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Differential activity of human leukocyte extract on systemic immune response and cyst growth in mice with *Echinococcus multilocularis* infection after oral, subcutaneous and intraperitoneal routes of administration

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Article info

Summary

Alveolar echinococcosis (AE) caused by the larval stage of *Echinococcus multilocularis* is serious parasitic diseases associated with the host´s immunosuppression. The effects of human non-immune dialyzable leukocyte extract (DLE) on immune cells in blood and spleen and parasitic cysts weight in Balb/c mice after oral (PO), subcutaneous (SC) and intraperitoneal administration (IP) were compared. The reduction in cysts weight (p < 0.01) was recorded after PO route, whereas moderate reduction was found after SC and IP routes. The elevation of lymphoid populations in blood and spleen was found after PO administration (p < 0.01) in parallel with reduced myeloid population. Infection-elicited decline in B220+B cells was partially abolished by PO route, but DLE routes did not influence the CD3+ T cells. The proportions of CD3+CD4+Th lymphocytes were moderately upregulated, whereas CD3+CD8+Tc populations were reduced after all DLE routes (p < 0.01). PO administration increased CD11b+MHCII[high] blood monocytes, CD11b-SigleF+ cell, but not CD11b+Si-glecF+ eosinophils in the blood, stimulated after SC and IP routes. DLE induced downregulation of NO production by LPS-stimulated adherent splenocytes *ex vivo*. Con A-triggered T lymphocyte proliferation was associated with the elevated IFN-γ production and transcription factor Tbet mRNA expression. The alleviation of Th2 (IL-4) and Treg (TGF-β) cytokine production by lymphocytes *ex vivo* paralleled with downregulation of gene transcription for cytokines, GATA and FoxP3. Reduction of myeloid cells with suppressive activity was found. The SC and IP routes affected partially the cysts weights, diminished significantly gene transcription, NO levels and Th2 and Treg cytokines production. Results showed that PO route of DLE administration was the most effective in ameliorating immunosuppression via stimulation of Th1 type, reducing Th2 and Treg type of immunity and CD3+CD8+Tc lymphocytes in the blood and spleens during *E. multilocularis* infection in mice.
Keywords: administration routes; DLE; *Echinococcus multilocularis*; mice; blood white cells; spleen

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Introduction

Echinococcosis is an almost cosmopolitan zoonosis caused by adult or larval stages of tapeworms (*Cestoda*) belonging to the genus *Echinococcus* (family *Taeniidae*) (Wen *et al*., 2019). There are two species of these tapeworms circulating in Europe, *Echinococcus granulosus* and *Echinococcus multilocullaris*, the occurrence of which has also been confirmed in patients in Slovakia (Šnábel *et al*., 2000; Antolová *et al*., 2009). In humans, *E. multilocularis* causes severe disease, alveolar echinococcosis (AE), which is

characterized by progressive liver damage due to growing cysts, chronic inflammation and pathology (Vuitton, 2003; Kern, 2006). Proliferating cells of the metacestode tissue frequently detach and disseminate via lymph nodes and blood vessels, thus leading to metastasis in large parenchymatous organs. Without treatment, AE is fatal in more than 80 % of cases (Wilson *et al.*, 1995). The currently available curative treatment for AE is invasive surgical resection, but success is limited by the ability to remove a parasite cyst (He *et al.*, 2015; Hillenbrand *et al.*, 2017; Rasib *et al.*, 2021). Surgery involves administration of benzimidazole anthelmintics albendazole (ABZ) or mebendazole. Chemotherapy is the only treatment option if surgery is not possible. Although benzimidazoles are highly effective against adult non-proliferating stages of helminths, their activity against alveolar echinococcosis (AE) and cystic echinococcosis (CE) is primarily parasitostatic (Schantz *et al.*, 1983; Vuitton *et al.*, 2010). Moreover, therapy is usually complicated by generalized immunosuppression. The immune response during AE is characterized by an early Th1 response which is gradually replaced with the cellular and humoral components of Th2 and T regulatory immunity, due to specific immunomodulatory activity of molecules secreted by the parasites (Vuitton *et al.*, 2006; Vuitton & Gottstein, 2010; Mendes *et al.*, 2013; Ma *et al.*, 2014). Several approaches, aiming to improve the outcome of therapy are under investigation. For example, combining the two drugs with different mode of action, such as praziquantel and albendazole (Skuhala *et al.*, 2019; Wani *et al.*, 2021), nanotechnology using drug carriers for benzimidazoles (Abulaihaiti *et al.,* 2015; Shnawa *et al.*, 2022) or combination therapy where an immunomodulatory compound is used as the adjuvant to enhance drug´s efficacy via attenuation of immunosuppression (Gottstein & Hemphill, 2008; Siles-Lucas *et al.*, 2018). Therefore, selecting a suitable immunomodulatory agent and understanding its effects on the cellular and humoral immunological compartments is essential for more effective chemotherapy. Moreover, the specific route of administration delineates the downstream axis of the immunomodulatory effect (Zitvogel *et al.*, 2008).

Dialyzable leukocyte extract (DLE), available under the commercial name IMMODIN (ImunaPharm, Ltd. Slovakia) is the cell homogenate prepared from desintegrated blood leukocytes of healthy human donors. In our previous studies we have shown that this type of DLE administered as an adjuvant to ABZ enhanced the drug´s efficacy via stimulation of Th1 and attenuation of Th2/Treg biased immune responses in mice infected with model metacestodes of *Mesocestoides vogae* (Mačak Kubašková *et al.*, 2018). DLEs of human and animal origin typically consist of various biologically active components, which include cyclic nucleotides, nicotinamide, purine bases, histamine, ascorbates, prostaglandins, serotonin, amino acids, proteins, etc. (Doelker & Anderer, 1992; Arnaudov & Kostova, 2015). In the Zuniga-Navarrete *et al.,* (2021) study, the authors detected forty-eight unique human proteins, which were classified according to their principal function into three major groups. The largest group (14 proteins, 50 %) was

formed by proteins associated with immune response, which can be directly or indirectly involved in the regulation of immune response, including secretion of cytokines, such as IFN-γ and TNF-α and chemokines, to recruit and instruct immune cell types. In addition, the receptors for parasites or microbes were also identified, which can recognize the invaders and initiate their inhibition or elimination. The second family of proteins (9 proteins, 32.1 %) is involved in the inflammatory responses and tissue repair and the smallest group of proteins (5 proteins, 17.9 %) participates in the regulating cell growth. In general, molecules in non-immune DLEs, including IMMODIN, can normalize the specific cellular immunity of the recipient during infections, allergies, cancer and in the patients with other immunodeficiencies (Viza *et al.*, 2013). Several experimental studies showed that DLE is effective also in the treatment of parasitic infections, such as acute leishmaniasis (Delgado *et al.*, 1981) and alveolar echinococcosis (Dvorožňáková *et al.,* 2009). In addition, administration of DLE derived from crocodile lymphoid tissue reduced pathological changes in the ileum, liver, spleen and brain associated with the inflammatory process in mice infected with *Toxoplasma gondii* and also decreased the parasite load (Fuentes-Castro *et al.,* 2017). Promising antiviral activity was demonstrated against herpes simplex virus-1 (HSV-1) and herpes zoster (Viza *et al.,* 1983). The study by Fernández-Ortega *et al.* (2004) revealed the ability of DLE to inhibit *in vitro* replication of HIV in MT4 cells; to reduce TNF-α secretion and delay *in vivo* progression to AIDS in the early stage of HIV infection.

The present study was undertaken to compare the effects of DLE (IMMODIN) on systemic cellular immunity in blood and spleen after different routes of administration as well as on the reduction of *E. multilocularis* cysts growth in mice.

Material and Methods

Infection and experiment design
Infection with metacestode *E. multilocularis* is maintained under pathogen-free conditions by intraperitoneal passage through gerbils (*Meriones ungulatus*) at the Institute of Parasitology of the Slovak Academy of Sciences animal facilities. Protoscoleces for mice infection were recovered from peritoneal cysts of gerbils three months after infection. The experiment was carried out on 8-week-old male BALB/c mice and animals were housed in a temperature-controlled light cycle room with food and water ad libitum. Mice were infected intraperitoneally with 2000 protoscoleces. In our study we used human dialyzable leukocyte extract (DLE) registered as an immunomodulatory product under the commercial name IMMODIN® produced by the corporate pharmaceutical company (SevaPharma, Ltd. Prague, Czech Republic) and ImunaPharm Ltd., Šarišské Michaľany, Slovakia), batch No. 01-0419. Infected mice were divided into four groups, each comprising 5 animals: an untreated control, mice treated with DLE *per os* (PO), mice treated with DLE intraperitoneally (IP), the group treated with DLE subcutaneously (SC) and healthy mice served as intact

control. The medication (DLE administration) started after 4 weeks post-infection (p.i.) and DLE was given in 9 doses, two times per week at the dose of 0.2 ml/mouse. Blood samples, spleens and cysts from the peritoneal cavities were obtained from infected mice the next day after termination of the therapy (week 9 p.i.). Blood was obtained from the retro-orbital plexus of infected and uninfected mice under weak anesthesia into heparinized tubes and used for flow cytometry analyses and total white blood cell counts. Then mice were sacrificed by cervical dislocation and the abdominal cavity was opened. The parasitic cysts collected from the peritoneal cavities of mice were weighted.

Total white blood cells count
For total leukocyte count a blood sample dilution 1:20 in Türk solution was prepared and cells were counted using an optical microscope Olympus BX51 (Japan) in s Bürker counting chamber. The final counts for 1 ml were calculated by multiplication by 10 times.

Phagocytic assay
In vitro phagocytic activity was determined using the Phagotest kit (BD Biosciences, San Jose, CA, USA). The principle of the test is that whole blood is incubated with opsonized (by complement and immunoglobulin) *E. coli* that are labelled by fluorescein (fluorescein isothiocyanate, FITC). Bacteria are ingested by phagocytes generating a green fluorescence signal that can be quantified by flow cytometry. Samples were analyzed by flow cytometry using BD FACS Canto (Becton Dickinson Biosciences, USA) and data were analyzed using FACS Diva software. The results were expressed as the percentage of labelled cells from the total population of phagocytes (neutrophils and monocytes) and the mean fluorescence intensity (MFI).

Phenotypic analysis of white blood cells by flow cytometry
We analyzed the proportions of lymphocyte subpopulations, myeloid cells subpopulations, including eosinophils in the peripheral blood. Fifty microliters of heparinized blood were plated into a round bottom tube and stained with monoclonal antibodies for 30 minutes at room temperature in dark. Then cells were fixed with BD FACS Lysing Solution (BD Biosciences, San Jose, CA, USA), washed with PBS and resuspended in 100 µl of FACS buffer. Samples of blood cells were stained with monoclonal anti-mouse antibodies CD3-FITC (clone 17A2) and CD45R (B220, clone RA3-6B2) which were analyzed from lymphocyte population determined by forward and side scatter. Other samples were stained with antibodies to CD3-PerCTeFluor710 (clone C 17A2), CD4-FITC, (clone GK1.5) and CD8a-PE (clone 53–6.7). Myeloid sub-populations were detected using antibodies to CD45.2 PE-Cyanine 7 (clone 104), CD11b-FITC (clone M1/70), MHCII-PE Cyanine 7 (clone M5/114.15.2) and CD170 SiglecF-PerCPeFluor 170 (clone 1RNM44N). The analyses were performed on BD FACS Canto (Becton Dickinson Biosciences) flow cytometer.

Isolation of lymphoid and myeloid populations from spleens
Suspensions of splenocytes were prepared by the method described previously (Mačak Kubašková *et al.*, 2021). Cells were counted and samples were used to determinate of the proportions of lymphoid and myeloid populations by flow cytometry utilizing forward and side scatter analysis. Then splenocyte suspension from each mouse was plated onto plastic dish and myeloid cells were allowed to adhere for 4h at 37 °C and 5 % CO_2 atmosphere. Non-adherent cells were collected into Falcon tubes, washed and counted in Trypan blue solution to assess the viability and subsequently used for the proliferation assay and production of cytokines *ex vivo*. The remaining samples were immersed in RNAlater solution (Invitrogen, Carlsbad, CA, USA) and stored at -80 °C for RNA isolation. The microscopic analysis of adherent splenocytes confirmed the presence of myeloid cells, mostly dendritic cells with round shape and macrophages having spindle like shape. Adherent cells were then washed with PBS and incubated with warm Accutase solution (Sigma-Aldrich St. Louis, USA) for 15 – 20 min at 37 °C and mixed several times with a pipette. Then, detached cells were collected, washed three times with PBS, resuspended in RPMI medium (Biochrom, Berlin, Germany) containing 2 mM of stable glutamine. Medium was supplemented with 10 % heat-inactivated bovine fetal serum (Biochrom, Berlin, Germany), 100 U/ml of penicillin, 100 µg/ml of streptomycin, 10 µg/ml of gentamicin, and 2.5 µg/ml of amphotericin B and is further termed as complete medium (CM) (all from Sigma-Aldrich, St. Louis, USA). Viability was checked using Trypan blue exclusion assay and live cells were diluted to the concentration of 1×10^6 cells/ml and were utilized for NO production *ex vivo*.

Proliferation of splenocytes ex vivo
The suspensions of lymphoid spleen cells from naive and infected/treated mice were diluted to the concentration of 2×10^6 cells/ml in CM and plated (200 µl/well) into 96-well plates (Corning. Inc., USA) in triplicates for unstimulated and Con A (3 µg/ml) stimulated T lymphocytes and LPS (1 µg/ml) stimulated B lymphocytes. After incubation for 52 h at 37 °C and 5 % CO_2, the BrdU solution was added to the cell suspensions at 5 µM final concentrations (BrdU ELISA cell proliferation kit, Roche Diagnostics GmbH, Mannheim, Germany). Cells were further incubated for 18 h, then were centrifuged at 360 *g* for 10 min. After removing the supernatants, plates were dried and used for colorimetric determination of BrdU in cells using Kit mentioned above. Stimulation of cell proliferation termed the proliferation index (PI) was determined as the ratio of absorbance of stimulated versus unstimulated cells for each cell sample for each mouse/group.

Cytokine concentrations in the supernatants of Con A stimulated splenocytes
Samples of the lymphoid population from spleens diluted to 2×10^6 cells/ml in CM were plated and stimulated by Con A as is described for BrdU proliferation assay. After 70 hrs of incubation plates were

Table 1. List of oligonucleotides and their sequences.

Gene	Orientation	Sequence
GAPDH	forward	5'-AGGTCGGTGTGAACGGATTTG-3'
	reverse	5'-TGTAGACCATGTAGTTGAGGTCA-3'
IFN-γ	forward	5'-TCAAGTGGCATAGATGTGGAAGAA-3'
	reverse	5'- TGGCTCTGCAGGATTTTCATG-3'
TGF-β	forward	5'-TGACGTCACTGGAGTTGTACGG-3'
	reverse	5'-GGTTCATGTCATGGATGGTGC-3'
IL-4	forward	5'-TCAACCCCCAGCTAGTTGTC-3'
	reverse	5'-TTCAAGCATGGAGTTTTCCC-3'
FoxP3	forward	5'-AATAGTTCCTTCCCAGAG-3'
	reverse	5'-GATTTCATTGAGTGTCCT-3'
T-bet	forward	5'-GCCAGGGAACCGCTTATATG-3'
	reverse	5'-TGGAGAGACTGCAGGACGAT-3'
GATA3	forward	5'-GAAGGCATCCAGACCCGAAAC-3'
	reverse	5'-ACCCATGGCGGTGACCATGC-3'

centrifuged as described previously and supernatants were collected and stored at -20 °C until used for determination of cytokine production. The concentration of cytokines interferon gamma (IFN-γ), interleukin-4 (IL-4) and transforming growth factor beta (TGF-β) were determined by ELISA kits (eBioscience, Thermo Fisher Scientific, USA). Results were expressed in pg/ml.

Real-time quantitative analysis
Total RNA from lymphoid spleen cells was extracted with Ribo-Zol™ RNA Extraction Reagent (Invitrogen, Carlsbad, CA, USA) after homogenization in Tissue lyzer (Qiagen, USA) for 30 sec. The concentration and purity of RNA were determined using AstraGene spectrophotometer (Harston, Cambridge, UK). After that,

3 μg of total RNA/sample was reverse-transcribed to produce cDNA utilizing RevertAid H Minus M-MuLV Reverse Transcriptase, 100 pmol of oligo dT primers and the reaction included 20 U of RNase inhibitor and 1mM dNTPmix (final) (all from ThermoScientific, Burlington, ON, Canada). In the present study the mRNA levels of IFN-γ, TGF-β, IL-4 and corresponding cytokine transcription factors T bet, GATA3 and FoxP3 were determined. GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as the house-keeping gene. The list of primers is summarised in Table 1. In the qPCR reactions cDNAs served as templates using iQ SYBR green master mix (BioRad, Hercules, CA, USA) and oligonucleotides pairs for individual genes. The reactions were run in duplicates on CFX96 thermocycler (BioRad, Hercules, CA, USA).
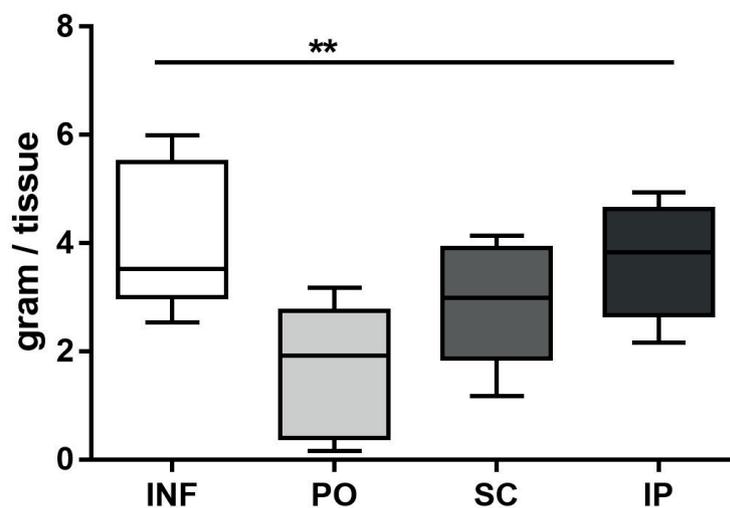

Fig. 1. The effects of DLE administration by PO, SC and IP routes on the parasite cysts weights in the peritoneal cavities of infected mice.
The numbers represent means ± SD of (n = 5 mice/ group). Significantly different values between infected and treated mice are indicated by connecting line, **p < 0.01.
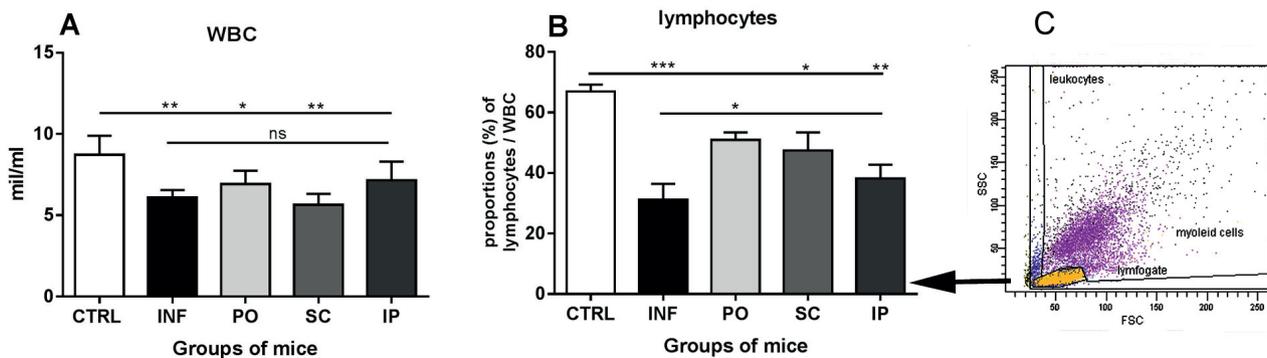
Fig. 2 A-B. The effects of DLE administration by PO, SC and IP routes on: **A** – total numbers of leukocytes in the peripheral blood; **B** – proportions of the lymphoid populations within WBC. **C** – dot plot showing forward and side analysis. Data are expressed as the means ± SD. Significantly different values were calculated between **a** – control and infected and infected treated mice; **b** – infected and treated mice, and are indicated by connecting lines,* p < 0.05, **p < 0.01, *** p < 0.001, ns – nonsignificant.

Melting-curve analysis was used to confirm amplification of the single product. Ct values were normalized to Ct values for the housekeeping gene and relative gene expression was calculated by the $2^{-\Delta\Delta Ct}$ method using data for healthy mice as calibration value.

NO production

To measure the production of nitric oxide (NO) by adherent myeloid cells from spleens, cell suspensions (1×10^6 cells/ml) were cultured in 24-well plates in duplicates (Corning) in CM in the presence or absence of lipopolysaccharide (LPS) at the concentration of 1 µg/ml. The plates were incubated for 72 h at 37 °C and 5 % CO_2. Immediately after assay termination, the concentration of NO in the culture supernatants was determined as nitrite (NO_2-) using Griess reagent. Briefly, 50 µl of a solution containing 1 % sulphanilamide and 5 % H_3PO_4 was incubated with 50 µl of supernatants in a 96-well plate for 10 min at room temperature. Subsequently, 50 µl of a second solution (0.1 % N-(1-naphthyl) ethylenediamine dihydrochloride) was added to the mixture and the absorbance was measured at 550 nm using ELISA reader. Nitrite concentration was determined from the standard curve of 0.1 M $NaNO_2$ dilutions run in parallel with samples. After that, cells were lysed with lysis solution (0.1 % NaOH, 0.1 % Triton-X100) overnight at 4 °C and used to determine cell proteins with Bradford protein reagent (BioRad, Hercules, CA, USA). Finally amount of NO was calculated for 1 mg of cell proteins.

Statistical analyses

All data were calculated as mean ± standard deviation (SD). Statistical analyses were performed using one-way ANOVA followed by Tukey's post-hoc test. Data were evaluated by GraphPad Prism (version 7) (GraphPad Software, Inc., San Diego, CA, USA) and the differences were considered significant at p < 0.05.

Results

Effect of treatment on reduction of parasites mass in the peritoneal cavities of mice

The effect of DLE treatment administered intraperitoneally (IP), subcutaneous (SC) and per os (PO) was compared with untreated mice and the mean weights of parasite cysts are shown in Figure 1. The significantly lower weight of parasite cysts was found in PO - treated group compared to the untreated control (p < 0.01). The slight nonsignificant reduction was seen after SC and IP application of DLE.

Table 2. The total numbers of white blood cell subpopulations in 1 ml of peripheral blood.

Groups of mice/ cell subpopulations	Routes of DLE administration				
	CTRL	INF	PO	SC	IP
White blood cells	8.72 ± 1.17	6.10 ± 0.46	7.02 ± 0.82	5.65 ± 0.67	7.06 ± 1.05
Lymphocytes/WBC	5.86 ± 0.94	1.79± 0.55	3.54±0.67	2.61±0.46	2.66 ± 0.49
CD3+/lymphocytes	3.41 ± 0.62	1.41 ± 0.43	2.42 ± 0.51	2.02 ± 0.38	2.04 ± 0.23
B220+/lymphocytes	2.33 ± 0.29	0.34 ± 0.13	1.03 ± 0.24	0.56 ± 0.09	0.52 ± 0.11
CD11b+/WBC	3.71 ± 0.71	4.21 ± 0.55	4.55 ± 0.85	3.95 ± 0.43	5.23 ± 0.99

Legend: Lymphocytes and CD11b+ cell subpopulations were calculated from total white blood cell numbers based on their proportions determined by flow cytometry. Counts of T lymphocytes (CD3+) and B lymphocytes (B220+) were calculated from subpopulation of lymphocytes.

The effects of DLE on white blood cells and the proportions of lymphocytes in the peripheral blood

The total numbers/ml of leukocytes in INF (p < 0.01), PO (p < 0.05) and SC-treated (p < 0.01) groups were lower than in the healthy control group (Fig. 2A). The IP administration of DLE had no significant effect on total white blood cell counts in infected mice. We then analyzed changes in the proportions of subpopulations of leukocytes by flow cytometry and total counts were then calculated for white blood cells (Table 2). The infection resulted in the reduction of total lymphocytes in blood (Fig. 2B) and adminis-

tration of DLE partially restored their proportions, the most after PO application (p < 0.05). The representative dot plot is shown in Fig. 2C. In comparison with healthy mice the proportions of CD3+ T lymphocytes were elevated in infected untreated mice (79.1 ± 2.4 %) (p < 0.001) and treated mice (Fig. 3A). PO route of DLE administration decreased the proportions of CD3+T cells (p < 0.01) when compared to infected group. The opposite trend was observed in the proportions of B220+ B lymphocytes for all infected groups (Fig. 3B), which declined significantly (p < 0.001) compared to healthy mice (40.3 ± 3.6 %). PO given treatment resulted
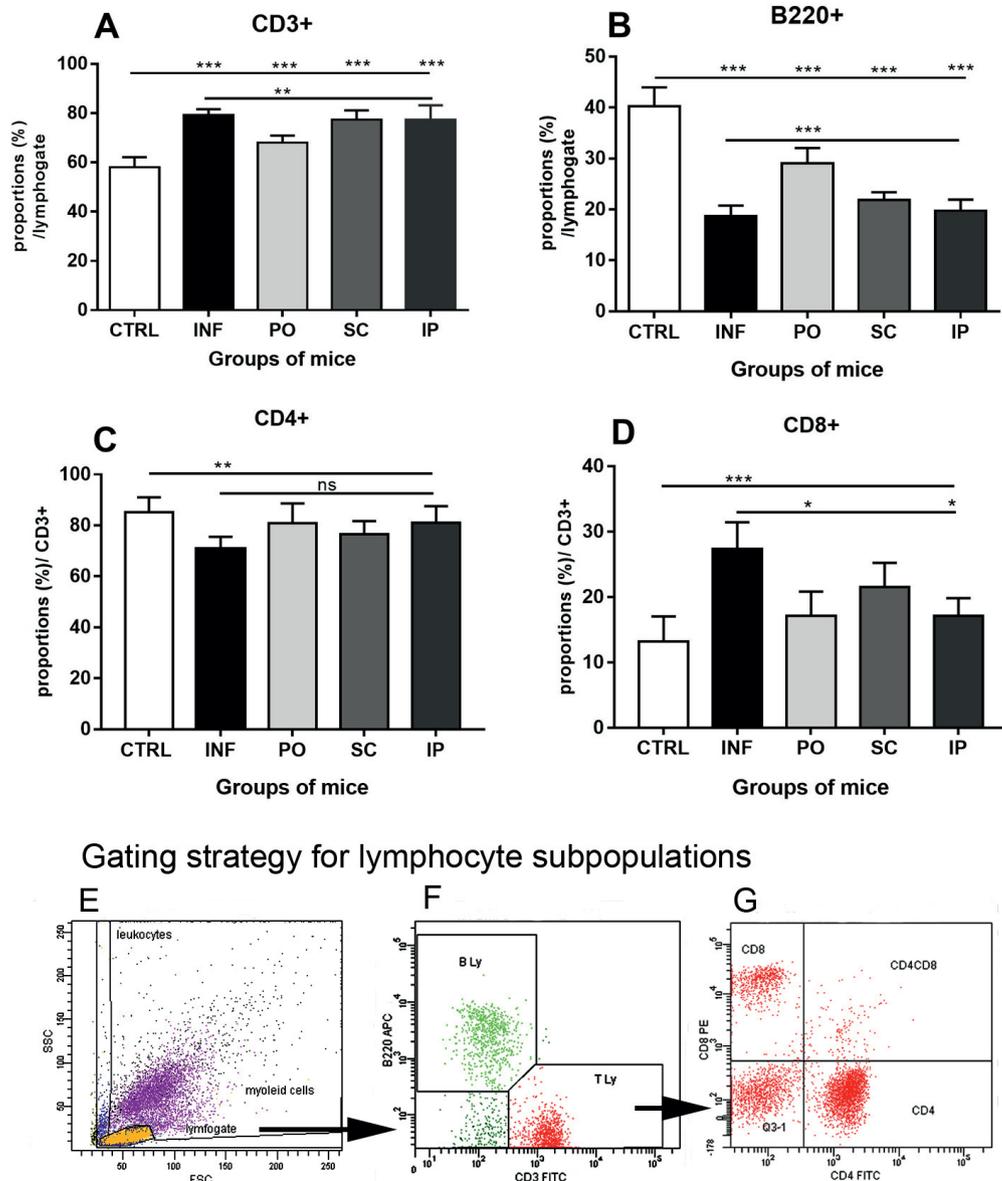

Fig. 3 A-D. The proportions of lymphoid cells subpopulations in the peripheral blood of control, infected and infected and treated mice were analyzed by flow cytometry. **A** – lymphoid populations were gated on CD3+T lymphocytes and **B** – B220+ B lymphocytes; **C** – CD4+T helper and **D** – CD8+ cytotoxic lymphocytes were gated within CD3+ cells. Data are expressed as the means ± SD. Significantly different values were calculated between **a** – control and infected and infected+treated mice; **b** – infected and treated mice, and are indicated by connecting lines,* p < 0.05, **p < 0.01, *** p < 0.001, ns-nonsignificant.

in the elevation (29.1 ± 2.9 %) (p < 0.001) compared to infected untreated group (18.8 ± 2.3 %). Examining the DLE effect on lymphocyte subpopulations, CD3+T lymphocytes were gated on CD4+T helper cells (Fig. 3C) and CD8+ cytotoxic T cells (Fig. 3D). Compared to healthy mice proportions of CD4+ lymphocytes were lower in infected untreated mice (70.8 ± 4.7 %) (p < 0.01) and DLE administration did not influence their proportions. The proportions of CD8+T lymphocytes were significantly higher in infected group (p < 0.001) compared to the healthy control (Fig. 3D) and were diminished after PO and IP routes (p < 0.05) as compared to the infected untreated group. The gating strategy (representative dot plots) for analysis of lymphocytes subpopulations is shown in Fig. 3 E-G.

Phagocytic activity
The DLE effect on the innate immune cells was studied by means of the phagocytosis of blood neutrophils and monocytes (Fig. 4A). Phagocytic activity (PA) was measured as the average number of fluorescein-labelled *E. coli* engulfed per phagocyte. In comparison with infected untreated mice DLE did not significantly affect PA. Mean fluorescence intensity (MFI) was suppressed in infected untreated group (p < 0.05) and only IP administration route elevated this phagocyte function (p < 0.05).

The effect of therapy on the proportions of blood myeloid populations
Myeloid-derived populations of white blood cells were evaluated using antibodies to CD11b, which is part of the surface complement receptor detected on monocytes, macrophages, NK cells, granulocytes, mostly neutrophils and on some populations of eosinophils during inflammation (Stevens *et al.*, 2007; Lee *et al.*, 2012). Eosinophils were identified with antibodies to SiglecF, a specific marker for all mouse eosinophils (Lee *et al.*, 2012). In comparison with healthy control, the proportions of CD45.2+CD11b+ cells significantly increased in all infected groups (p < 0.001 and p < 0.01 for PO therapy) (Fig. 5A). The DLE administration did not modulate their proportions substantially. We next analyzed the proportions of antigen presenting cells using antibodies to the MHCII surface receptor (Fig. 5B). Consistent with well described suppressive effect of *E. multilocularis* infection on the immune system, the proportions of CD11b+MHCII^{high} cells significantly lowered (5.52 ± 0.78 %) in comparison with healthy mice (p < 0.05). Only PO application alleviated this suppressive effect (p < 0.01) and representative histograms are in Fig. 4 C and D. While analyzing SiglecF positive cells, we discovered two sub-populations, the more abundant population of CD11b-SiglecF+ cells (Fig. 5E) and the smaller subpopulation of CD11b+SiglecF+ cells (Fig. 5F). The gating strategy and representative dot plots are shown in Fig. 5G-I. Infection significantly downregulated the proportions of CD11b-SiglecF+ cells (18.2 ± 2.35 %) in comparison with healthy mice (51.0 ± 1.92 %) (p < 0.001) and PO therapy partially abolished this suppression (31.5 ± 2.68 %). By contrast, the different behaviour of CD11b+SiglecF+ eosinophils in blood was observed. In comparison with healthy mice the infection-elicited decrease in eosinophils (p < 0.05) was reduced by SC and IP administration of DLE (p < 0.05), thus suggesting a different role of both populations in immune defence against *E. multilocularis* metacestodes.

Effect of DLE administration on the proportions of splenocytes
The proportions of lymphoid and myeloid sub-populations in the suspensions of splenocytes was evaluated by flow cytometry by means of FSC and SSC analysis. Similarly, as in the case of the lymphoid population in blood, we observed the significant decrease of lymphoid cells (33.17 ± 6.5 %) in infected mice compared to the
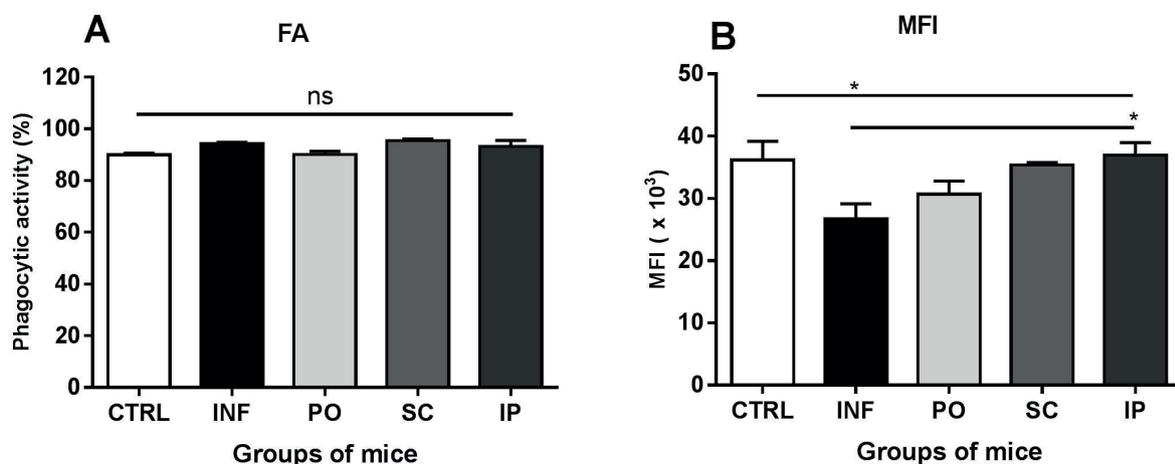

Fig. 4 A-B. The effects of DLE administration on the **A** – phagocytic activity and **B** – MFI of monocytes and neutrophils in the peripheral blood of mice. Significantly different values were calculated between **a** – control and infected and infected+ treated mice; **b** – infected and treated mice, and are indicated by connecting lines.* p < 0.05, ns – nonsignificant.
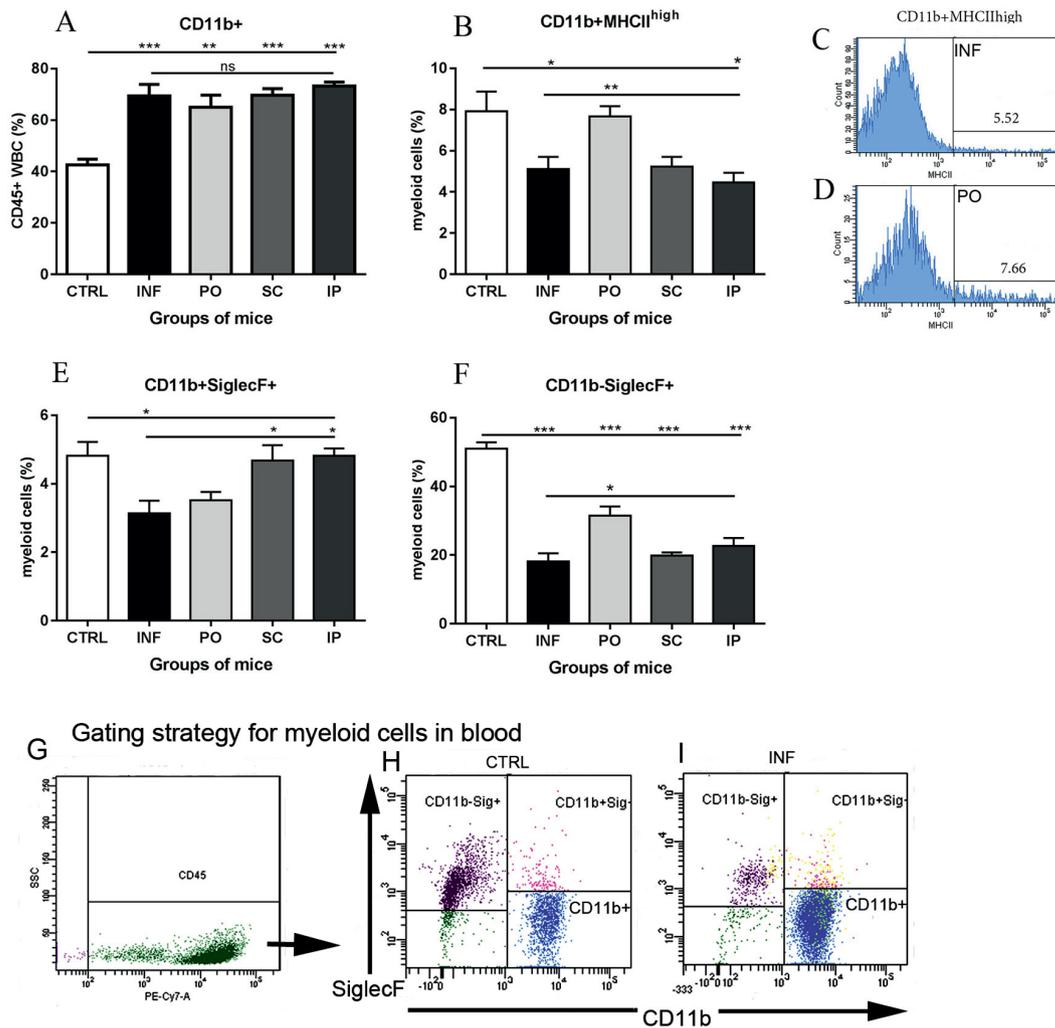
Fig. 5 A-F. Phenotypic analysis of myeloid-derived populations in peripheral blood of mice was performed by flow cytometry. Cells in control, infected and infected and treated mice were gated on **A** – CD11b+ cells and **B** – CD11b+MHCIIhigh monocytes, **C-D** – the representative histograms showing expression of MHCII in infected and PO-treated group. **E** – the proportions of CD11b-SiglecF+ eosinophil-like cells, **F** – the proportions of CD11b+SiglecF+ eosinophils. Significantly different values were calculated between a/control and infected and infected+treated mice; b/ infected and treated mice, and are indicated by connecting lines.
* $p < 0.05$, **$p < 0.01$, *** $p < 0.001$; ns-nonsignificant.

healthy group ($p < 0.001$). Of all three administration routes, only PO given DLE attenuated significantly ($p < 0.01$) suppressive parasitic effect on lymphocyte proportions (46.01 ± 4.3 %) (Fig. 6A). However, the behaviour of the myeloid population following therapy showed the opposite trend (Fig. 6B) and the proportions found in the infected untreated group (68.12 ± 7.0 %) declined after PO therapy (53.4 ± 4.4 %) ($p < 0.05$).

Proliferation of T and B lymphocytes
T and B lymphocytes were separated from myeloid cells after their adherence on the plastic surface and used to examine proliferation ability *ex vivo*. Proliferation of T lymphocytes (both CD4 and CD8) (Dwyer & Johnson, 1981) was triggered by Con A and B lymphocytes by LPS. The proliferation indexes for T cells and B cells

are shown in Fig. 7A and B, respectively. Compared with control group, proliferation index of T cells declined due to infection and PO route partially abolished this suppressive effect ($p < 0.05$). In comparison with infected mice B cells proliferation was stimulated in PO treated ($p < 0.05$) and IP treated groups ($p < 0.001$) in comparison with infected mice.

Cytokine concentrations in supernatants of Con A stimulated lymphocytes ex vivo
Production of cytokines by T cells from all groups was examined in the supernatants of Con A stimulated lymphocytes after 70 h of incubation *ex vivo*. We analyzed the impact of treatment on the concentrations of IFN-γ, IL-4 and TGF-β by ELISA tests (Fig. 8). In comparison with healthy mice elevated levels of IFN-γ were de-
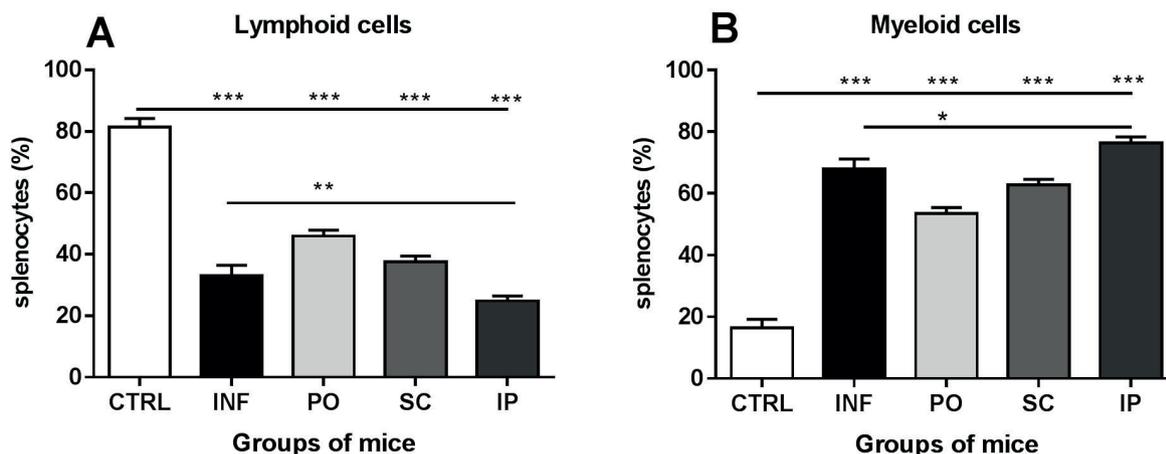
Fig. 6. The proportions of: **A** – lymphoid and **B** – myeloid cell populations in the spleens of control, infected and infected and treated mice were analysed by flow cytometry. Data are expressed as means ± SD. Significantly different values were calculated between **a** – control and infected and infected+treated mice; **b** – infected and treated mice, and are indicated by connecting lines.* p < 0.05, **p < 0.01, *** p < 0.001.

tected in infected group, PO and SC-treated groups. In PO group concentrations were significantly higher in comparison with infected (p < 0.05) group (Fig. 8A). The lowest levels were detected after IP administration (p < 0.001). Levels of IL-4 were elevated (p < 0.001) in infected untreated group and all three routes of DLE administration resulted in significant cytokine´s decline. The largest drop was observed in PO and SC-treated groups (p < 0.001) (Fig.8B). Similarly, the highest values of TGF-β were detected in the T cells supernatants from infected group (p < 0.001) comparing to healthy mice. DLE administration reduced concentration of this suppressive cytokine, the most in PO-treated group (p < 0.01) (Fig. 8C). These data might indicate that parasites and their E/S products increased populations of T cells producing Th2/Treg cytokines. It seems that proteins and other molecules present in DLE counteracted cellular systemic immunity by different mechanisms

following different routes of DLE administration.

mRNA expression in spleen´s lymphocytes

We next studied the effect of infection and DLE on IFN-γ, IL-4 and TGF-β cytokines and transcription factors T bet (Th1), GATA (Th2) and FoxP3 (Treg) on mRNA levels, which are essential for T helper cells differentiation. Data summarizing relative gene expression are shown in Fig. 9A-F. The activity of the gene encoding IFN-γ was very low in the untreated group, whereas PO (p < 0.001) and SC routes of therapy (p < 0.01) stimulated this gene expression. The similar pattern of gene expression was found for T bet. On the contrary, mRNAs encoding Th2 type cytokine IL-4 and transcription factor GATA were upregulated in infected group compared to healthy mice. Suppression of gene expression for IL-4 in splenic unstimulated lymphocytes was found after all three routes of DLE administration (p < 0.001), the most after PO therapy. How-
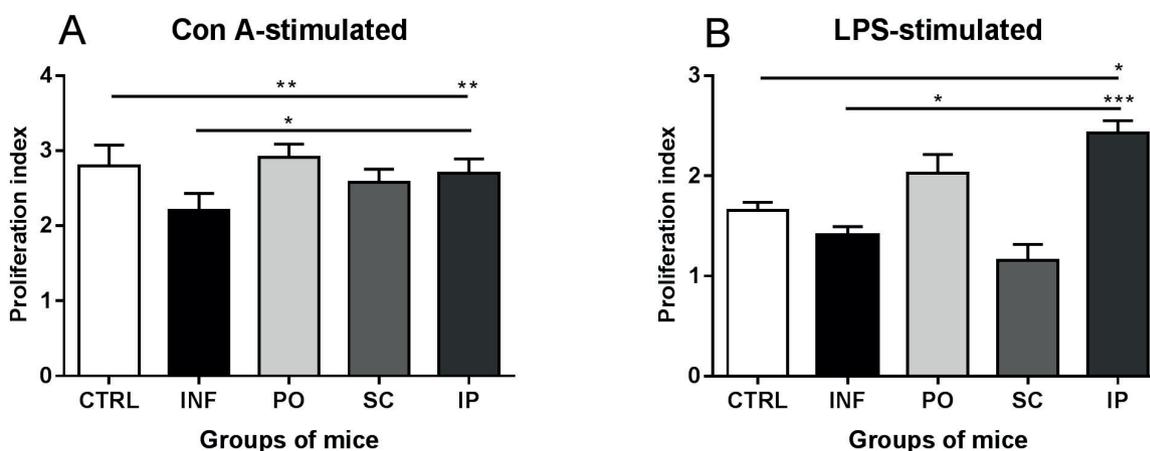

Fig. 7 A-B. The proliferation index of: **A** – Con A stimulated T lymphocytes and **B** – LPS stimulated B lymphocytes, isolated from the spleens of control, infected and infected and treated mice. Data are expressed means ± SD of triplicates/mouse/group after 70 hrs of incubation *ex vivo*. Cell proliferation was evaluated using BrdU Cell Proliferation ELISA Kit. Significantly different values were calculated between **a** – control and infected and infected+treated mice; **b** – infected and treated mice, and are indicated by connecting lines.* p < 0.05, **p < 0.01, *** p < 0.001.

ever, GATA expression was down-regulated only after PO and SC administration. IP administration resulted in the GATA elevation (p < 0.05) in comparison with infected mice. Transcripts of TGF-β cytokine involved in Treg immunity as well as the corresponding FoxP3 factor were strongly elevated in infected group (p < 0.001). Significant downregulation of both genes was found after PO and SC administration of DLE. However, IP therapy was unable to abolish the infection - induced elevated transcription of TGF-β and FoxP3 in spleen lymphocytes.

NO production by LPS stimulated myeloid spleen cell lineage ex vivo
Next, we examined the NO production by the enzyme iNOS, which utilizes L-arginine as a substrate and competes for the same substrate with arginase. Adherent splenocytes were incubated for 72 h *ex vivo* and NO was determined in LPS-stimulated cells (Fig. 10). NO levels were significantly elevated in infected untreated mice in comparison with healthy mice (p < 0.001). DLE administration significantly suppressed NO production by LPS-stimulated myeloid cell lineage (p < 0.001), more after SC and IP applications.

Discussion

The infection of the intermediate hosts by the metacestodes stage of *Echinococcus* species, especially *E. multilocularis*, appears to be an excellent model to unveil the key mechanisms of the host–parasite interplay which lead to the anergy in AE (Vuitton & Gottstein, 2010). This information would provide a scientific basis for the development of novel and more effective immunotherapeutic approaches employing biologicals, vaccines or natural compounds.
In the present study we investigated immunomodulatory effects of human DLE (IMMODIN) consisting of biologically active mixture of small protein and non-protein molecules after three routes of administration to the Balb/c strain of mice with *E. multilocularis* infection. In clinical and experimental studies DLE preparations are

given via different routes, which can influence the effects on immunity and pathology. Non-immune human DLE used in the present study contains a large number of small molecules of protein and non-protein character up to 10 kDa having various biological and immunomodulatory functions. During absorption the molecule´s size and chemical properties seem to have an important role. Orally administered drugs after crossing the intestinal epithelia pass directly to the hepatic portal vein, which carries them to the liver and afterwards to the systemic circulation (DeSesso & Jacobson, 2001). The SC delivery is a commonly utilised administration route and size of molecules is a crucial determinant of the relative roles of the blood and lymphatic absorptive pathways. It should be acknowledged that increasing the size is likely to eventually retard movement through the interstitial space and thus restrict lymphatic drainage (Mclennan *et al.,* 2005). Experimental studies indicate that after intraperitoneal administration small to medium size molecules (MW < 5000 Da) and fluids are predominantly absorbed by visceral peritoneum, where diffusion through the splenic, inferior and superior mesenteric capillaries drain those molecules into the portal vein (Wiliams & White, 1986). On the other hand, large molecules (MW > 5000 Da), proteins, blood and immune cells are taken up by the lymphatic system (Surbone & Myers, 1986).
In present study when compared to untreated group, a significant reduction of peritoneal cyst weight was found after per os application of nine DLE doses. Subcutaneous and intraperitoneal routes reduced parasite cyst growth only moderately. In the patients with echinococcosis, therapy outcome is routinely evaluated by white blood cell proportions, cytokines, chemokines and other markers. To explain the observed outcome of DLE administration after different routes we evaluated the effect on white blood cells and splenocytes as these compartments interact closely with each other (Bronte *et al.,* 2013). Lymphoid populations in the blood and spleen decreased significantly in infected untreated mice compared to healthy mice. The significant elevation of the cell proportions was observed only after oral route. In the patients with cellular deficiencies associated with different pathologies, DLE (IM-
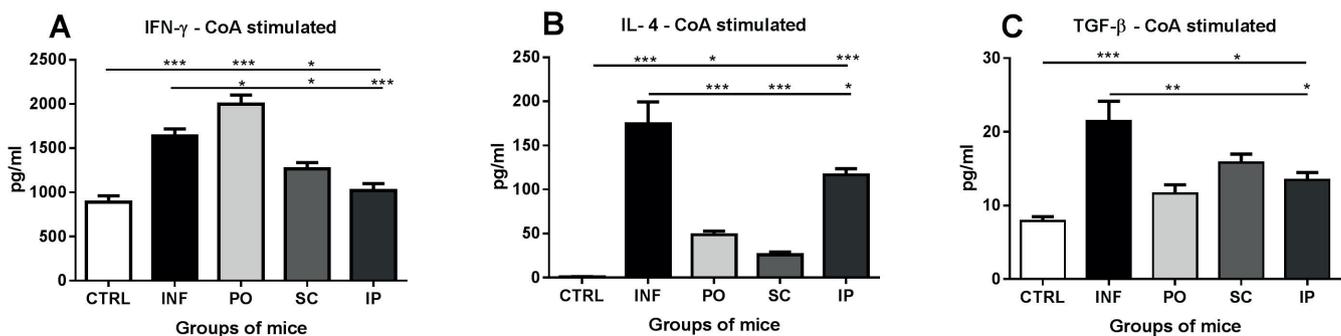

Fig. 8 A-C. The production of cytokines by Con A stimulated T lymphocytes isolated from the spleens of control, infected and infected and treated mice *ex vivo*. Lymphocytes were cultured for 70 hrs and cytokines were determined in the supernatants by ELISA tests. Data are expressed means ± SD of duplicates/mouse/group in pg/ml. Significantly different values were calculated between **a** – control and infected and infected+treated mice; **b** – infected and treated mice, and are indicated by connecting lines.* p < 0.05, **p < 0.01, *** p < 0.001.
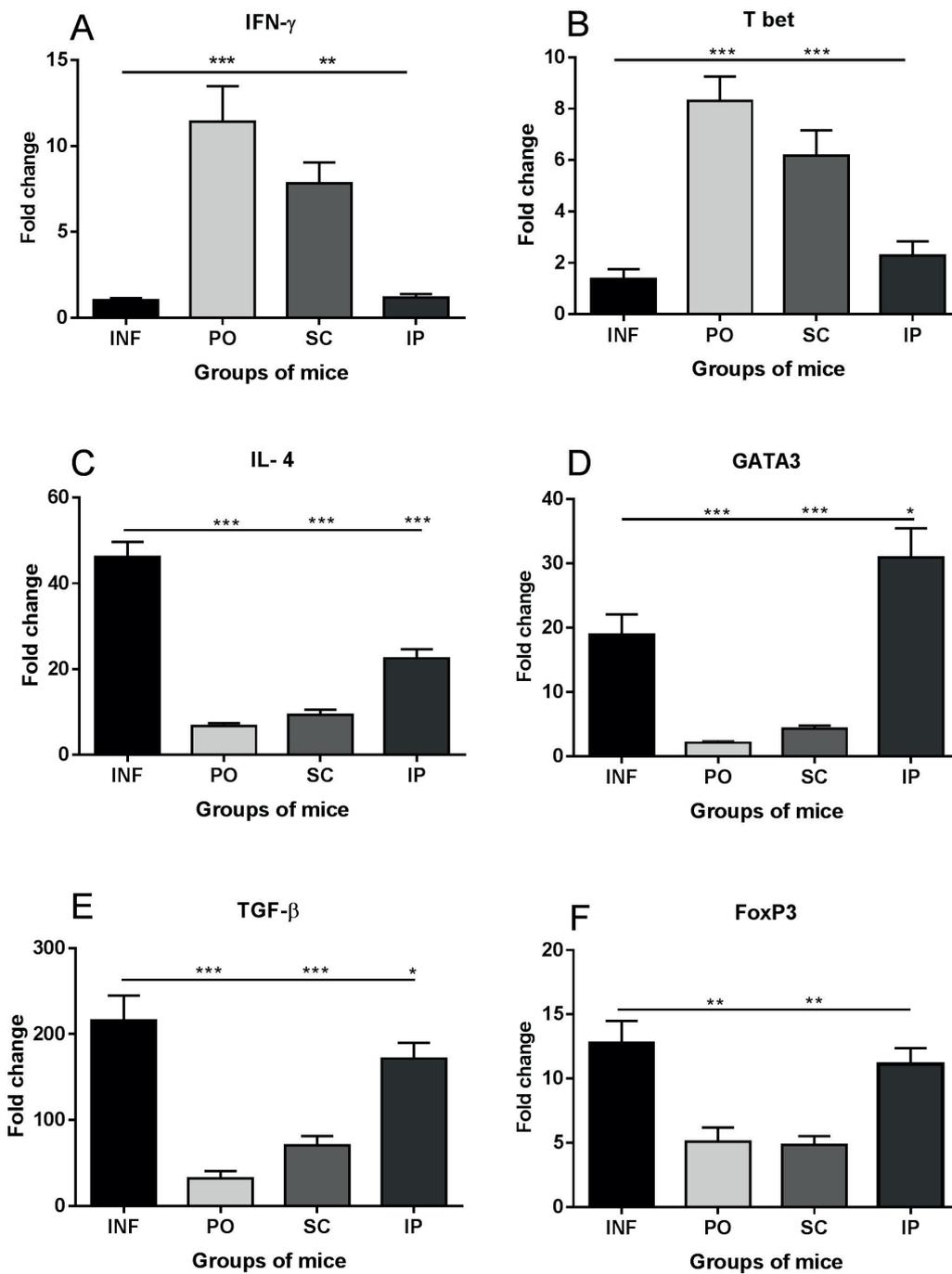
Fig. 9 A-F. Relative m-RNA expression of cytokines IFN-γ, IL-4 and TGF-β and corresponding transcriptions factors T bet, GATA3 and FoxP3 (shown as the fold change) was examined by qRT-PCR in the samples of unstimulated lymphocytes isolated from the spleens of control, infected and infected and treated mice. Relative gene expression was calculated by $2^{-\Delta\Delta Ct}$ method using data for control healthy mice as calibrator following normalisation to GAPDH. Data are expressed means ± SD of duplicates/mouse/group. Significantly different values between infected and treated mice are indicated by connecting line, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.
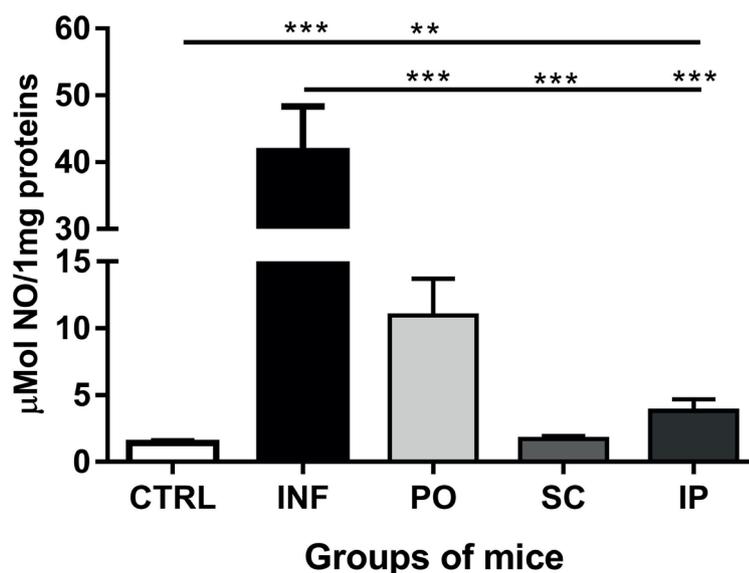
Fig. 10. The concentration of NO determined in the culture supernatants of LPS-stimulated adherent myeloid cells (1x10^{6}) isolated from the spleens of control, infected and infected and treated mice *ex vivo*. NO was determined as nitrite (NO_2) using Griess reagent. Significantly different values were calculated between **a** – control and infected and infected+treated mice; **b** – infected and treated mice, and are indicated by connecting lines.**p < 0.01, *** p < 0.001.

MODIN) was able to normalise numbers of CD4+ and CD8+ white blood cells and to increase suppressed expression of HLA-DR receptor on monocytes (Rauová *et al.,* 2014). In comparison with healthy mice, the populations of CD3+ T cells were significantly elevated in all infected groups and diminished after PO route. However, CD3+CD4+ population of T helper lymphocytes declined in infected group, what was partially eliminated in all treated groups. On contrary, populations of CD3+CD8+ T cytotoxic lymphocytes in blood were significantly elevated in infected group and decline was observed after all three routes of application. CD4+ T helper lymphocytes are polarised upon the contact with parasitic antigens to individual subsets which are defined by the functional abilities and cytokine profiles determining the progress of the disease. In humans a T helper 2- biased immunity is augmented with increased susceptibility to disease leading to chronic AE and progressive course in patients, while activation of Th1-type has been linked to reduced or abrogated metacestode proliferation in patients with the cured disease (Vuitton, 2003; Vuitton *et al.,* 2006). Meanwhile, the reduced proportions of B220+ B cells following infection in blood were elevated only after PO route, indicating that individual routes of DLE application influenced pathways by which molecules in DLE reached different compartments of the immune system.

To examine further the effects of DLE on the properties of lymphocyte subpopulations on the functional level, we assessed proliferation index (PI) after Con A and LPS stimulation and cytokine levels in the supernatants of stimulated cells. Con A is a potent mitogen for lymphocytes and can induce rapid cell proliferation, preferentially of T lymphocytes, including helper and suppressor subsets (Dwyer & Johnson, 1981). As is shown in Fig. 7A, infection resulted in suppression of PI, which was partially abrogated after all

three routes of DLE administration, however significant elevation was found only in PO treated group. The proliferation suppression of Con A- stimulated splenocytes after incubation with protoscoleces of *E. multilocularis* was reported by Kizaki *et al.* (1993), where depletion of CD4+ suggested the involvement of CD8+ T cells. Moreover, the proportions of CD8+ cells markedly increased in these Con A stimulated cultures. The results of our study might indicate that DLE partially reduced the activation and proliferation of CD8+ T-suppressor cells generated during infection. In line with this, it was reported that AE patients with progressive parasitic lesions displayed increased activation of predominantly CD8+ T cells compared to healthy controls and AE patients with the cured disease (Manfras *et al.*, 2004). The fact that the four cured AE patients (i.e., complete surgical resection of parasite) had normal levels of activated CD8+ T cells within the range of healthy control subjects provides further strong evidence for the interpretation that the parasite indeed triggers activation of CD8+ T cells.

Significant PO-stimulated proliferation of T cells observed in our study correlated with the highest reduction of cyst weight in the peritoneal cavities, suggesting that this route was the most effective in the activating antiparasitic Th1-biased CD4+ T cell subsets. LPS stimulates proliferation of B lymphocytes via the TLR4 receptor and we observed the significantly higher proliferative response after PO and IP administration compared to infected mice. Dvorožňáková *et al.* (2009) demonstrated on mice infected with *E. multilocularis* that orally given non-immune porcine DLE (Imunor, CZ) reduced cyst growth, stimulated the proliferative response of Con A-activated splenocytes along with the elevation of IFN-γ production by these cells. These effects were strongly enhanced when DLE was administered to mice in combination with albenda-

zole. The effects of IP-administered DLE alone and as the adjuvant with albendazole were investigated during infection of mice with proliferating metacestodes of *Mesocestoides vogae*. DLE increased the larvicidal effect of albendazole on the parasites in the livers and the peritoneal cavities, stimulated the proliferation of T lymphocytes, and modified the serum cytokine profiles towards the balanced Th1/Th2 type (Mačak Kubašková *et al*., 2018).

Cytokines are the key players in the process of naïve lymphocyte polarization. In *E. multilocularis* infection the Th1 cytokines promote initial cell recruitment around the metacestodes. They are also involved in the chronicity of the cell infiltrate leading to the fully organized periparasitic granulomas and their final consequences- fibrosis and necrosis. The Th2 cytokines, on the other hand, could be responsible for the inhibition of a successful parasite killing especially because of the 'anti-inflammatory' potency of IL-10 and TGF-β (Vuitton, 2003). Regarding the roles of individual CD4+T helper cell subsets, Tuxun *et al*. (2012) and Wang *et al*. (2017) showed that FoxP3 T regulatory lymphocytes are quantitatively upregulated in human AE and this rise was associated with the suppression of the Th1/Th17-type of immunity and decreased secretion of pro-inflammatory cytokines. Exploring further if different routes of DLE administration were able to modulate the proportions of Th1, Th2 and Treg CD4+ T helper lymphocytes, we assessed production of corresponding cytokines IFN-γ, IL-4 and TGF-β in the supernatants of Con A stimulated lymphocytes. We also assessed mRNA expression of these cytokines and corresponding transcription factors T bet, GATA3 and FoxP3 in unstimulated lymphocytes. Transcription factor T bet directs Th1 lineage commitment (Szabo *et al*., 2000). Beyond the commitment to early T cell lineage, GATA3 promotes the development of CD4+ Th2 cells (Gao *et al*., 2015) and transcription factor FoxP3 is critical in specifying regulatory CD4+CD25+ Treg lineage, which is central to the maintenance of immunological tolerance (Hill *et al*., 2007). The concentration of TGF-β in supernatants of Con A stimulated lymphocytes and transcripts of TGF-β were significantly elevated following infection and were strongly reduced after PO and SC administration of DLE, suggesting downregulation of Treg type polarization. A very similar effect was found for FoxP3 transcripts. IL-10 and TGF-β are major cytokines released by Treg lymphocytes. Wang *et al*. (2017) in their study demonstrated that reduced parasite growth in mice was accompanied with low expression of TGF-β after depletion of FoxP3 in mice.

We showed here that the production of the canonical cytokine of Th1 type of immunity IFN-γ was increased by lymphocytes from infected mice compared to healthy mice. DLE given by SC and IP routes significantly reduced concentrations of this cytokine, while PO route resulted in considerably higher concentrations. The similar pattern was observed on mRNA levels for IFN-γ and T bet genes. IL-4 cytokine concentrations and mRNA levels were highly upregulated in infected mice and DLE strongly reduced this Th2 dominant cytokine after all three routes. However, mRNA expression of GATA3 factor was upregulated after IP route, but not after

SC and PO routes. In AE patients Ma *et al*. (2020) confirmed the presence of 20 Th1 and Th2 cytokines in the serum, which formed a more complicated network than in the healthy individuals. Thus, it seems that the specific molecules in DLE apparently regulated mRNA transcription for cytokine genes without directly affecting the lineage-specific transcription factors.

Immunosuppression induced by metacestodes of *Echinococcus* spp. is a very complex process where individual myeloid lineage cells are strongly influenced at the functional and phenotypic levels. Here we compared the phagocytic activity of blood monocytes and neutrophils via the uptake of FITC-*E. coli*. As shown in Fig. 4A-B, neither infection nor DLE administration modulated the proportions of phagocytosing cells compared to healthy mice. In line with our results, in *E. granulosus* infected mice, the percentage of phagocytosing white blood cells was not changed in the early stage of infection but an increase was observed in the later stage (Wangoo *et al*., 1989). To address whether DLE therapy changed the proportions of monocytes and eosinophils, blood samples were stained with antibodies to the surface markers CD11b, MHCII and SiglecF, which is considered as the specific eosinophil marker. Mice responded to infection at week 9 p.i. by significantly higher proportions of CD11b+ myeloid cells. The proportions of mature CD11b+MHCII^high monocytes levelled down compared to healthy mice. Within the one year of infection with closely related *E. granulosus*, the gradual and profound elevation of CD11b+/Gr-1+ myeloid suppressor cells in the blood and spleen was observed. These cells downregulated expression of MHCII, indicating impairment of antigen-presenting capacity (Pan *et al*., 2013). Neither way of DLE administration significantly changed the proportions of CD11b+ myeloid cells in blood, but PO and SC routes showed stimulatory effects towards CD11b+MHCII^high cell proportions. Elevated numbers of eosinophils in the blood are often seen during many helminth infections. Very low numbers are detected in the blood of mice with *E. multilocularis* infection what is maintained by the proteolytic effects of E/S molecules towards eotaxin which triggers mobilization of these cells from gastro intestinal compartment (Mejri & Gottstein, 2009). Eosinophils express multiple surface markers such as SiglecF (Zhang *et al*., 2004) and also CD11b (integrin), which is the part of C3 complement receptor. In our study anti-SiglecF antibody recognized two populations: CD11b-SiglecF+ and CD11b+SiglecF+ cells by flow cytometry. Both were significantly lower in infected mice than in the healthy mice as shown in Fig. 5C and D. Stevens *et al*. (2007) described two populations in the lung parenchyma of normal/non-inflamed and OVA sensitized mice, specifically CD11b+Siglec-F+ eosinophils and CD11b-Siglec-F+ pulmonary macrophages. In the naïve mice blood monocytes represent only 2-6 % of WBC, therefore, we speculate, based on the relatively high CD11b-SiglecF+ proportions, that anti-SiglecF antibodies could bind to other cell types lacking CD11b marker, like lymphocytes or CD11b-SiglecF+ is the population of phenotypically different eosinophils. Per orally given DLE significantly elevated this population in the blood, suggesting

that this type of eosinophils probably contributed to the highest cyst reduction. The CD11b+SiglecF+ eosinophils formed much lower populations in the blood, similarly as was detected in another study on this infection model (Yuan *et al*., 2016). These cells were elevated after SC and IP routes, but not after PO route. Finally, we examined ability of LPS-stimulated adherent splenic myeloid cells to produce NO *ex vivo* (Fig. 10) to see if DLE therapy also modulated this inflammatory mediator. NO plays an important role in the immunosuppression in mice with *E. multilocullaris* infection (Dai & Gottstein, 1999) and high production of NO was linked to the complete suppression of splenic cells proliferation. The reconstitution of the lymphoproliferative response after treatment with iNOS inhibitors indicates a crucial role of macrophage derived NO in immunosuppression during murine AE (Brehm *et al*., 1999). In our study adherent splenocytes in infected mice produced high levels of NO, compared to the cells from healthy mice and all three routes of DLE administration significantly reduced NO concentrations, more after SC and IP routes.

In conclusion, the results of present study indicate that the kinetics of intestinal absorption of molecules in DLE after PO administration in the course of *E. multilocularis* infection in mice favoured their more effective interactions with cells in different compartments of the immune system, at least with white blood cells and splenocytes. The highest reduction of cyst weight seems to be linked to the significant stimulation of Th1 type CD4+T lymphocyte proliferation, production of IFN-γ via activation of T bet transcription factor. Concomitantly it was associated with the alleviation of Th2/Treg types of cellular and cytokine responses and the reduction NO excretion by myeloid cells, probably with the suppressive activity. We suppose that SC and IP administration and absorption of some large protein-like molecules could be slowed down in the local lymph nodes. This probably led to the moderate stimulation of Th1 type response and lymphocyte proliferation in spleens and blood, however, therapy with DLE significantly diminished Th2 and Treg cytokines production on mRNA and protein levels, NO production and elevated eosinophil proportions in the blood.

Conflict of interest

Authors have no potential conflict of interest.

Ethical Approval

The study was conducted according to the guidelines for the care and use of experimental animals, No. 289/2003 in Slovak Republic. The experiment was approved by Ethics Committee the State Veterinary and Food Administration of the Slovak Republic.

Acknowledgment

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