View Article Online
View Journal

Food & Function

Linking the chemistry and physics of food with health and nutrition

Accepted Manuscript

This article can be cited before page numbers have been issued, to do this please use: D. Gajic, T. Saksida, I. Koprivica, L. Senerovic, I. Mori, K. Katarina Savikin, N. Menkovic, N. Pejnovic and I. Stojanovi, *Food Funct.*, 2020, DOI: 10.1039/D0FO00946F.

This is an Accepted Manuscript, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this Accepted Manuscript with the edited and formatted Advance Article as soon as it is available.

You can find more information about Accepted Manuscripts in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this Accepted Manuscript or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

rsc.li/food-function

View Article Online
DOI: 10.1039/D0FO00946F

Immunomodulatory activity and protective effects of chokeberry fruit extract on *Listeria monocytogenes* infection in mice

Dragica Gajić*[a], Tamara Saksida*[a], Ivan Koprivica[a], Lidija Šenerović[b], Ivana Morić[b], Katarina Šavikin[c], Nebojša Menković[c], Nada Pejnović[a], Ivana Stojanović#[a]

[a]Department of Immunology, Institute for Biological Research „Siniša Stanković"- National Institute of Republic of Serbia, University of Belgrade, Bulevar despota Stefana 142, 11060 Belgrade, Serbia

[b]Institute of Molecular Genetics and Genetic Engineering, University of Belgrade, Vojvode Stepe 444a , 11042 Belgrade 152 , Serbia

[c]Institute for Medicinal Plants Research "Dr Josif Pančić", Tadeuša Košćuška 1, 11000 Belgrade, Serbia

*equally contributed

#corresponding author

Ivana Stojanovic, PhD

Department of Immunology

Institute for Biological Research „Siniša Stanković"- National Institute of Republic of Serbia, University of Belgrade

Bulevar despota Stefana 142

11060 Belgrade

Serbia

View Article Online
DOI: 10.1039/D0FO00946F

Abstract

Chokeberry (*Aronia melanocarpa*) fruit extracts (CE) are rich in polyphenols and usually exhibit immunomodulatory, anti-viral and anti-bacterial effects. We have previously shown that the CE used in this study activated macrophages and stimulated effector T cell differentiation *in vitro*. When applied orally to healthy mice, CE increased the proportion of $CD11c^+$ dendritic cells in the gut-associated lymphoid tissue. CE-pretreated BALB/c mice readily eradicated orally ingested *Listeria monocytogenes* as evidenced by a slighter decrease in body weight and number of bacteria recovered from the spleen and reduced spleen size compared to the control infected mice. CE pretreatment in infected mice resulted in higher proportions of $CD11b^+$ macrophages and $CD8^+$ cytotoxic T cells both in the gut and the spleen. Phagocytosis, reactive oxygen species production and the proportions of activated $CD86^+$ macrophages ($CD11b^+$) and dendritic cells ($CD11c^+$) was also enhanced in CE-pretreated infected mice. Further, the expression of inducible nitric oxide synthase and IL-6 was increased in CE-pretreated infected mice and the similar results were obtained in peritoneal macrophages *in vitro*. This effect of CE was associated with increased phosphorylation of IκB and Notch1 production. Finally, CE pretreatment elevated the proportion of perforin-producing cells in the spleen compared to control infected mice. This study demonstrates that prophylactic treatment with CE leads to more rapid eradication of bacterial infection with *L. monocytogenes* predominantly through increased activity of myeloid cells in the gut and in the spleen.

View Article Online
DOI: 10.1039/D0FO00946F

47 Abbreviations

48 CE – Chokeberry extract

49 CFU – Colony-forming unit

50 DHR – Dihydrorhodamine 123

51 FCS – Fetal Calf Serum

52 FCS buffer – Flow Cytometry Staining buffer

53 GALT – Gut-associated lymphoid tissue

54 IFN-γ – Interferon-γ

55 IL-17 –Interleukin-17

56 IL-1β – Interleukin-1β

57 IL-6 – Interleukin-6

58 MLN – Mesenteric lymph nodes

59 NF-κB – Nuclear factor κB

60 NO – Nitric oxide

61 PBS – Phosphate-buffered saline

62 PP – Peyer's patches

63 ROS – Reactive oxygen species

64 TNF – Tumor necrosis factor

View Article Online
DOI: 10.1039/D0FO00946F

Introduction

Chokeberry (*Aronia melanocarpa)* fruit is characterized by high content of phenolic constituents such as procyanidins, anthocyanins and phenolic acids known for their antioxidative, anti-bacterial and immune-modulating properties.[1,2] Therefore, chokeberry has been traditionally used in the treatment of common cold.[3,4,5] Chokeberry extracts have also exhibited strong bacteriostatic and antiviral activity *in vitro*.[6] In humans, the oral consumption of chokeberry juice significantly reduced antibiotic intake in nursing home residents with urinary infections.[7] In addition, chokeberry was shown to exert beneficial effects in cardiovascular diseases, diabetes, hypertension, hypercholesterolemia, indigestion and malignant diseases.[8,9]

Chokeberry may affect the functions of immune cells by exerting anti-inflammatory effects or, in some conditions, stimulating immune reactions. Anti-inflammatory activities of chokeberry in animal models of ulcerative colitis and neuroinflammation were mediated by down-regulation of pro-inflammatory nitric oxide (NO) production, cyclooxygenase-2 expression and prostaglandin E2 production, along with reduction of interleukin-6 (IL-6) and tumor necrosis factor (TNF).[10,11,12,13] However, chokeberry extract was also shown to stimulate NO secretion from macrophages and dendritic cells and stimulate T helper 1 lymphocyte differentiation, thus evidently exerting pro-inflammatory effects.[14]

Listeria monocytogenes is an opportunistic pathogen causing infection after ingestion of contaminated food. Immunocompromised patients, neonates, pregnant women and elderly persons may develop fatal complications such as meningitis or sepsis as a result of their impaired cell-mediated immunity.[15] In healthy people *L. monocytogenes* can cause acute, self-limiting febrile gastroenteritis.[16] Oral mouse models which mimic foodborne *L.*

View Article Online
DOI: 10.1039/D0FO00946F

monocytogenes infection are relevant for studying natural dissemination of bacteria from the gut to the spleen, liver, and brain.[17]

Pathogen-associated molecular patterns from *L. monocytogenes* engage pattern recognition receptors on epithelial cells, macrophages and dendritic cells in the gut mucosa and trigger intracellular signaling pathways (e.g. the nuclear factor κB - NF-κB pathway and others) that lead to the activation of both the innate and adaptive arm of the immune response.[15] Cells of the innate immunity eliminate *L. monocytogenes* by producing reactive oxygen species (ROS) and NO that are toxic to bacteria and by serving as antigen-presenting cells that activate the cells of adaptive immunity. This is achieved through the up-regulation of co-stimulatory molecules on antigen-presenting cells, such as CD80, CD86 and CD40.[18] Additionally, these cells produce pro-inflammatory cytokines, interleukin-1β (IL-1β) and interleukin-18 that promote the development of the adaptive immune response.[19] *L. monocytogenes* induces a strong type 1 immune response in which interferon-γ (IFN-γ), a cytokine generally essential for host resistance to intracellular bacteria, has a key role. Activated $CD8^+$ cells are involved in *L. monocytogenes* clearance by both mechanisms of direct bacteria killing via perforin and granzymes and by IFN-γ production. [20]

We have previously shown that 7-day long oral application of chokeberry extract (CE) changed the frequencies of immune cells within the intestine in healthy C57BL/6 mice.[14] So far, there are no data whether these CE-mediated immunoregulatory effects could aid the immune response against bacterial infection. Therefore, the objective of this study was to delineate the effects of CE on the immune response in the gut-associated lymphoid tissue (GALT) and spleen before and after the infection with *Listeria monocytogenes* in BALB/c mice. Also, we aimed to determine the potential molecular mechanisms underlying CE effects on the anti-bacterial immune response *in vivo* and *in vitro*.

View Article Online
DOI: 10.1039/D0FO00946F

Material and methods

Chokeberry extract preparation

In this work, we used an extract that we produced in our previous study.[14] Briefly, chokeberry was provided by Conimex Trade Ltd. from the organic cultivation field, immediately dried at 40°C for 48 h and grinded by a laboratory mill. Grinded material was extracted using 50% ethanol and after ethanol evaporation under vacuum, water residue was freeze-dried.[21,**Error! Bookmark not defined.**] Chokeberry extract (CE) contained cyanidin-3-galactoside (0.34 mg/g), cyanidin-3-arabinoside (0.16 mg/g), cyanidin-3-glucoside (0.06 mg/g), quercetin-3-glucoside (0.15 mg/g), quercetin-3-galactoside (0.31 mg/g), quercetin-3-rutinoside (0.28 mg/g), chlorogenic acid (3.53 mg/g) and proanthocyanidins (36.5 mg CE/g in total).[14] Moreover, CE was free of viable bacteria (tested in the Brain Heart Infusion broth), and no endotoxin was detected (tested by PYROGENTTM-5000 Kinetic Turbidimetric LAL Assay, Lonza Netherlands B.V., Geleen, The Netherlands).

Preparation of *L. monocytogenes* for infection

Bacteria were grown in BHI broth at 37°C on a rotary shaker at 180 rpm. After 20 h, bacteria were diluted 50 times in BHI broth and cultured until mid-log-phase growth was reached. The optical density of the bacterial suspension was read with a spectrophotometer, and the numbers of *L. monocytogenes* CFU were extrapolated from a standard growth curve. To prepare the inoculum for the mice infection, appropriate dilutions were made in sterile phosphate-buffered saline (PBS) to achieve the desired bacterial concentration (5.5×10^7 cell/ml). The actual number of CFU in the inoculum was verified by plating on BHI agar.

Mice

View Article Online
DOI: 10.1039/D0FO00946F

BALB/c mice were bred and kept under standard conditions with free access to standard pelleted diet and tap water at the Animal Facility of the Institute for Biological Research "Siniša Stanković"- National Institute of Republic of Serbia, University of Belgrade. All experiments, approved by the Ethical Committee of the Institute for Biological Research "Siniša Stanković" (App.No 02-5/19-01-1036), were in accordance with the Directive 2010/63/EU and complied with the ARRIVE guidelines.

CE pretreatment and infection with *L. monocytogenes*

Two months old male BALB/c mice were separated into four groups in a blind fashion. The first group received 100 µl of water for 7 days by oral gavage (control group for CE - 6 mice). The second group orally received 100 µl of CE (50 mg/kg bw) for 7 days (6 mice). The third group received 100 µl of water for 7 days by oral gavage and on the 8th day was orally infected with *L. monocytogenes* (5×10^6 CFU in 100 µl of PBS – 18 mice). The fourth group was treated orally every day with 100 µl of CE (50 mg/kg bw) for 7 days and on the 8th day infected with *L. monocytogenes* (5×10^6 CFU in 100 µl of PBS - 18 mice). The animals were euthanized by CO_2 asphyxiation. The *ex vivo* analysis of immune cells for the first and the second group was performed 24 h after the final treatment with either CE or water, while for the third and the fourth group it was performed 48 h after the infection with *L. monocytogenes*. The third and the fourth group consisted of 18 mice, 6 for *ex vivo* analysis and an additional 12 mice were used for the monitoring of body mass and animal appearance up to 7 days after the infection and the initial (preliminary) analysis of splenic bacterial burden 48 h after the infection. Body weight gain in % was determined by the following formula $100-(X_{current}-X_0)*100$, where $X_{current}$ is the measured weight of the mouse on the specific day (precision scales PCE-DS600, PCE Group, Meschede, Germany) and X_0 the body weight of the mouse at the beginning of the experiments.

View Article Online
DOI: 10.1039/D0FO00946F

Recovery of *L. monocytogenes* from the tissues of infected mice

The spleen was removed aseptically from infected mice 48 h after the infection. The weight of the spleens was determined using precision scale (Mettler AE 200, Mettler-Toledo GmbH, Giessen, Germany) and the spleens were homogenized in sterile PBS. Two-fold dilutions were prepared and plated in triplicate on BHI agar. The plates were incubated at 37°C for 24 h and the colonies were counted. The data were expressed as the mean ± standard deviation of *L. monocytogenes* CFU per gram of tissue (wet weight).

Immune cell isolation

Spleen, mesenteric lymph nodes (MLN) and small intestines were collected 48 h after the infection. Spleens were passed through a cell strainer with pores of 40 µm. RBC Lysis Buffer (eBioscience, San Diego, CA, USA) was used in order to remove red blood cells, and after the reaction was stopped with PBS 3% Fetal Calf Serum (FCS - Sigma-Aldrich, St. Louis, MO, USA) and centrifugation, spleen cells were counted and prepared for further manipulation. Peyer's patches (PP) were excised from the small intestine and PP cells as well as MLN cells were obtained after passage through a 40 µm cell strainer. Cells were counted on LUNA-II™ Automated Cell Counter (Logos Biosystems, Gyeonggi-do, Korea) and used for further analysis. Peritoneal cells from healthy BALB/c mice were collected by injecting cold PBS and recovering the fluid from the peritoneum using a pipette. Cells were centrifuged at 500 g, resuspended in RPMI medium supplemented with 5% FCS, 1% penicillin and streptomycin, 0.02 mM Na-pyruvate, 25 mM HEPES, and 2 mM L-glutamine (Sigma-Aldrich), counted and 2.5×10^6 cells were placed in 24-well adherent cell plates (Sarstedt, Numbrecht, Germany). After 2 h incubation at 37°C, cells were rinsed with warm PBS twice and the remaining adherent cells were predominantly macrophages. CE (25 µg/ml) was added and NO

View Article Online
DOI: 10.1039/D0FO00946F

production was measured and protein or mRNA isolation was performed after 24 h of incubation.

Flow cytometry

Viable spleen, MLN and PP cells were stained for detection of surface molecules. The following anti-mouse antibodies were used: CD4-FITC (rat IgG2b,κ), CD8-PE-Cy5.5 (rat IgG2a,κ), CD11b-FITC (rat IgG2b,κ), CD11c-PE-Cy5.5 (Armenian hamster IgG), CD11c-FITC (Armenian hamster IgG) and CD86-PE-Cy5.5 (rat IgG2a,κ) (all from ThermoFisher Scientific, Waltham, MA, USA). The staining was performed for 45 min at 4°C, with the antibodies dissolved in Flow Cytometry Staining buffer (FCS buffer, eBioscience). The samples were washed twice and resuspended in FCS buffer for analysis. For intracellular staining, cells were stimulated for 4 h with Cell Stimulation Cocktail (1:500, eBioscience) and stained for extracellular markers as described. Cells were then fixed in 2% paraformaldehyde, permeabilized with permeabilization buffer (PB, eBioscience), after which the following anti-mouse antibodies were used: IFN-γ-PerCP-Cy5.5 (rat IgG1,κ), Granzyme B-FITC (rat IgG2a,κ), IL-17-PE (rat IgG2a) (all from Thermo Fisher Scientific). Staining was performed for 45 min at 4°C, washed once with PB, once with PBS and finally resuspended in FCS buffer. For Perforin-PE (rat IgG2a,κ, eBioscience) staining, the FoxP3 permeabilization protocol was used, according to the manufacturer's instructions. Cells were detected by Partec CyFlow Space and analysed by FlowMax software (Partec, Görlitz, Germany). In all experiments isotype-matched controls (eBioscience) were included.

Phagocytosis assay

PP and spleen cells were incubated for 1 h at 37°C in 24-well plate (1×10^5 cells/well) and the nonadherent cells were then washed away with PBS. PE-labelled amine-modified polystyrene latex beads (mean particle size 1 μm, Sigma-Aldrich) were pre-opsonised in PBS 50% FCS

for 1 h at 37°C and then incubated with adhered PP and spleen cells for 1 h at 37°C (10 beads/cell). After washing, cells were resuspended in FCS buffer and the proportion of phagocytic cells and phagocytic activity were analyzed with flow cytometry.

Measurement of reactive oxygen species

Immediately after isolation, PP and spleen cells (5×10^5) were exposed to 5 µM dihydrorhodamine 123 (DHR) (Sigma-Aldrich) for 20 min at 37°C. Cells were then washed with PBS and finally resuspended in FCS buffer. The proportion of reactive oxygen species (ROS)-producing cells and a measure of intracellular production of ROS were detected by flow cytometry.

NO secretion

Peritoneal macrophages (2×10^5) were cultured in 96-well plates and treated with CE (25 µg/ml) for 24 h. Nitrite accumulation, an indicator of NO production, was measured in cell-culture supernatants using the Griess reagent.[14]

Immunoblot

In vitro cultured peritoneal cells (2.5×10^6) or *ex vivo* isolated spleen cells were lysed with a buffer containing 62.5 mM Tris–HCl (pH 6.8), 2% SDS, 50 mM DTT, 10% glycerol, with the Protease Inhibitor Cocktail (all from Sigma-Aldrich). All samples were boiled with the 4× SDS sample loading buffer. Sample electrophoresis was performed on 12% SDS–polyacrylamide gel. By using a semi-dry blotting system (Semi-Dry Transfer Unit, GE Healthcare, Buckinghamshire, England), the protein samples were electro-transferred from the gel onto polyvinylidene difluoride membranes. The membranes were then blocked with PBST (PBS 0.1% Tween-20, Sigma-Aldrich) containing 5% BSA and probed with specific antibodies diluted in PBST 1% BSA, according to the manufacturer's instructions. The following primary antibodies were used: rabbit anti-mouse phospho-IκB (1:1000, Sigma-

Aldrich) rabbit anti-mouse IκB (1:1000, Cell Signalling Technology, Danvers, MA, USA), rabbit anti-mouse Notch1 (1:300, Sigma-Aldrich) and mouse anti-mouse β-actin (1:1000, Sigma-Aldrich). Appropriate HRP conjugated secondary antibodies were used: anti-rabbit IgG (1:5000, Invitrogen, Carlsbad, CA, USA) or anti-mouse IgG (1:5,000, Invitrogen). Detection was achieved with Immobilon Western Chemiluminescent HRP Substrate (Millipore, Billerica, MA, USA), and the signal was captured with X-ray film (Kodak, Rochester, NY, USA). Densitometry was performed with Fiji, an open-source software for biological image analysis, and the production of specific proteins was presented relative to the production of either their non-phosphorylated protein forms or of β-actin.

Reverse transcription and real-time PCR

TriReagent (Metabion, Martinsried, Germany) was used for dissolving the samples and centrifugation with chloroform at 12000 g was performed subsequently. After RNA isolation from the aqueous layer and precipitation with isopropanol, reverse transcription was performed. Samples (1 μg) were incubated with random hexamer primers and RevertAid™ M-MuLV Reverse Transcriptase (Fermentas, Vilnius, Lithuania). Target sequences of cDNA were amplified using SYBRGreen PCR master mix (Applied Biosystems, Woolston, UK) in Real-time PCR machine (Applied Biosystems). Primer pairs for iNOS were: forward 5′-CTG CAG CAC TTG GAT CAG GA-3′ and reverse 5′-GCC AGA AAC TTC GGA AGG GA-3′ (NM_001313922.1), TNF forward 5′-CCACGTCGTAGCAAACCAC-3′ and reverse 5′-TGGGTGAGGAGC ACGTAGT-3′ (NM_013693.3), for IL-1β forward 5′-GCTGAAAGCTCTCCACCTCAA-3′ and reverse 5′- TGTCGTTGCTTGGTTCTCCTTG-3′ (NM_008361.4), for IL-6 forward 5′-TTG CCT TCT TGG GAC TGA TGC T-3′ and reverse 5′-GTA TCT CTC TGA AGG ACT CTG G-3′ (NM_031168.2), and for β-actin they were 5′-GACCTGACAGACTACC-3′ and 5′-GGCATAGAGGTCTTTACGG-3′ (NM_007393.2). Gene expression was determined as $2^{-(Ct-Ca)}$, where Ct is target gene cycle threshold and Ca is

β-actin cycle threshold. SDS2.1 software (Applied Biosystems) was used to analyse the obtained cycle thresholds.

Statistical Analysis

Data are presented as mean ± SD. A two tailed Student's t test was used for determination of significance of differences between experimental groups, or a Mann-Whitney non-parametric test for the analysis of the data that were not normally distributed. If $p < 0.05$, the differences were considered as statistically significant. Statistical analyses were performed using GraphPad Prism 8 software (GraphPad Software, Inc., La Jolla, CA, USA).

View Article Online
DOI: 10.1039/D0FO00946F

Results

Pretreatment with chokeberry extract accelerates eradication of *L. monocytogenes* infection in BALB/c mice

BALB/c mice were treated with CE or vehicle for 7 days orally and then infected with *L. monocytogenes* through the oral route. The results showed that after the infection, the control mice which received water instead of CE during pretreatment exhibited significant weight loss (Fig. 1A). In contrast, body weight of CE-pretreated mice did not significantly change compared to their weight on day 0. This is concordant with the number of CFU found in the spleen two days after the infection; CE-pretreated mice had significantly lower bacterial burden in the spleen compared to the vehicle-pretreated mice (Fig. 1B). Also, the relative spleen weight was higher in the infected control mice (Fig. 1C).

The effect of CE pretreatment on immune cells in the GALT

The more successful eradication of *L. monocytogenes* observed in the CE-pretreated mice might be the consequence of altered immune response within GALT that includes PP and MLN. Therefore, we compared the immune cell composition in PP of vehicle and CE-treated mice with or without infection. Results showed that CE treatment in non-infected animals did not change the proportions of $CD11b^+$ macrophages, $CD8^+$ cytotoxic T lymphocytes and $CD4^+$ T helper lymphocytes while it significantly increased the proportion of $CD11c^+$ dendritic cells (Table 1). After the infection, CE-pretreated mice had significantly increased proportions of $CD11b^+$ (Fig. 2A) and $CD8^+$ (Fig. 2C) cells compared to the control infected mice, while $CD11c^+$ (Fig. 2B) and $CD4^+$ (Fig. 2D) cell proportions remained the same.

View Article Online
DOI: 10.1039/D0FO00946F

The effect of CE pretreatment on immune cells in the spleen

Lower bacterial burden found in the spleen of CE-treated animals prompted us to investigate the effect of CE on cells important for bacterial clearance. CE pretreatment did not change the proportions of splenic macrophages ($CD11b^+$), dendritic cells ($CD11c^+$) or $CD8^+$ lymphocytes, while the proportion of $CD4^+$ lymphocytes was down-regulated (Table 1) compared to the untreated animals (before the infection). Importantly, after the infection, in CE-pretreated animals the proportion of macrophages (Fig. 3A) and $CD8^+$ lymphocytes (Fig. 3C) were up-regulated. However, the proportion of $CD11c^+$ dendritic cells in spleens of infected CE-pretreated mice was lower compared to control infected mice (Fig. 3B), while the proportion $CD4^+$ (Fig. 3D) remained the same.

The effect of CE on myeloid cells - mechanism of action

Next, we wanted to examine the possible mechanism of action of CE pretreatment on bacterial infection and therefore we investigated the activities of macrophages and dendritic cells. CE pretreatment increased both the proportion of actively phagocytic cells within the PP (Fig. 4A) and their phagocytic activity in the PP and spleen (Fig. 4A, C), determined by mean fluorescence intensity of ingested fluorescent latex beads. Further, the proportion of cells that produced ROS was higher in PP of CE-pretreated infected animals compared to infected controls (Fig. 4B). ROS-producing cells proportion and ROS production in the spleen was the same between the treatment groups (Fig. 4D). The higher expression of the co-stimulatory molecule CD86 on myeloid cells coincided with their increased activity. We found that CE pretreatment up-regulated the proportion of macrophages that express CD86 in PP (Fig. 5A) and spleen (Fig. 5B) and the proportions of macrophages and dendritic cells expressing CD86 in MLN (Fig. 5C). In addition, spleen cells obtained from CE-pretreated infected mice

expressed higher levels of iNOS (Fig. 6A) and IL-6 mRNA (Fig. 6B) compared to the cells of infected control mice. IL-1β expression remained similar between the groups (0.19±0.09 vs 0.10±0.01, p=0.387, infection vs CE+infection) as well as TNF expression (0.037±0.015 vs 0.022±0.009, p=0.652, infection vs CE+infection). *In vitro* studies on peritoneal macrophages isolated from BALB/c mice showed a significant stimulation of NO production after CE treatment (25 μg/ml) (Fig. 6C), accompanied by the increase in iNOS (Fig. 6D) and IL-6 mRNA (Fig. 6E) and comparable levels of IL-1β mRNA (0.479±0.393 vs 0.323±0.238, p=0.677, infection vs CE+infection) and TNF (0.057±0.048 vs 0.249±0.142, p=0.091, infection vs CE+infection).

In order to investigate the CE effect on signaling pathways involved in iNOS and IL-6 expression, we measured the activation of NF-κB through IκB phosphorylation and Notch1 protein expression in macrophages *in vitro*. Results indicate that CE activated NF-κB (Fig. 6F) and induced Notch1 expression (Fig. 6G) in macrophages 24 h after the treatment.

The effect of CE pretreatment on $CD8^+$ T cell-derived cytotoxic proteins

The eradication of intracellular bacteria depends upon cytotoxic mediators such as perforin, granzyme and IFN-γ, a cytokine that drives anti-bacterial immune response. CE-pretreated infected mice exhibited similar proportions of cells that produced perforin, granzyme and pro-inflammatory cytokine IFN-γ or interleukin 17 (IL-17) at intestinal draining sites, PP and MLN (Table 2). The effect of CE was only observed in the increased proportion of $perforin^+$ cells in the spleen (Table 2).

View Article Online
DOI: 10.1039/D0FO00946F

Discussion

This study shows that CE pretreatment results in faster eradication of *L. monocytogenes* infection. In this study CE application was ceased one day prior to infection, making the direct anti-bacterial effect not probable, but reliant on the modulation of immune response. CE effect was predominantly mediated by the increased phagocytic ability of macrophages and bacterial destruction through NO and ROS production. Furthermore, the increased expression of the co-stimulatory molecule CD86 on myeloid cells (macrophages and dendritic cells) and IL-6 was associated with enhanced activation of the adaptive immunity, predominantly $CD8^+$ cytotoxic T cells. Comparable results were observed *in vitro* where CE significantly up-regulated iNOS and IL-6 expression and NO production in peritoneal macrophages, further supporting the conclusions from the *in vivo* model. The stimulatory effect of CE on macrophage was probably mediated by the activation of NF-κB transcription factor and Notch1 transcription regulator.

Orally ingested *L. monocytogenes* enters the mucosa of the intestinal tract via enterocytes or indirectly through invasion of Peyer's patches and then disseminates to the spleen, liver and brain.[15,17] The ability of myeloid cells to phagocytize bacteria is of great importance for successful eradication of bacteria.[15] The faster elimination of *L. monocytogenes* observed in CE-pretreated mice was most probably a result of CE-mediated changes in the immune system before the infection. The most evident changes were observed in the gut immune system where CE increased the proportion of dendritic cells and, after the infection, the phagocytic ability of myeloid cells. This CE effect is similar to the activity of a protein fraction from garlic which was shown to augment *in vitro* macrophage cytotoxicity and phagocytosis and activate macrophages to stimulate lymphocyte proliferation.[22] Flavonoid constituents of CE such as quercetin could be responsible for the stronger induction of

View Article Online
DOI: 10.1039/D0FO00946F

phagocytosis observed in CE-pretreated mice or in macrophages *in vitro*.[14,23] As quercetin is identified as an mTORC1 inhibitor[24] and mTORC1 suppression is associated with increased macrophage pro-inflammatory status,[25,26] the observed activation of myeloid cells may be mediated by quercetin-directed mTORC1 inhibition. Enhanced phagocytosis is probably one of the reasons for lower number of bacteria recovered from the spleen of CE-pretreated mice.

After ingestion of bacteria, myeloid cells produce NO and ROS that mediate bacterial killing.[27] Although CE contains high levels of polyphenols (chlorogenic acid, proanthocyanidins) which generally exhibit anti-oxidant activity and decrease NO,[28,29] our study has shown that CE significantly increased NO production in macrophages *in vitro* and iNOS expression in splenocytes *in vivo*. The observed stimulatory effect on NO production is similar to the studies that tested an ethanol fraction of chokeberry on endothelial cells *in vitro*[30] or its juice *in vivo*.[31] The stimulatory effect on NO is not solely a feature of CE. There is also evidence of macrophage activation by other plant crude extract applications. For example, hot water extract of *Herba pogostemonis* elicited altered morphology and elevated iNOS mRNA in macrophages *in vitro* and thereby protected mice against *Salmonella typhimurium*-induced liver damage and mortality.[32] Additionally, *Echinacea purpurea* extract was shown to activate the innate immune response, stimulating the production of IL-6, TNF, IL-12, and NO from macrophages *in vitro*.[33] Specific flavonoid compounds, proanthocyanidins for example, have been shown to induce moderate levels of NO in macrophages.[34] Also, they have stimulated NO production in endothelial cells.[35] The effect of quercetin on NO was more thoroughly investigated, showing that it can induce NO production and increase NO longevity through suppression of ROS generating enzymes and subsequent reduction of superoxide and peroxynitrite which neutralize NO.[36] However, the observed increased ROS production after CE application suggested that this cannot be the mechanism of NO up-regulation. According to our results, CE influenced signaling pathways

responsible for NO generation, i.e. it enhanced the activation of iNOS mandatory transcription factor NF-κB[37] and subsequently stimulated iNOS mRNA expression.

Along with increased ROS and NO, the CE extract stimulated IL-6 expression both *in vitro* and *in vivo*. IL-6 can be regulated either by canonical NF-κB pathway alone[38] or in combination with Notch1, a protein that enables deactivation of specific repressors of RNA transcription.[39,40] Therefore, the observed increase in NF-κB and Notch1 in macrophages upon CE treatment was most likely responsible for the up-regulated IL-6 mRNA expression.

After myeloid cell-mediated digestion of bacteria, the myeloid cells commonly up-regulate their co-stimulatory molecules CD80, CD86 or CD40, which mediate the activation of adaptive immune cells.[27] In concordance with the previous findings that CE stimulated CD86 expression in macrophages *in vitro*,[14] CE-pretreated mice up-regulated the expression of CD86 on dendritic cells and macrophages in the gut, MLN and spleen. Increased CD86 could be responsible for the observed IL-6 up-regulation as it has been previously shown that CD86 crosslinking stimulate IL-6 in dendritic cells through NF-κB and Notch1 signaling.[41] The observed increase of CD86 on macrophages and dendritic cells was associated with the increase of $CD8^+$ T cell proportions. However, only perforin-secreting cells which mediate direct bacterial killing[28] were found to be increased in spleens of CE-pretreated and infected mice, while the proportions of cells producing other pro-inflammatory mediators such as IFN-γ, IL-17 or granzyme B were unchanged between the groups. This suggests that the increased $CD8^+$ proportions accomplished a more efficient *L. monocytogenes* clearance. This is in contrast with, for example, a garlic extract that stimulated IFN-γ-producing cells *in vivo* in the presence of *Mycobacterium tuberculosis*, while no changes were observed in the proportion of macrophages, dendritic cells, $CD4^+$ and $CD8^+$ lymphocytes.[42]

Conclusions

View Article Online
DOI: 10.1039/D0FO00946F

Since the bacterial resistance to commonly used antibiotics is increasing worldwide, the wide spectra of antimicrobial research are geared towards the discovery and development of novel agents that would preferably stimulate the anti-bacterial immune response. This study demonstrates the immunostimulatory effects of CE. Prophylactic treatment with CE resulted in enhanced eradication of *L. monocytogenes* in mice. Mechanisms of CE action include the activation and enhanced function of gut-associated myeloid cells relevant in the fight against *L. monocytogenes* infection and increased $CD8^+$ T and perforin-producing cells in spleen. Further investigation on the determination of the relevant constituent from CE that promotes this activity will be performed in the future.

Authors' contribution

IS designed the experiments, analyzed data and wrote the manuscript, DG and TS performed *in vivo* and *ex vivo* experiments and analyzed data, IK and NP performed *ex vivo* experiments, KS and NM provided the extract, LS and IM provided the bacteria and performed *ex vivo* measurement of *L. monocytogenes* CFU from the spleen homogenate. All authors proofread the manuscript and approved the submission.

Conflicts of interest

The authors have declared no conflicts of interest.

Acknowledgements

This work was supported by Ministry of Education, Science and Technological Development of Republic of Serbia (grants: #451-03-68/2020-14/200007, 451-03-68/2020-14/200003, 451-03-68/2020-14/200042).

View Article Online
DOI: 10.1039/D0FO00946F

Figure legends

Figure 1. The effect of CE pretreatment on *L. monocytogenes* infection in BALB/c mice. (A) Body weight change (%) during the days post infection in CE- and control-pretreated infected mice. The total number of mice per group was 8. (B) Bacterial burden in the spleen, observed through the number of colony forming units (CFU), and relative spleen weight (C), measured 48 h after the infection. The experiment was performed twice and the results of both experiments are shown. The total number of mice per group was from 10 to 12. *$p < 0.05$ represents a statistically significant difference *vs.* the control infection group.

Figure 2. The effect of CE pretreatment on immune cells in the GALT. The proportion of immune cells was determined in PP of infected and CE-treated infected mice with *L. monocytogenes* 48 h after the infection. The following immune cells were analyzed by flow cytometry: (A) $CD11b^+$ macrophages, (B) $CD11c^+$ dendritic cells, (C) $CD8^+$ cytotoxic T lymphocytes and (D) $CD4^+$ T helper cells. Representative dot plots are shown below the graphs. *Ex vivo* experiments were performed on 6 mice per group. Values of *p* are given where significant differences were found.

Figure 3. The effect of CE pretreatment on immune cells in the spleen. The immune cell proportion was observed in the spleen of infected and CE-treated infected mice with *L. monocytogenes* 48 h after infection. The following immune cells were analyzed by flow cytometry: (A) $CD11b^+$ macrophages, (B) $CD11c^+$ dendritic cells, (C) $CD8^+$ cytotoxic T lymphocytes and (D) $CD4^+$ T helper cells. Representative dot plots are shown on the right-hand side. *Ex vivo* experiments were performed on 6 mice per group. Values of *p* are given where significant differences were found.

Figure 4. CE pretreatment effect on phagocytosis and ROS production. The proportion of phagocytic cells (%) and phagocytic activity, shown through the mean fluorescence intensity

View Article Online
DOI: 10.1039/D0FO00946F

(MFI), were analyzed in the Peyer's patches (A) and the spleens (C) of CE or vehicle pretreated infected mice two days after *L. monocytogenes* infection. The proportion of ROS-producing cells and their MFI in Payer's patches (B) and spleens (D) of CE or vehicle pretreated infected mice two days after *L. monocytogenes* infection. Representative dot plots are shown on the right-hand side. *Ex vivo* experiments were performed on 6 mice per group. Values of *p* are given where significant differences were found.

Figure 5. CE pretreatment effect on expression of myeloid cells co-stimulatory molecules. The proportion (%) of CD86 expressing macrophages ($CD86^+CD11b^+$ cells) and dendritic cells ($CD86^+CD11c^+$ cells) was determined in the PP (A), spleen (B) and MLN (C) of infected mice and CE-pretreated and infected mice 48 h after the infection. Representative dot plots are shown on the right-hand side. *Ex vivo* experiments were performed on 6 mice per group. Values of *p* are given where significant differences were found.

Figure 6. CE effect on expression of pro-inflammatory mediators and transcription regulators. iNOS mRNA expression (A) and IL-6 mRNA expression (B) was determined in spleen cells of infected and CE-pretreated and infected mice 48 h after the infection with *L. monocytogenes*. *Ex vivo* experiments were performed on 6 mice per group. Peritoneal macrophages were *in vitro* treated with CE (25 μg/ml) and 24 h after the treatment NO production (C), iNOS expression (D) and IL-6 expression (E) were determined by RT-PCR. The level of IκB phosphorylation (pIκB) was normalized to total IκB production in macrophages (F), while Notch1 production was normalized to the level of β-actin (determined by western blot) (G). Appropriate membrane images are presented. The experiment was performed in quadruplets. Values of *p* are given where significant differences were found.

View Article Online
DOI: 10.1039/D0FO00946F

References

[1]M.P. Kähkönen, A.I. Hopia and M. Heinonen, Berry phenolics and their antioxidant activity, *J Agric Food Chem,* 2001, **49**, 4076–4082.

[2]X. Wu, L. Gu, R.L. Prior and S. McKay, Characterization of anthocyanins and proanthocyanins in some cultivars of *Ribes*, Aronia, and *Sambucus* and their antioxidant capacity, *J Agric Food Chem,* 2004, **52**, 7846–7856.

[3]P. Denev, M. Číž, M. Kratchanova and D. Blazheva, Black chokeberry (*Aronia melanocarpa*) polyphenols reveal different antioxidant, antimicrobial and neutrophil-modulating activities, *Food Chem*., 2019, **284**, 108-117.

[4]T. Jurikova, J. Mlcek, S. Skrovankova, D. Sumczynski, J. Sochor, I. Hlavacova, L. Snopek and J. Orsavova, Fruits of Black Chokeberry *Aronia melanocarpa* in the Prevention of Chronic Diseases, *Molecules*, 2017, **22**, 944.

[5]I. Banjari, A. Misir, K Šavikin, S. Jokić, M, Molnar, H.K.S. De Zoysa and V.Y. Waisundara, Antidiabetic Effects of *Aronia melanocarpa* and Its Other Therapeutic Properties, *Front Nutr*, 2017, **4**, 53.

[6]S.V. Valcheva-Kuzmanova and A. Belcheva, Current knowledge of *Aronia melanocarpa* as a medicinal plant, *Folia Med (Plovdiv),* 2006, **48**, 11–17.

[7]M. Handeland, N. Grude, T. Torp and R. Slimestad, Black chokeberry juice (*Aronia melanocarpa*) reduces incidences of urinary tract infection among nursing home residents in the long term--a pilot study, *Nutr Res*, 2014, **34**, 518-525.

[8]A. Kokotkiewicz, Z. Jaremicz and M. Luczkiewicz, Aronia plants: a review of traditional use, biological activities, and perspectives for modern medicine, *J Med Food*., 2010, **13**, 255-269.

[9]N Ćujić, K. Šavikin, Z. Miloradović, M. Ivanov, U.J. Vajić, D. Karanović, J. Grujić-Milanović, D. Jovović and N. Mihailović-Stanojević, Characterization of dried chokeberry fruit extract and its chronic effects on blood pressure and oxidative stress in spontaneously hypertensive rats, *J Funct Foods,* 2018, **44**, 330-339.

[10]S.H. Kang, Y.D. Jeon, K.H. Moon, J.H. Lee, D.G. Kim, W. Kim, H. Myung, J.S. Kim, H.J. Kim, K.S. Bang and J.S. Jin, Aronia Berry Extract Ameliorates the Severity of Dextran Sodium Sulfate-Induced Ulcerative Colitis in Mice, *J Med Food*, 2017, **20**, 667-675.

View Article Online
DOI: 10.1039/D0FO00946F

[11]D.C. Montrose, N.A. Horelik, J.P. Madigan, G.D. Stoner, L.S. Wang, R.S. Bruno, H.J. Park, C. Giardina and D.W. Rosenberg, Anti-inflammatory effects of freeze-dried black raspberry powder in ulcerative colitis, *Carcinogenesis*, 2011, **32**, 343-50.

[12]D.A. Martin, R. Taheri, M.H. Brand, A. Draghi, F.A. Sylvester and B.W. Bolling, Anti-inflammatory activity of Aronia berry extracts in murine splenocytes, *J Funct Foods,* 2014, **8**, 68–75.

[13]K.P. Lee, N.H. Choi, H.S. Kim, S. Ahn, I.S. Park and D.W. Lee, Anti-neuroinflammatory effects of ethanolic extract of black chokeberry (*Aronia melanocapa* L.) in lipopolysaccharide-stimulated BV2 cells and ICR mice, *Nutr Res Pract*, 2018, **12**, 13-19.

[14] D. Gajic, T. Saksida, I. Koprivica, M. Vujicic, S. Despotovic, K. Savikin, T. Jankovic and I. Stojanovic, Chokeberry (*Aronia melanocarpa*) fruit extract modulates immune response *in vivo* and *in vitro, J Funct Foods*, 2020, **66**, 103836.

[15]M. Schuppler and M.J. Loessner, The Opportunistic Pathogen *Listeria monocytogenes*: Pathogenicity and Interaction with the Mucosal Immune System, *Int J Inflam*, **2010**, 704321.

[16]S.T. Ooi and B. Lorber, Gastroenteritis due to *Listeria monocytogenes*, *Clin Infect Dis*, 2005, **40**, 1327-1332.

[17]M.G. Pitts and S.E.F. D'Orazio, A Comparison of Oral and Intravenous Mouse Models of Listeriosis, *Pathogens*, 2018, **7**.

[18]D.S. Damlund, L. Christophersen, P.Ø. Jensen, M. Alhede, N. Høiby amd C. Moser, Activation of pulmonary and lymph node dendritic cells during chronic *Pseudomonas aeruginosa* lung infection in mice, *APMIS*, 2016, **124**, 500-507.

[19]E.M. Creagh and L.A. O'Neill, TLRs, NLRs and RLRs: a trinity of pathogen sensors that co-operate in innate immunity, *Trends Immunol,* 2006, **27**, 352-357.

[20]J.T. Harty and V.P. Badovinac, Influence of effector molecules on the CD8(+) T cell response to infection, *Curr Opin Immunol*, 2002, **14**, 360-365.

[21]N. Ćujić, K. Šavikin, T. Janković, D. Pljevljakušić, G. Zdunić and S. Ibrić, Optimization of polyphenols extraction from dried chokeberry using maceration as traditional technique, *Food Chem*, 2016, **194**, 135-42.

View Article Online
DOI: 10.1039/D0FO00946F

[22]M.S. Butt, M.T. Sultan, M.S. Butt and J. Iqbal, Garlic: nature's protection against physiological threats, *Crit Rev Food Sci Nutr*, 2009, **49**, 538-551.

[23] C.S. Yu, K.C. Lai, J.S. Yang, J.H. Chiang, C.C. Lu, C.L. Wu, J.P. Lin, C.L. Liao, N.Y. Tang, W.G. Wood and J.G. Chung, Quercetin inhibited murine leukemia WEHI-3 cells in vivo and promoted immune response. *Phytother Res*, 2010, **24**, 163-168.

[24] T. Weichhart, G. Costantino, M. Poglitsch, M. Rosner, M. Zeyda, K. M. Stuhlmeier, T. Kolbe, T. M. Stulnig, W. H. Hörl, M. Hengstschläger, M. Müller, M.D. Säemann, The TSC-mTOR signaling pathway regulates the innate inflammatory response. *Immunity,* 2008, **29**, 565–577.

[25] E. Vergadi, E. Ieronymaki, K. Lyroni, K. Vaporidi and C. Tsatsanis, Akt Signaling Pathway in Macrophage Activation and M1/M2 Polarization. *J Immunol,* 2017, **198**, 1006-1014.

[26] T. Weichhart, M. Haidinger, K. Katholnig, C. Kopecky, M. Poglitsch, C. Lassnig, M. Rosner, G. J. Zlabinger, M. Hengstschläger, M. Müller, W.H. Hörl, and M.D. Säemann, Inhibition of mTOR blocks the anti-inflammatory effects of glucocorticoids in myeloid immune cells. *Blood*, 2011, **117**, 4273–4283.

[27] S.E.F. D'Orazio, Innate and Adaptive Immune Responses during Listeria monocytogenes Infection. *Microbiol Spectr*, 2019, **7**, 10.1128/microbiolspec.GPP3-0065-2019.

[28]N. Yahfoufi, N. Alsadi, M. Jambi and C. Matar, The Immunomodulatory and Anti-Inflammatory Role of Polyphenols, *Nutrients*, 2018, **10**, 1618.

[29] S.H. Kim, S.Y. Park, Y.L. Park, D.S. Myung, J.S. Rew, Y.E. Joo, Chlorogenic acid suppresses lipopolysaccharide‐induced nitric oxide and interleukin‐1β expression by inhibiting JAK2/STAT3 activation in RAW264.7 cells. *Mol Med Rep*, 2017, **16**, 9224-9232.

[30] C.E. Varela, E. Fromentin, M. Roller, F. Villarreal and I. Ramirez-Sanchez, Effects of a natural extract of Aronia Melanocarpa berry on endothelial cell nitric oxide production. *J Food Biochem*, 2016, **40**, 404-410.

[31] (J.H. Kim, C. Auger, I. Kurita, E. Anselm, L.O. Rivoarilala, H.J. Lee, K.W. Lee and V.B. Schini-Kerth, Aronia melanocarpa juice, a rich source of polyphenols, induces endothelium-dependent relaxations in porcine coronary arteries via the redox-sensitive activation of endothelial nitric oxide synthase. *Nitric Oxide*, 2013, **35**, 54-64.

[32]S.P. Kim, E. Moon, S.H. Nam and M. Friedman, Composition of *Herba Pogostemonis* water extract and protection of infected mice against *Salmonella Typhimurium*-induced liver damage and mortality by stimulation of innate immune cells, *J Agric Food Chem*, 2012, **60**, 12122–12130.

View Article Online
DOI: 10.1039/D0FO00946F

[33]A.M. Sullivan, J.G. Laba, J.A. Moore and T.D. Lee, Echinacea-induced macrophage activation, *Immunopharmacol Immunotoxicol*, 2008, **30**, 553–574.

[34] H. Kolodziej, O. Kayser, A.F. Kiderlen, H. Ito, T. Hatano, T. Yoshida and L.Y. Foo, Proanthocyanidins and related compounds: antileishmanial activity and modulatory effects on nitric oxide and tumor necrosis factor-alpha-release in the murine macrophage-like cell line RAW 264.7. *Biol Pharm Bull*, 2001, **24**, 1016-1021.

[35] R. Furuuchi, I. Shimizu, Y. Yoshida, Y. Hayashi, R. Ikegami, M. Suda, G. Katsuumi, T. Wakasugi, M. Nakao and T. Minamino, Boysenberry polyphenol inhibits endothelial dysfunction and improves vascular health. *PLoS One*, 2018, **13**, e0202051.

[36] G. López-López, L. Moreno, A. Cogolludo, M. Galisteo, M. Ibarra, J. Duarte, F. Lodi, J. Tamargo, F. Perez-Vizcaino, Nitric oxide (NO) scavenging and NO protecting effects of quercetin and their biological significance in vascular smooth muscle. *Mol Pharmacol*, 2004, **65**, 851-859.

[37] F. Aktan F, iNOS-mediated nitric oxide production and its regulation. *Life Sci*, 2004, **75**, 639-653.

[38] T. Kishimoto, The biology of interleukin-6. *Blood*, 1989, **74**, 1-10.

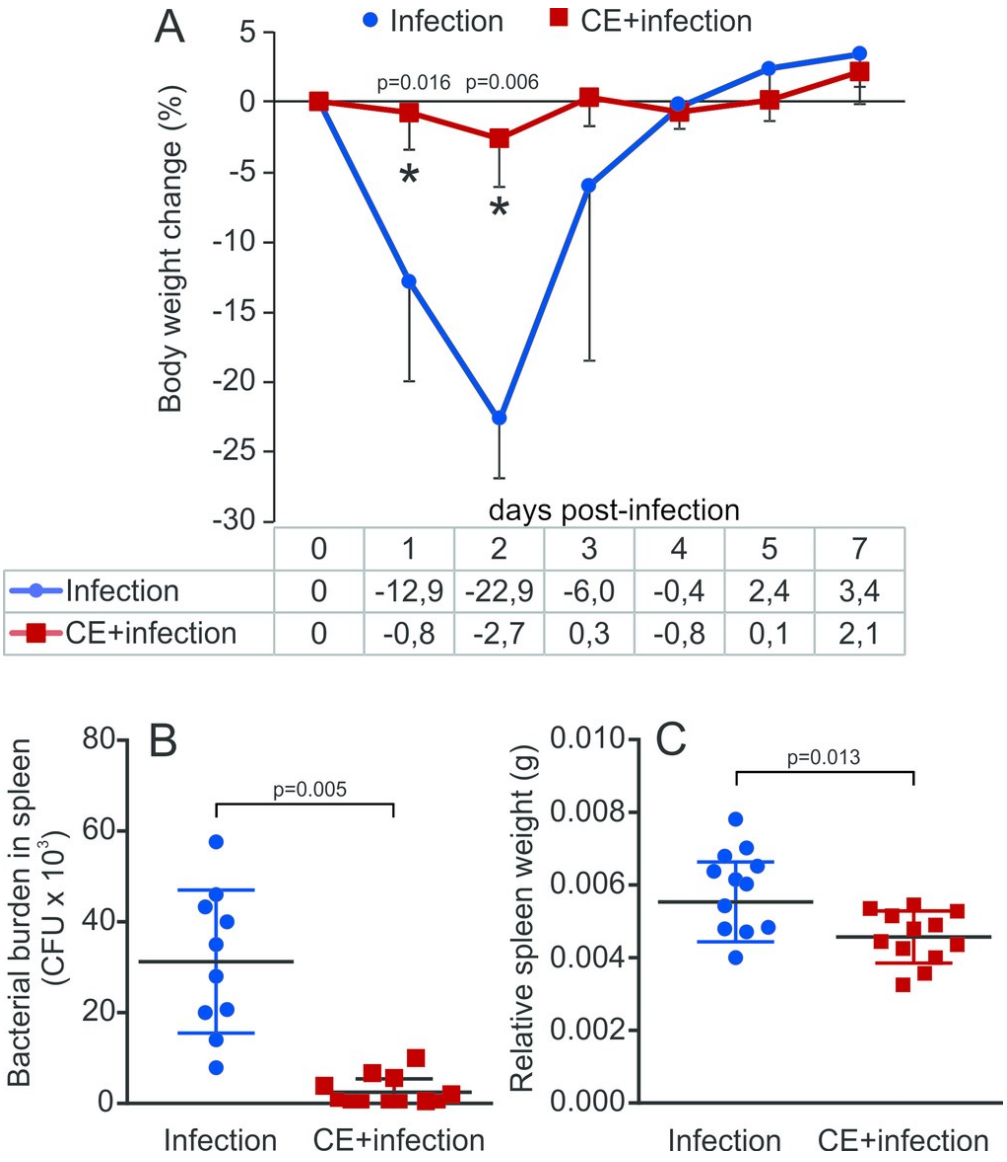
[39] W. Wongchana and T. Palaga, Direct regulation of interleukin-6 expression by Notch signaling in macrophages. *Cell Mol Immunol*, 2012, **9**, 155-162.

[40] G.D. Hurlbut, M.W. Kankel, R.J. Lake and S. Artavanis-Tsakonas, Crossing paths with Notch in the hyper-network. *Curr Opin Cell Biol*, 2007, **19**, 166-175.

[41] C. Koorella, J.R. Nair, M.E. Murray, L.M. Carlson, S.K. Watkins and K.P. Lee, Novel regulation of CD80/CD86-induced phosphatidylinositol 3-kinase signaling by NOTCH1 protein in interleukin-6 and indoleamine 2,3-dioxygenase production by dendritic cells. *J Biol Chem*, 2014, **289**, 7747-7762.

[42]V.P. Dwivedi, D. Bhattacharya, M. Singh, A. Bhaskar, S. Kumar, S. Fatima, P. Sobia, L.V. Kaer and G. Das, Allicin enhances antimicrobial activity of macrophages during *Mycobacterium tuberculosis* infection, *J Ethnopharmacol*, 2019, **243**, 111634.

View Article Online
DOI: 10.1039/D0FO00946F



	0	1	2	3	4	5	7
Infection	0	-12,9	-22,9	-6,0	-0,4	2,4	3,4
CE+infection	0	-0,8	-2,7	0,3	-0,8	0,1	2,1

Figure 1. The effect of CE pretreatment on L. monocytogenes infection in BALB/c mice. (A) Body weight change (%) during the days post infection in CE- and control-pretreated infected mice. The total number of mice per group was 8. (B) Bacterial burden in the spleen, observed through the number of colony forming units (CFU), and relative spleen weight (C), measured 48 h after the infection. The experiment was performed twice and the results of both experiments are shown. The total number of mice per group was from 10 to 12. *p < 0.05 represents a statistically significant difference vs. the control infection group.

85x98mm (300 x 300 DPI)

View Article Online
DOI: 10.1039/D0FO00946F

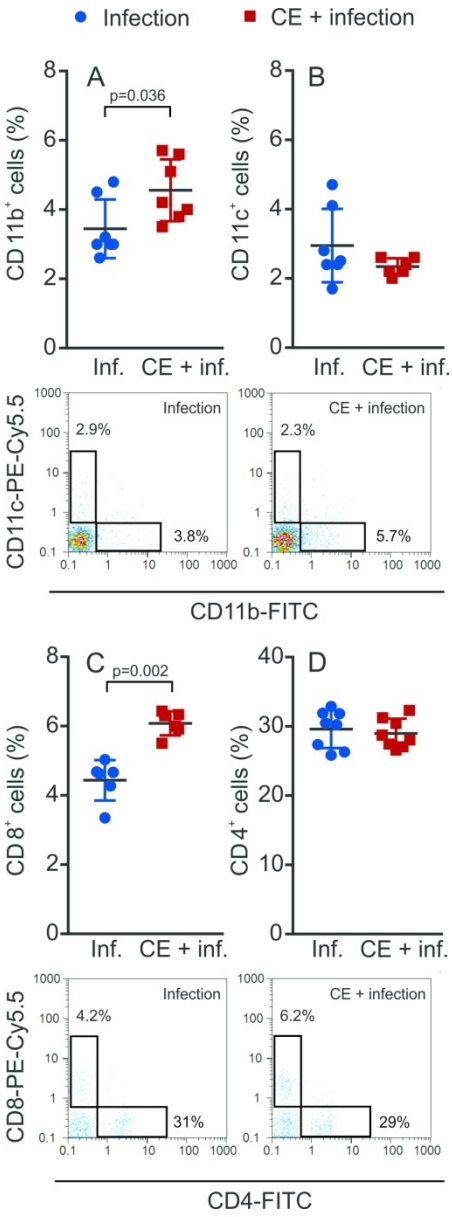


Figure 2. The effect of CE pretreatment on immune cells in the GALT. The proportion of immune cells was determined in PP of infected and CE-treated infected mice with L. monocytogenes 48 h after the infection. The following immune cells were analyzed by flow cytometry: (A) CD11b+ macrophages, (B) CD11c+ dendritic cells, (C) CD8+ cytotoxic T lymphocytes and (D) CD4+ T helper cells. Representative dot plots are shown below the graphs. Ex vivo experiments were performed on 6 mice per group. Values of p are given where significant differences were found.

81x220mm (300 x 300 DPI)

View Article Online
DOI: 10.1039/D0FO00946F

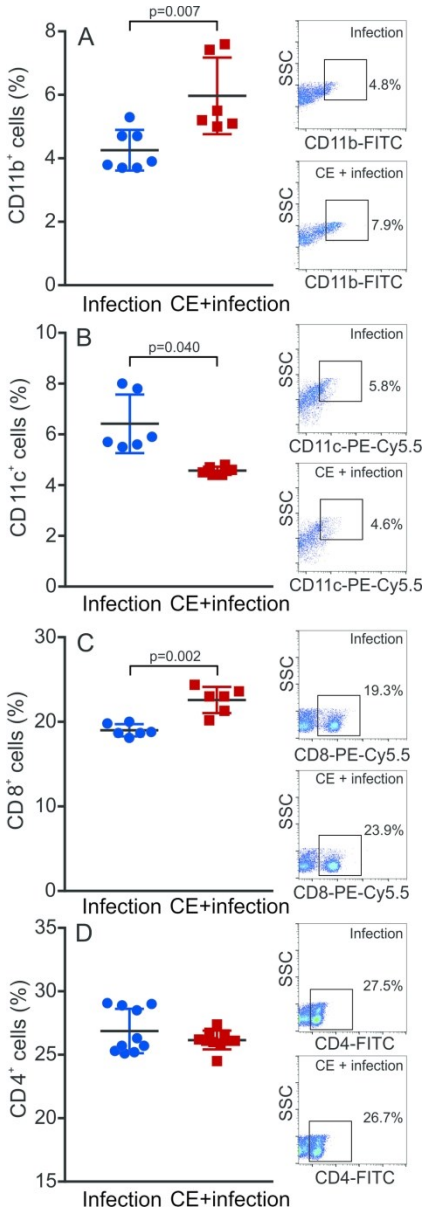


Figure 3. The effect of CE pretreatment on immune cells in the spleen. The immune cell proportion was observed in the spleen of infected and CE-treated infected mice with L. monocytogenes 48 h after infection. The following immune cells were analyzed by flow cytometry: (A) CD11b+ macrophages, (B) CD11c+ dendritic cells, (C) CD8+ cytotoxic T lymphocytes and (D) CD4+ T helper cells. Representative dot plots are shown on the right-hand side. Ex vivo experiments were performed on 6 mice per group. Values of p are given where significant differences were found.

83x240mm (300 x 300 DPI)

View Article Online
DOI: 10.1039/D0FO00946F

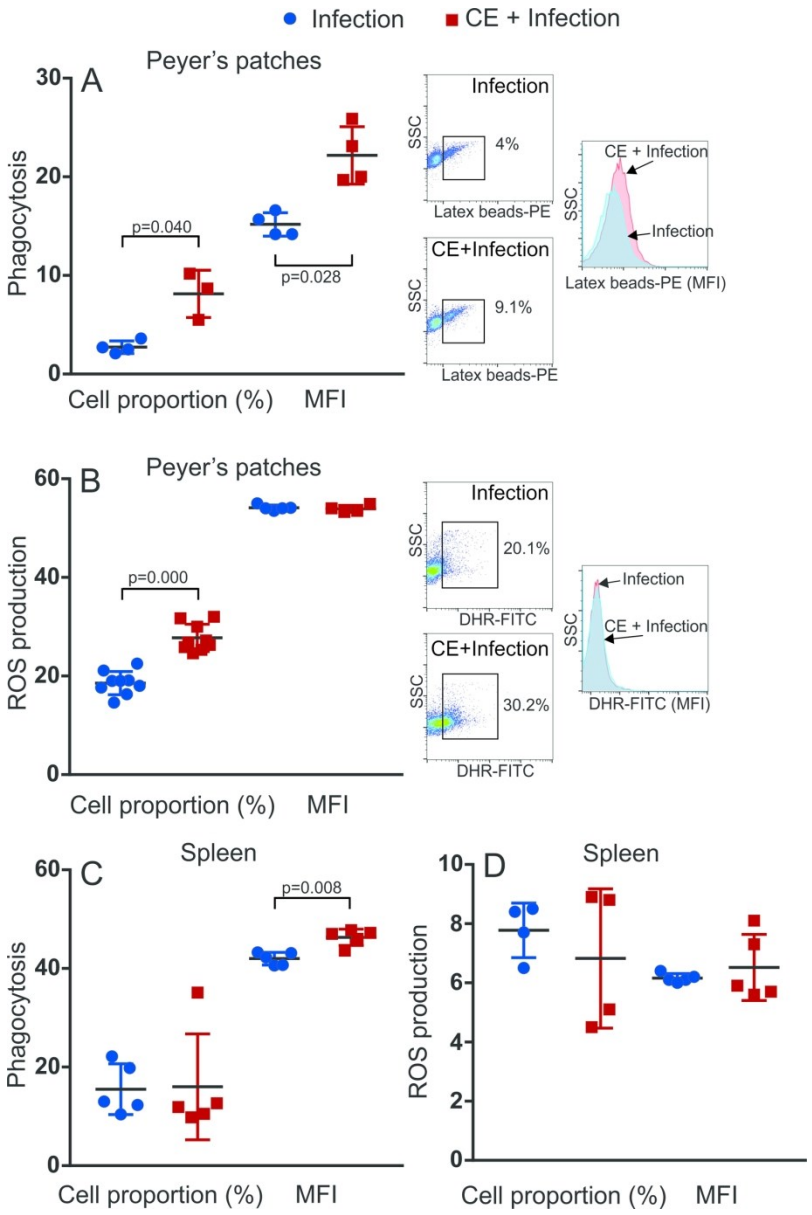


Figure 4. CE pretreatment effect on phagocytosis and ROS production. The proportion of phagocytic cells (%) and phagocytic activity, shown through the mean fluorescence intensity (MFI), were analyzed in the Peyer's patches (A) and the spleens (C) of CE or vehicle pretreated infected mice two days after L. monocytogenes infection. The proportion of ROS-producing cells and their MFI in Payer's patches (B) and spleens (D) of CE or vehicle pretreated infected mice two days after L. monocytogenes infection. Representative dot plots are shown on the right-hand side. Ex vivo experiments were performed on 6 mice per group. Values of p are given where significant differences were found.

137x206mm (300 x 300 DPI)

View Article Online
DOI: 10.1039/D0FO00946F

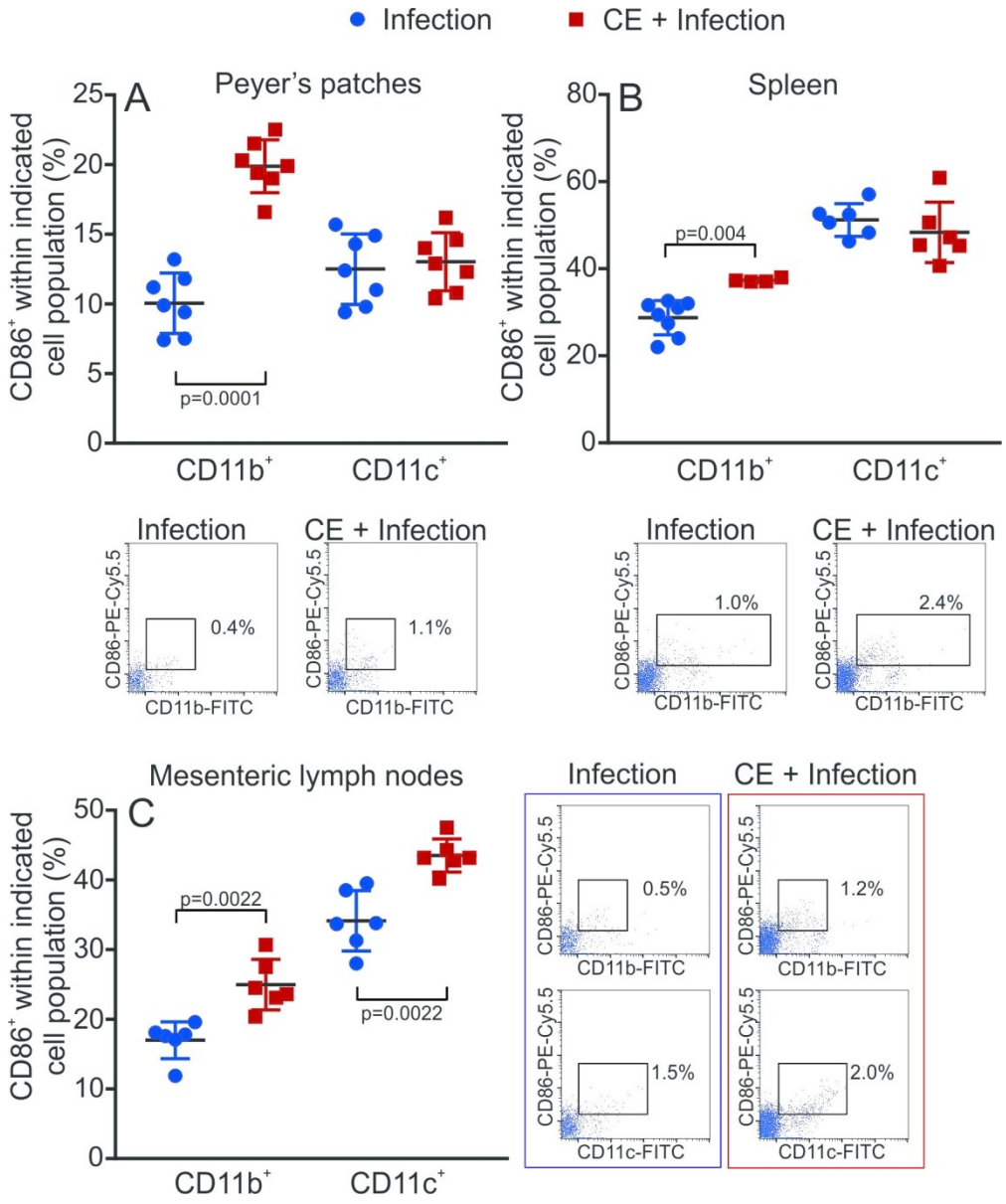


Figure 5. CE pretreatment effect on expression of myeloid cells co-stimulatory molecules. The proportion (%) of CD86 expressing macrophages (CD86+CD11b+ cells) and dendritic cells (CD86+CD11c+ cells) was determined in the PP (A), spleen (B) and MLN (C) of infected mice and CE-pretreated and infected mice 48 h after the infection. Representative dot plots are shown on the right-hand side. Ex vivo experiments were performed on 6 mice per group. Values of p are given where significant differences were found.

145x173mm (300 x 300 DPI)

View Article Online
DOI: 10.1039/D0FO00946F

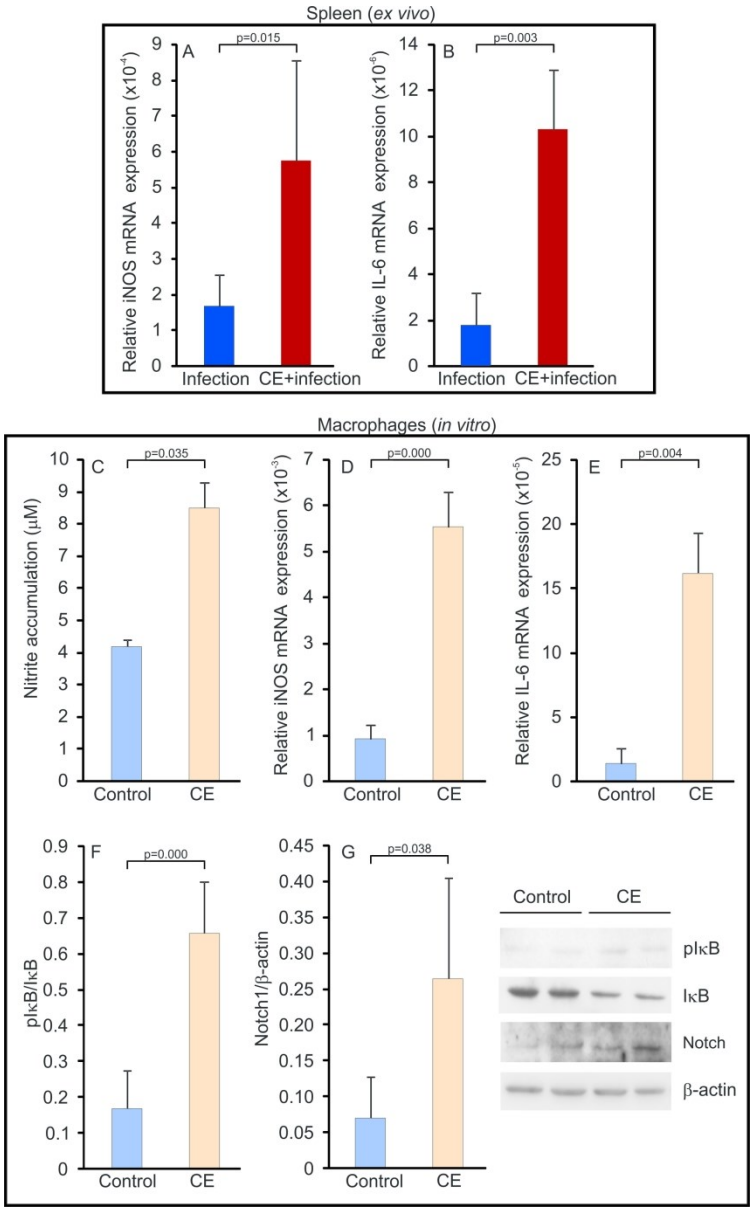


Figure 6. CE effect on expression of pro-inflammatory mediators and transcription regulators. iNOS mRNA expression (A) and IL-6 mRNA expression (B) was determined in spleen cells of infected and CE-pretreated and infected mice 48 h after the infection with L. monocytogenes. Ex vivo experiments were performed on 6 mice per group. Peritoneal macrophages were in vitro treated with CE (25 μg/ml) and 24 h after the treatment NO production (C), iNOS expression (D) and IL-6 expression (E) were determined by RT-PCR. The level of IκB phosphorylation (pIκB) was normalized to total IκB production in macrophages (F), while Notch1 production was normalized to the level of β-actin (determined by western blot) (G). Appropriate membrane images are presented. The experiment was performed in quadruplets. Values of p are given where significant differences were found.

175x282mm (300 x 300 DPI)

View Article Online
DOI: 10.1039/D0FO00946F

Table 1. The effect of CE on immune cell distribution in PP and spleen of healthy BALB/c mice.

Lymphoid tissue	Control (% cells)	CE-treated (% cells)
PP		
$CD11b^+$	1.2±0.7	1.6±0.8
$CD11c^+$	3.1±0.4	4.6±1.3*
$CD8^+$	3.7±0.7	3.9±0.9
$CD4^+$	22.6±3.6	21.5±2.4
Spleen		
$CD11b^+$	4.3±0.2	3.9±0.2
$CD11c^+$	2.5±0.3	2.5±0.1
$CD8^+$	13.6±0.6	13.5±1.0
$CD4^+$	22.8±1.7	20.0±1.3*

Healthy BALB/c mice were treated orally either with water (control) or with CE (50 mg/kg bw) for 7 days. The frequency of immune cells was determined in PP and spleen 24 h after the end of the treatment by flow cytometry.*$p < 0.05$ represents a statistically significant difference between CE-treated vs. the control group.

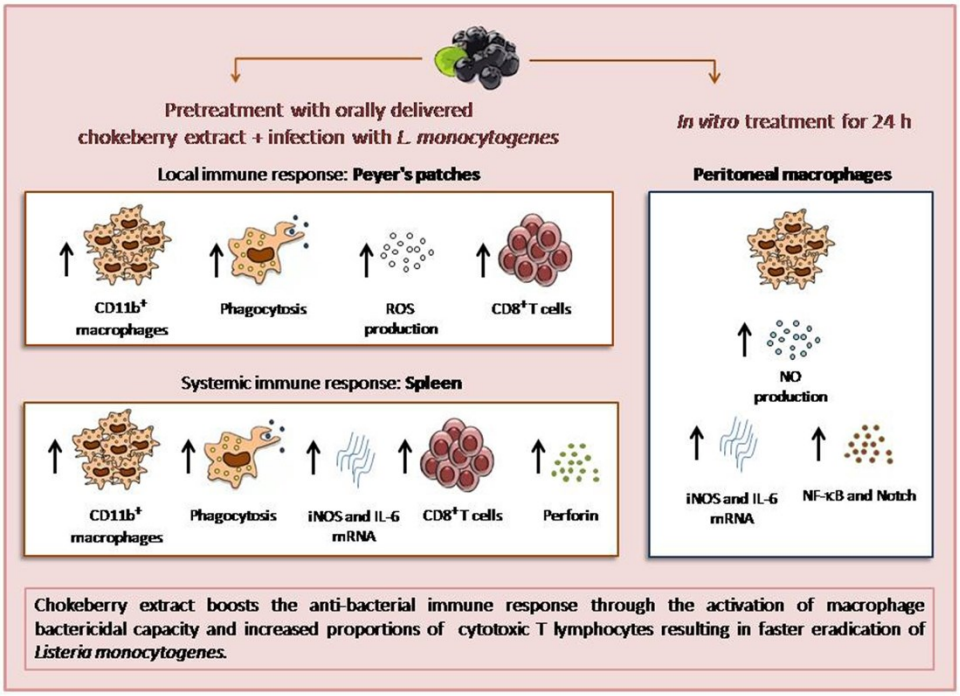
View Article Online
DOI: 10.1039/D0FO00946F

Table 2. The effect of CE pretreatment on the cells producing pro-inflammatory mediators against *L. monocytogenes* in spleen, PP and MLN.

Lymphoid tissue	Infected mice (% cells)	CE-pretreated +infected mice (% cells)
Spleen		
Granzyme^{+}	1.1±0.5	0.4±0.2
Perforin^{+}	1.1±0.2	1.9±0.6*
IFN-γ^{+}	2.5±0.9	2.2±0.9
PP		
Granzyme^{+}	0.4±0.1	0.4±0.1
Perforin^{+}	0.7±0.2	0.6±0.2
IFN-γ^{+}	0.2±0.0	0.2±0.1
MLN		
Granzyme^{+}	0.3±0.0	0.1±0.0
Perforin^{+}	0.4±0.1	0.3±0.1
IL-17^{+}	0.4±0.0	0.3±0.1

Healthy BALB/c mice were treated orally either with water or with CE (50 mg/kg bw) for 7 days. 24 h after the last CE treatment, all mice were infected with *L. monocytogenes*(5×10^{6} CFU in 100 µl ofPBS). The frequency of immune cells was determined in lymphoid tissues of infected or CE-pretreated and infected mice48 h after the infectionby flow cytometry. *$p < 0.05$ represents a statistically significant difference between CE-pretreated and infected mice vs. the infected control group.

View Article Online
DOI: 10.1039/D0FO00946F



53x40mm (1000 x 1000 DPI)