamster IgG), CTLA-4-PE (Armenian hamster IgG), CD27-PE (Armenian hamster IgG), CCR5-PE (Armenian hamster IgG), CCR9-FITC (mouse IgG2a, κ) (all from Thermo Fisher Scientific) and CCR5-AlexaFluor® 488 (Armenian Hamster IgG; BioLegend, San Diego, CA, USA), perforin-PE (rat IgG2a, κ), granzyme B-FITC (rat IgG2a, κ). Antibodies were dissolved in the flow cytometry staining buffer (FCS buffer, eBioscience) and staining was performed for 45 min at 4℃. After washing, cells were resuspended in the FCS buffer and analyzed.

For detection of intracellular cytokines, cells were incubated with the cell stimulation cocktail (1:500, eBioscience) for 4 h at 37℃ before staining. Staining for extracellular markers was performed as described, after which the cells were fixed in 2 % paraformaldehyde for 15 min and left overnight at 4℃. After permeabilization with permeabilization buffer (PB, eBioscience), antibodies were dissolved in PB and the staining for intracellular proteins was performed for 45 min at 4℃. The following anti-mouse antibodies were used: IFN-γ-PE (rat IgG1, κ), IFN-γ-PE-Cy5 (rat IgG1, κ) and TNF-PE (rat IgG1, κ) (all from Thermo Fisher Scientific). After washing, cells were resuspended in the FCS buffer and analyzed.

For detection of Treg cells, the cells were stained for extracellular markers, permeabilized using the protocol and reagents from the Mouse Regulatory T cell Staining Kit (eBioscience) and then stained with anti-mouse FoxP3-APC (rat IgG2a, κ; eBioscience).

For detection of both extracellular and intracellular markers, cells were analyzed on PartecCyFlow Space (Partec, Görlitz, Germany) by FlowMax software or on BD FACSAria™ III Sorter (BD Life Sciences, San Jose, CA, USA) and analyzed on FlowJo software (FlowJo LLC, Ashland, OR, USA).

2.8. ELISA for detection of cytokines

Cytokine concentration in the 24-hour SPC culture supernatants and sera was determined 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 IFN-γ, TNF, IL-1β, and IL-17 (eBioscience) and absorbance was measured by LKB microplate reader (LKB Instruments, Vienna, Austria) at 450 and 670 nm (for impurity corrections). A standard curve created from the known concentrations of appropriate recombinant cytokines was used to calculate concentration values of measured cytokines.

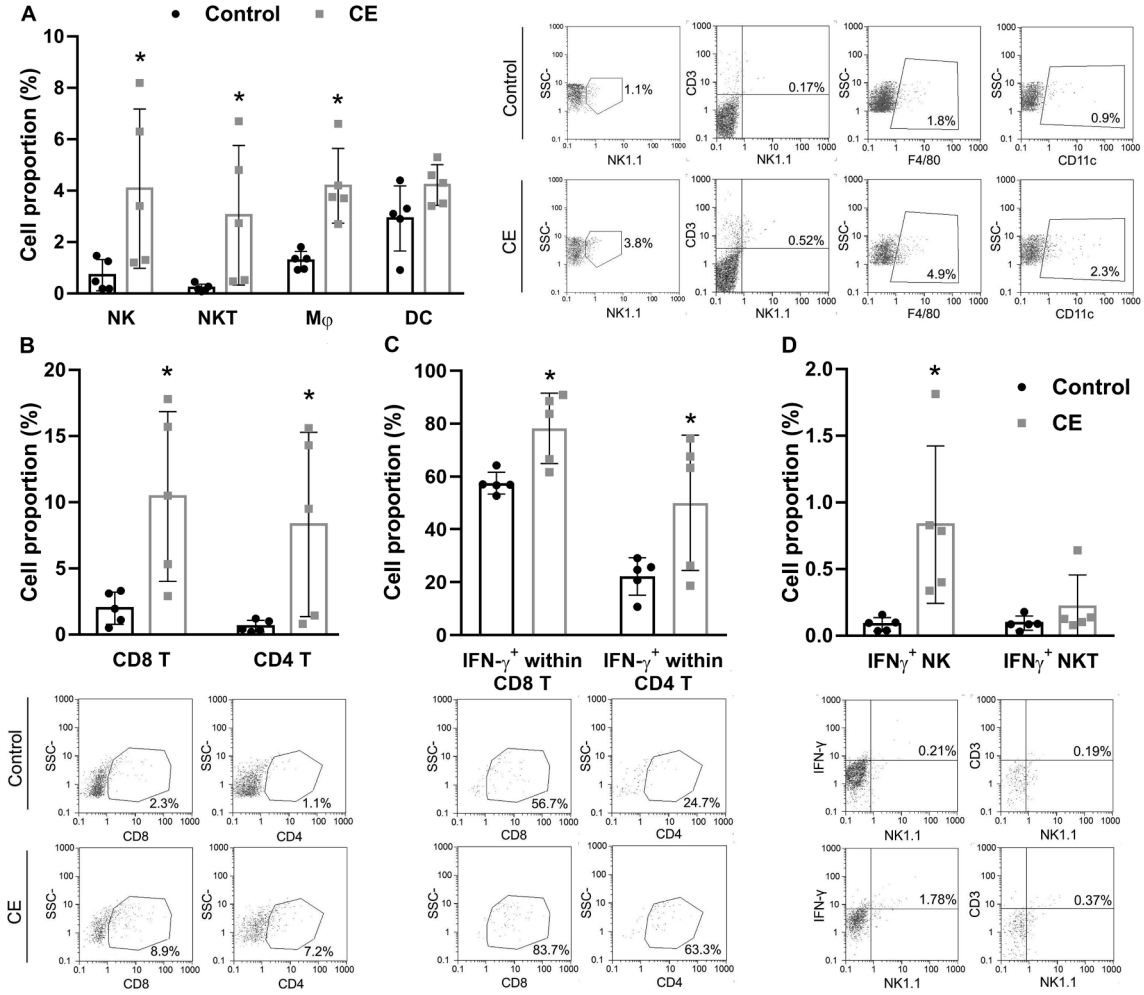

Fig. 2. ***In vivo*** **CE anti-tumor activity is mediated by the modulation of immune cells within the melanoma environment**. The tumor-infiltrating immune cells were analyzed by flow cytometry on the 13th day after the B16 inoculation in the CE-treated or control group. The percentages of NK cells ($NK1.1^+$), NKT cells ($CD3^+NK1.1^+$), Mφ ($F4/80^+$), DC ($CD11c^+$) (A) and representative dot plots shown on the right hand side; CD8 and CD4 T lymphocytes (B) and representative dot plots shown below; IFN-γ within the CD8 and CD4 T lymphocytes (C) and IFN-γ-expressing NK and IFN-γ-expressing NKT cells (D) and representative dot plots shown below. Cells shown in the dot plots are gated on the lymphocytic gate (based on FSC-A vs SSC-A scatter) followed by singlet populations (FSC-A vs FSC-W scatter). Numbers in the dot plots indicate the percentage of cells. The results are shown as mean ± SD for a representative experiment, n = 5. * $p < 0.05$ versus the control group.

2.9. Statistical analysis

Data are presented as mean ± SD. The presented results are a representative of four repeated experiments. For the *in vivo* experiments, statistical significance was estimated by the Mann-Whitney test. Unless stated otherwise, two tailed Student's *t*-test was used for determining the statistical significance. A p value<0.05 was considered significant. Statistical analyses were performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA).

3. Results

3.1. CE efficiently suppresses melanoma growth in vivo

The ability of CE to interfere with melanoma growth was tested by daily oral CE administration that started 7 days before the inoculation with B16 cells and continued until the end of the experiment. As in our previous experiments (Gajic et al., 2020), the mice received the CE dose of 50 mg/kg body weight or vehicle (potable water, control group) by oral gavage (Fig. 1A). Daily monitoring showed that animals receiving CE had significantly reduced tumor growth (Fig. 1B, D) and the occurrence of melanomas in CE-treated group was delayed compared to the vehicle-treated mice (Fig. 1C). *Ex vivo* analysis was performed on the 13th day after B16 cells inoculation. The volumes of excised tumors were lower in animals receiving CE (Fig. 1E) and this was accompanied with the increased infiltration of immune cells into the melanoma (Fig. 1F).

3.2. Attenuated tumor progression in CE-treated mice is associated with increased proportion of IFN-γ producing immune cells and decreased MDSC within the tumor microenvironment

Elimination of melanoma cells is achieved through anti-tumor activity of DC, Mφ, NK and NKT cells within the TME. They act through direct cytotoxicity, by activating the adaptive immune response via antigen presentation, and by secreting pro-inflammatory cytokines (TNF, IFN-γ). In order to delineate immune populations responsible for decreased tumor growth in the CE-treated group, a phenotypic analysis of tumor-infiltrating immune cells was performed. Animals which received CE had significantly higher proportions of NK, NKT cells and Mφ, while the proportion of DC within the tumor was not affected (Fig. 2A). NK cells have activating and inhibitory receptors on their

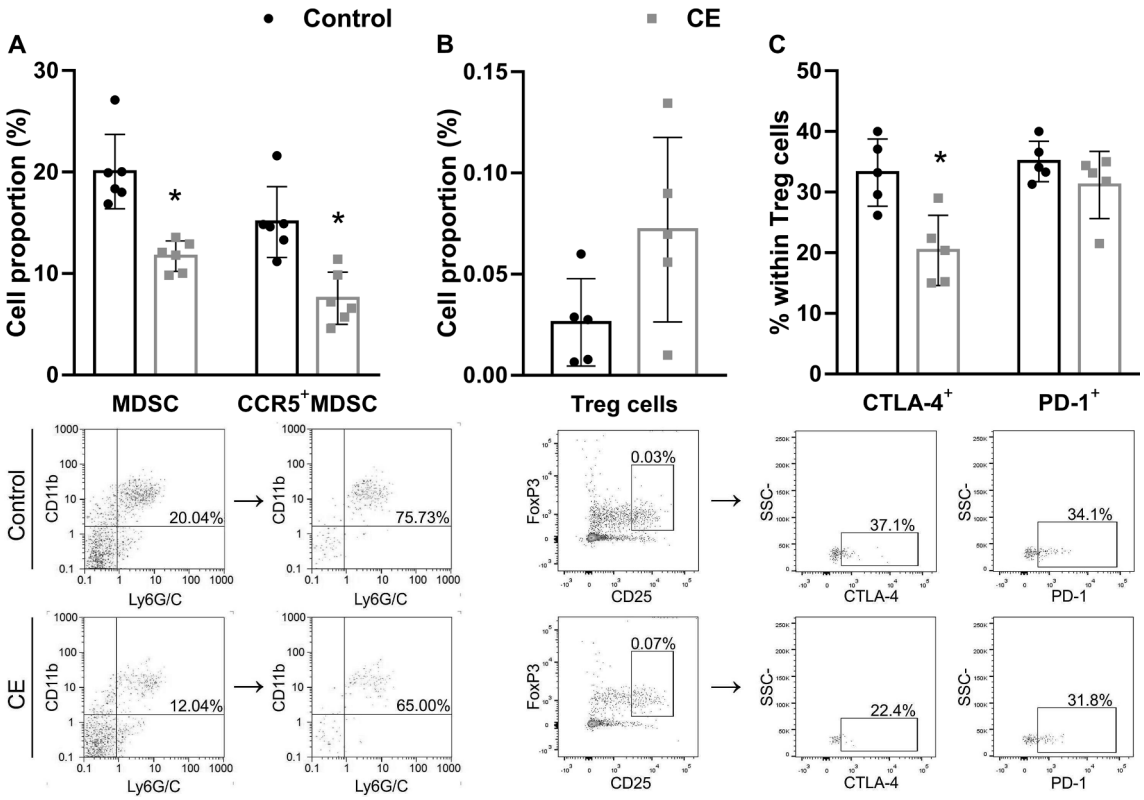

Fig. 3. CE treatment downregulates the frequency of MDSC within the tumor while not affecting the Treg frequency. The tumor-infiltrating immune cells were analyzed by flow cytometry on the 13th day after the B16 inoculation in the CE-treated or control group. MDSC ($CD11b^+Ly6G/C^+$) and CCR5-expressing MDSC are presented as the percentage of leukocytes (A). Treg cells ($CD25^{high}FoxP3^+$) are presented as percentages within the $CD3^+$ cells (B). CTLA-4- and PD-1-expressing cells are presented as percentages within the Treg cells. Numbers in the dot plots indicate the percentage of cells and representative dot plots shown below the graphs. The results are shown as mean ± SD for a representative experiment, n = 5. * $p < 0.05$ versus the control group.

surface, which determine their actions. CE treatment did not change the percentage of KLRG1 inhibitory receptor or the percentage of CD27 activating receptor, nor it affected CD11b adhesion receptor on the surface of NK cells (Suppl. Fig. 1A). Also, immune cells that produce TNF within the innate immune cells/non-lymphocyte cells were unaltered ($CD3^-TNF^+$ cells, Suppl. Fig. 1B). CE treatment equally increased both M1 ($F4/80^+CD40^+$) and M2 macrophages ($F4/80^+CD206^+$) in the melanoma, as their ratio was similar in observed groups (Suppl. Fig. 1C). Also, the percentage of antigen-presenting DC ($CD86^+MHCII^+CD11c^+$; Suppl. Fig. 1D) was unaltered by the CE treatment.

Effective eradication of melanoma cells is dependent on the action of T cells, both $CD4^+$ and $CD8^+$, which act via IFN-γ, TNF, perforin and granzyme B. CE treatment increased the frequency of CD4 and CD8 T cells within the tumors (Fig. 2B). Also, the proportion of cells that produce IFN-γ within the CD4 and CD8 T cell populations (Fig. 2C) and within the NK cells (Fig. 2D) was increased in the CE-treated animals. The frequency of TNF-expressing T lymphocytes ($CD3^+TNF^+$ cells, Suppl. Fig. 1B) or the frequency of granzyme B or perforin producing NK or CD8 T cells (Suppl. Fig. 1E, F) was not altered compared to the control group.

The immunosuppressive environment in the melanoma tissue is enabled by the presence and action of tumor suppressive immune cells, MDSC and Treg cells. CE treatment abrogated the infiltration of MDSC into the melanoma, as well as MDSC that express CCR5 on their surface, which display stronger immunosuppressive capacity (Fig. 3A). Despite the fact that CE treatment had no influence on the frequency of Treg cells (Fig. 3B), the proportion of Treg cells expressing CTLA-4 was decreased compared to the control group, while the proportion of Treg cells that express PD-1 on their surface remained the same (Fig. 3C).

3.3. The effects of CE on cell populations at the intestine's draining site

As animals received CE or vehicle via oral gavage, we investigated the immune cell populations within the lymph nodes that drain the intestine, the MLN. The frequency of NK, NKT cells, Mφ, CD4 T, CD8 T, and Treg cells were unaffected by the CE treatment (Suppl. Fig. 2A-B). However, CE treatment down-regulated the specific CCR9-expressing CD8 T cell population (Fig. 4A). The CCR9-expressing CD8 T cells are gut-homing T cells which, after priming in the MLN, migrate to the small intestine following the CCR9 ligand gradient. Importantly, cells that express IFN-γ were up-regulated in the MLN of CE-treated animals, compared to vehicle treated mice (Fig. 4B).

3.4. B16 cytotoxicity is mediated by IFN-γ action from the splenocytes of CE-treated animals

In order to test whether CE has direct cytotoxic activity towards B16 cells, B16 cells were exposed to the CE in the dose range 50–400 μg/ml and cell viability was measured with MTT assay. In all applied concentrations, CE did not show cytotoxicity towards B16 cells (Fig. 5A). Simultaneously, staining with Annexin V and PI revealed that in the highest applied doses of 200 and 400 μg/ml, there were no apoptotic or necrotic cells after the CE treatment (Fig. 5B-C).

In order to test if CE exerts its anti-tumor effects by affecting the cells of the immune system, tumor target cells were co-cultured with SPC obtained from the control or CE-treated animals. Ratios of 20:1 and 10:1 of effector (SPC) cells and target (B16) cells were used. The measured absorbance of LDH released into the culture medium from dead cells represents the cytotoxicity of SPC. The SPC obtained from CE-treated animals displayed higher cytotoxicity against target B16 cells in both 10:1 and 20:1 ratios, compared to those from the control group

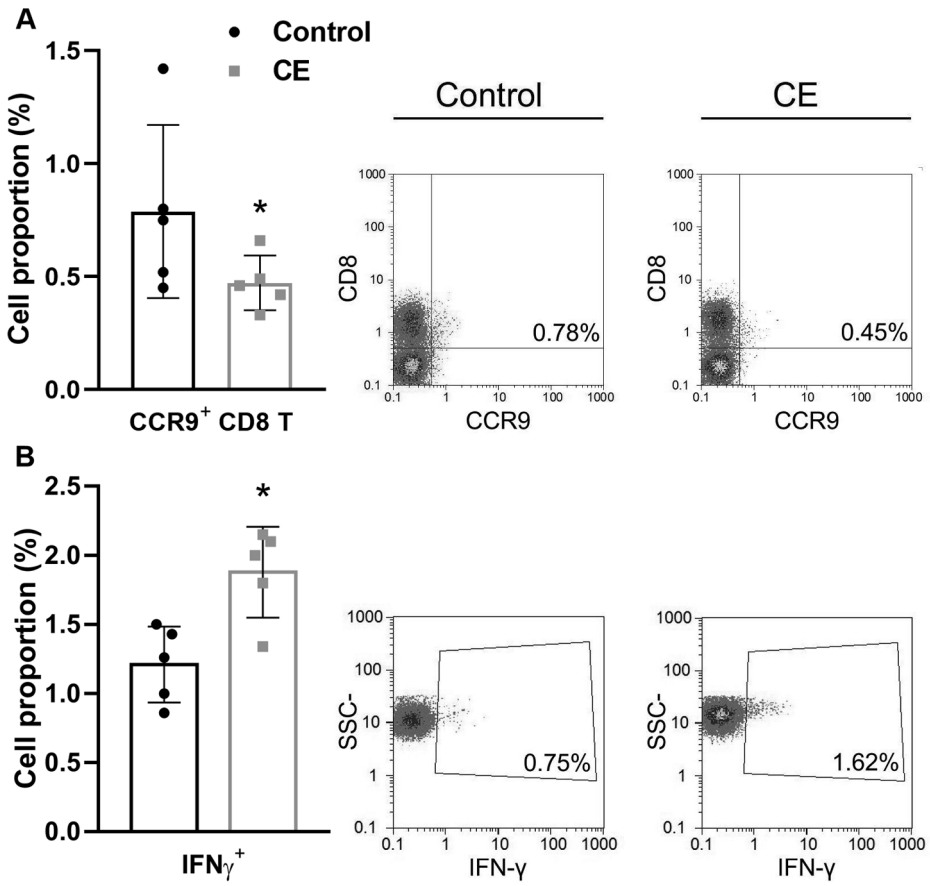

Fig. 4. The effects of CE on cell populations in the MLN. CCR9-expressing CD8 T cells (A) and IFN-γ-expressing cells (B) were analyzed by flow cytometry on the 13th day after the B16 inoculation in the MLN of CE-treated or control group. Numbers in the dot plots indicate the percentage of cells and representative dot plots shown on the right hand side. The results are shown as mean ± SD for a representative experiment, n = 5. * $p < 0.05$ versus the control group.

(Fig. 5D). In order to determine the molecules responsible for CE-mediated anti-tumor effects, we measured the concentration of cytokines in the SPC supernatants and sera. In the SPC cultures and sera of CE-treated animals, there were significantly higher levels of IFN-γ, while levels of IL-1β, IL-17 and TNF were not different from those in control animals (Fig. 6A, B). Furthermore, neutralization of IFN-γ during co-cultivation of B16 cells and SPC supernatants from CE-treated animals abrogated the cytotoxicity (Fig. 6C), indicating that IFN-γ is mainly responsible for the observed supernatant-provoked B16 cell death. Taken together, these results show that CE does not exert direct cytotoxicity towards B16 cells, but acts by enhancing the immune cells' cytotoxic effects which are mediated by IFN-γ.

4. Discussion

We have shown that CE-treated mice exhibit delayed tumor appearance, as well as abrogated tumor progression. In the TME, CE promoted the infiltration of immune cells, in particular NK, NKT, CD4 T and CD8 T cells, and most importantly, CE increased the percentages of IFN-γ-producing cells within these anti-tumor cell populations. Also, CE blocked the infiltration of tumor-supporting populations, like MDSC, while a direct *in vitro* cytotoxic effect on tumor cells was absent.

In the recent years, there are great efforts to develop various forms of immune-based therapies with the idea to mobilize the immune system to promote or restore an effective anti-tumor immune response. In addition, conventional therapy such as chemotherapy destroys rapidly dividing cancer cells, but also damages other cells, while immunotherapy has the power to target cancer cells selectively (Karimkhani et al., 2017; Prado et al., 2019; Singh & Salama, 2016).

In that sense, the checkpoint inhibitors are seen as a promising strategy (Carlino, Larkin, & Long, 2021). As such, monoclonal antibodies targeting CTLA-4 proved effective in clinical trials of melanoma (Grosso & Jure-Kunkel, 2013). Ipilimumab, a human IgG1κ anti-CTLA-4 monoclonal antibody, elicited potent tumor necrosis (Hodi et al., 2003), conferred a short-term survival benefit (Hodi et al., 2010) or even a long-term survival benefit in some patients (Schadendorf et al., 2015) and persistence of antitumoral immunity (Maio et al., 2015). PD-1 inhibition augmented antitumoral immunity and limited haematogenous seeding of B16 melanoma in mouse models (Iwai, Terawaki, & Honjo, 2005). Pembrolizumab and nivolumab, human IgG4 anti-PD-1 monoclonal antibodies, are the first FDA-approved PD-1-targeted therapeutics for refractory and unresectable melanoma (Gong, Chehrazi-Raffle, Reddi, & Salgia, 2018; Weber et al., 2017).

Although they are potent in their antitumor actions, the usage of checkpoint inhibitors has some adverse effects, because of inadequate control of immune effector mechanisms. There is the decline in naive T cells on one side but also the accumulation of overactive memory T cells that seed peripheral organs, such as the gastrointestinal tract and lungs, and cause inflammatory damage (Fritz & Lenardo, 2019). The anti-CTLA-4 therapy is more affecting the gastrointestinal tract and brain, and the usage of PD-1 axis-targeted therapies possess a higher risk of hepatoxicity, hypothyroidism and pneumonitis (Kumar et al., 2017).

Various natural, including plant-or fungi-derived compounds have shown promising results in the mouse models of melanoma (Ben Abdessamad, Bouhlel, Chekir-Ghedira, & Krifa, 2020; Tanaka et al., 2011; Xian et al., 2021). The mechanism of action has been attributed to direct anti-proliferative effects against melanoma cells (Li et al., 2021; Lj et al., 2008), but also to different effects on the immune system. Extract

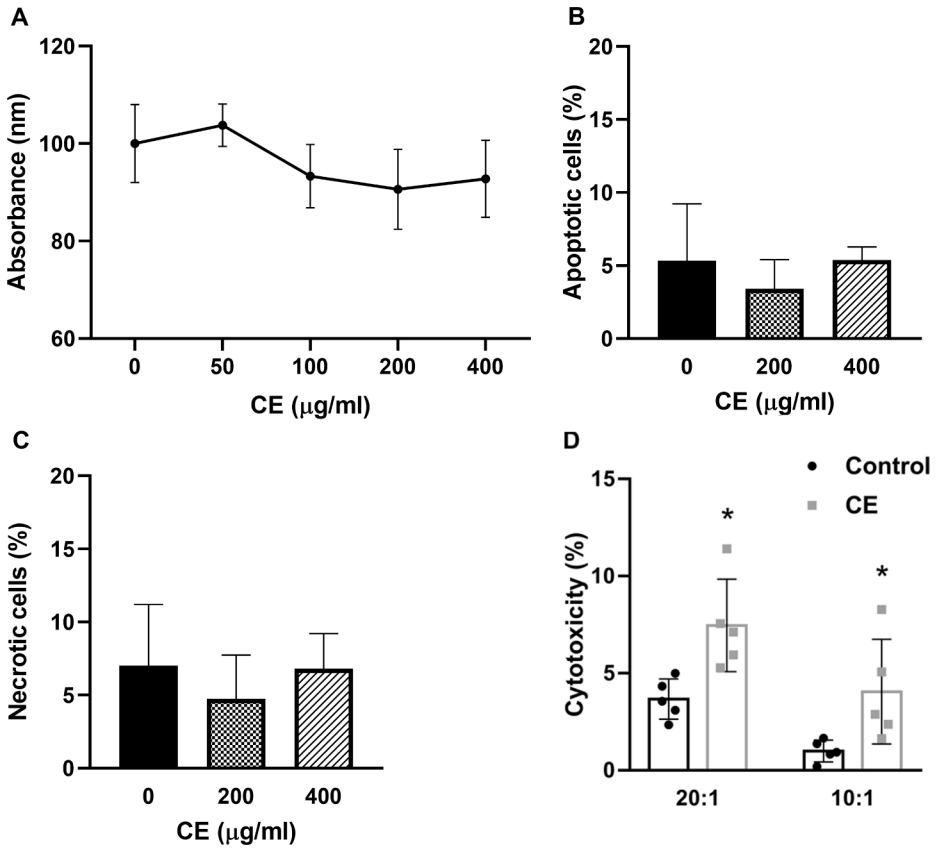

Fig. 5. CE acts by enhancing cytotoxicity of immune cells. Cellular viability of B16 cells determined by MTT (A), or flow cytometry results with Annexin V-FITC/PI staining presented as persentages of apoptotic cells (B) or necrotic cells (C) upon treatment in indicated CE concentrations after 24 h of cultivation. Cytotoxicity assay against B16 target cells (D) at effector:target cell ratios of 20:1 and 10:1. SPC from control or CE-treated animals were co-cultivated with B16 cells for 48 h. LDH determined in the cell supernatant was a measure of the cytotoxic action of SPC, using Triton X-100-treated B16 cells as a positive control and non-treated B16 cells as a negative control. The results are shown as mean ± SD from a representative experiment, n = 3. * $p < 0.05$ versus the control group.

of *Lentinula edodes mycelia* inhibited melanoma growth by restoration of melanoma reactive T cells, presumably by a mitigation of regulatory T cells-mediated immunosuppression (Tanaka et al., 2011). Anti-melanoma effects of *Daphne gnidium* aqueous extract were attributed to enhanced proliferation of splenocytes and enhanced cytotoxic activities of NK and cytotoxic lymphocytes (Chaabane et al., 2016). Chokeberry has also showed potential in cytotoxic effects against tumor cells. In particular, it was reported that CE can reduce the proliferation of HT-29 human colon cancer cells (Gill et al., 2021) and also, anthocyanins from CE decreased the proliferation of melanoma murine cell line B16-F10 in a dose dependent manner (Diaconeasa et al., 2017). This was not the case with the CE investigated in our study, as it did not affect the viability of B16 cells *in vitro*, judging by the comparable proportions of apoptotic and necrotic cells between CE-treated and untreated B16 cells. These results suggest that CE does not manifest a direct cytotoxic effect against melanoma cells and, having in mind the obtained *ex vivo* results, we could conclude that the anti-tumor effect of CE is achieved mainly by affecting the immune cells. Additionally, the CE was efficient in protecting against lung tumor development (Balansky et al., 2012). In detail, in a cigarette smoke-induced lung cancer model in Swiss ICR mice, the CE inhibited cigarette smoke-related body weight loss, cytogenetical damage, and lung adenomas. These results, along with our study, point to strong protective potential of chokeberry in different tumor models.

We have previously shown that CE possesses immunomodulatory capacity, both *in vitro* and *in vivo. In vitro,* CE-treated innate and adaptive immune cells acquired pro-inflammatory properties. CE given orally to healthy mice altered the proportions of Mφ, DC and T lymphocytes in the gut-associated lymphoid tissue (Gajic et al., 2020), while in an infection model with *Listeria monocytogenes,* CE-pretreatment accelerated the eradication of the bacteria by increasing the proportion of Mφ and $CD8^+$ T lymphocytes and promoting Mφ activity (Gajić et al., 2020). In this study, the CE-preconditioning of mice delayed tumor appearance and progression and enhanced the infiltration of immune cells into the TME, implying that the anti-tumor immune response is mainly concentrated in the TME.

Efficient anti-tumor immune response is mediated by cooperation of innate and adaptive immune cells (Marzagalli et al., 2019). Our results showed that CE treatment increased the proportions of NK and NKT cells, Mφ as well as CD4 and CD8 T lymphocytes in the TME. More importantly, CE stimulated the function of these cells as the proportions of IFN-γ-producing NK cells, CD4 and CD8 T lymphocytes were also increased after CE treatment, compared to the control group. These results suggest that CE promotes an anti-tumor immune response within the TME by increasing the infiltration of immune cells to fight against tumor cells, mostly by production of IFN-γ from both innate and adaptive immune cells. The contribution of other mediators of immune system, like perforin, granzyme B or TNF seems negligible in their contribution to CE's protective effects, as the levels of these molecules were comparable among groups. The direct effect of plant extracts and their components on the anti-tumor immune response has not been thoroughly investigated. There are only a few studies that address this issue. For example, an anti-tumor response is described in the study by Chaabane et al (Chaabane et al., 2016); where *Daphne gnidium* aqueous extract stimulated the cytotoxic activity of NK cells and CD8 T lymphocytes. Also, aqueous gall extract from *Limoniastrum guyonianum* exerted a strong anti-tumor response based on increased proliferation of splenocytes and enhanced activities in NK cells and CD8 T lymphocytes (Krifa et al., 2014). One study identified grape seed proanthocyanidins as major drivers of immunostimulation during the anti-tumor response which was mediated by the enhanced production of TNF by immune cells (Tong, Song, Sun, Sun, & Du, 2011). Although the CE possesses significant amounts of proanthocyanidins (Gajic et al., 2020), the change in TNF-expressing cells within the TME after CE treatment was not observed. Other constituents from CE could, in concert with proanthocyanidins, contribute to the observed immunomodulatory and

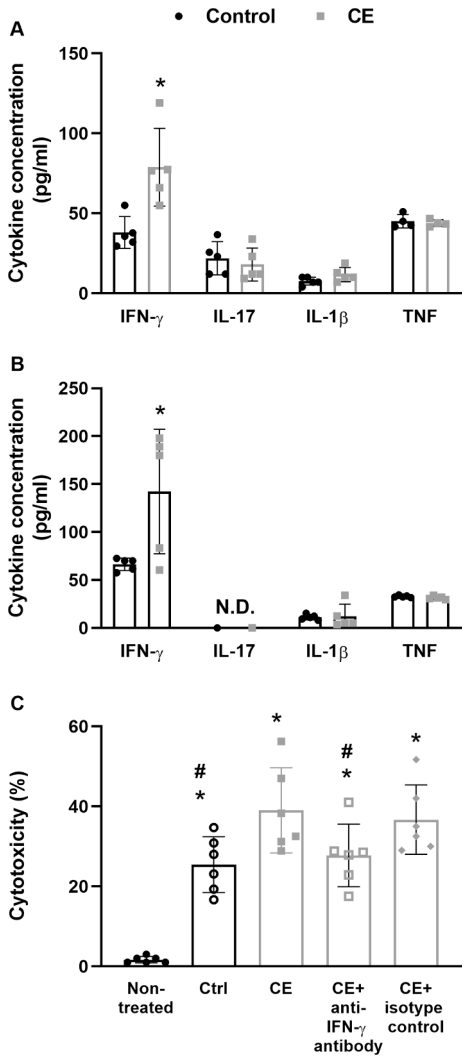

Fig. 6. Pro-inflammatory mediators in the spleen and sera. SPC supernatants after 24-hour cultures (A) or sera (B) from CE-treated animals or controls were analyzed for IFN-γ, IL-17, IL-1β and TNF with ELISA. The results are shown as mean ± SD for a representative experiment, n = 5. * $p < 0.05$ versus the control group. (C) B16 (target) cells were cultured in the presence of SPC supernatants obtained from control or CE-treated animals for 48 h and cytotoxicity was determined by LDH assay. Neutralization of IFN-γ in SPC cultures from CE-treated animals was accomplished with anti-IFN-γ antibody. Matching Isotype antibody served as a control. The results are shown as mean ± SD for a representative experiment, n = 5. * $p < 0.05$ versus the B16 (target) cells, # $p < 0.05$ versus SPC cultures from CE-treated animals. N.D. not detectable.

anti-tumor effects. In addition to the described components of CE, CE also contains high levels of polyphenols that generally exhibit anti-inflammatory activity (Yahfoufi, Alsadi, Jambi, & Matar, 2018). However, chlorogenic acid, the most abundant phenolic component in CE, can be accounted for the observed effects of CE as chlorogenic acid-containing liposomes have shown strong anti-tumor activity by stimulating CD4 and CD8 T cell infiltration and by inhibiting the expression of MDSC (Zhang et al., 2021). Similarly, our study showed that CE downregulated the infiltration of MDSC into the TME, and most importantly, we found decreased percentages of MDSC that express CCR5 in the TME of CE-treated animals. This finding is very important as these cells are marked as potent immunosuppressive cells that accumulate in the melanoma lesions and are associated with tumor progression (Blattner et al., 2018). The pro-inflammatory impact of CE described in our study could also result from the action of polysaccharides, as they are usually linked with the stimulation of the immune response. For example, polysaccharides isolated either from *Panax ginseng* or *Astragalus* have been shown to induce strong stimulation of NO synthesis in Mφ (Friedl, Moeslinger, Kopp, & Spieckermann, 2001; Zhou et al., 2017).

Orally administered drugs may affect gut microbiota composition, immune system in the gut and their interrelation (Buchta Rosean & Rutkowski, 2017). As there is an established causal relationship between the change in gut microbiota and cancer development (Derosa et al., 2021); we have evaluated the effect of CE on the gut immune system. Results imply that CE exerts immunostimulatory properties during pre-cancer development in the gut. Similar findings were obtained with a plant-derived traditional Chinese prescription which successfully attenuated the Treg response within the intestine and MLN during colorectal cancer progression in mice (Sui et al., 2020). We have previously shown that CE treatment in healthy animals alters the gut microenvironment by changing the abundance of Mφ, DC and T lymphocytes (Gajic et al., 2020). In accordance with these results, the pre-treatment with CE in melanoma-bearing animals enhanced the percentages of cells that produce IFN-γ in the gut's draining lymph nodes and downregulated the frequency of CCR9-expressing CD8 T cells. As CCR9 is a gut homing receptor (Hammerschmidt et al., 2008); the absence of cells with this marker may point to their migration to the TME, where indeed the frequency of cytotoxic CD8 T cells was enhanced.

In conclusion, the CE displayed significant anti-tumor effects in the melanoma model by enhancing the frequency of tumor-combating cells that produce IFN-γ in the TME and inhibiting the infiltration of tumor-supporting MDSC at the same time. This functional activation of the immune system by CE could be a potential strategy for melanoma treatment.

Ethics statement

All the procedures involving animals were approved by the Serbian Ministry of Agriculture, Forestry and Water Management, Veterinary Directorate (app. N° 323–07-01819/2020–05).

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CRediT authorship contribution statement

Dragica Gajić: Formal analysis, Investigation, Writing – original draft. **Ivana Stojanović:** Investigation, Writing – review & editing, Funding acquisition. **Ivan Koprivica:** Investigation, Writing – review & editing. **Nada Pejnović:** Writing – review & editing. **Katarina Šavikin:** Resources. **Nada Ćujić-Nikolić:** Resources. **Tamara Saksida:** Conceptualization, Formal analysis, Investigation, Writing – review & editing, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jff.2022.105185.

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