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Necrosis and apoptosis in *Trichinella spiralis*-mediated tumour reduction

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Abstract

It is known that infection with different pathogens, including helminths, can alter the progression of malignant or other diseases. We studied the effect of chronic Trichinella spiralis infection or muscle larvae excretory-secretory (ES L1) antigens on the malignant tumour growth in the mouse melanoma model system in vivo and in vitro. Our results confirmed that chronic infection with T. spiralis possesses the capacity to slow down the progression of tumour growth, resulting in an impressive reduction in tumour size. We found that the phenomenon could, at least partially, be related to a lower level of tumour necrosis compared to necrosis present in control animals with progressive malignancy course. An increased apoptotic potential among the low percentage of cells within the total tumour cell number in vivo was also observed. ES L1 antigen, as a parasitic product that is released during the chronic phase of infection, reduced the survival and slightly, but significantly increased the apoptosis level of melanoma cells in vitro. Our results imply that powerful Trichinella anti-malignance capacity does not rely only on necrosis and apoptosis but other mechanisms through which infection or parasite products manipulate the tumor establishment and expansion should be considered.

Key words: *apoptosis, excretory-secretory antigen, melanoma, necrosis, Trichinella spiralis, tumour.*

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Introduction

The progression of many diseases can be altered in the case of co-infection with different pathogens [1]. Some of the current research in the world considers the impact of helminth infections on the shaping of the immune response and protection of the infected host not only against reinfection, but also against other irrelevant diseases [2, 3].

It has been experimentally demonstrated that helminths could provide the protection or ameliorate the clinical signs of a number of diseases, such as autoimmune diseases [4-6], allergic inflammation [7-9], and even tumours [10, 11]. Among the other functions, products of helminth parasites have the capacity to modify signal transduction, resulting either in the host cell proliferation or cell death [12-14]. These kinds of modifications are observed in cancer cells in cases when tumours and parasite infection coincide or even more when the infection precedes tumour induction [15]. Antitumor effects have been described for infections with some parasites, like *Toxoplasma gondii* [16], *Trypanosoma cruzi* [17], and *Plasmodium yoelli* [18]. Among other parasites, *Trichinella spiralis* (*T. spiralis*) has been recognised as a helminth that can negatively influence tumour growth and prolong the life span of the host [19, 20]. Unfortunately, since these first findings about the potential of *Trichinella* spp. to affect tumour development, little has been done in this field of research. Only recently it was shown that *T. spiralis* infection, as well as treatment of mice with a mixture of crude extracts from *T. spiralis* adult parasites and newborn larvae, can slow down or even inhibit the progression of tumours induced by different tumour cell lines [10]. The same group of authors have found that adult crude extract had an *in vitro* anti-proliferative effect on the hepatoma cell line H7402. Based on these observations, the authors concluded that *T. spiralis* possesses powerful anti-tumour capacity. In further studies, Wang *et al.* [11] constructed a T7 phage display cDNA library of *T. spiralis* and showed that one recombinant protein, A200711, has a pro-apoptotic effect on the H7402 cell line, and hence it was proposed as a therapeutic agent in hepatocellular carcinoma treatment. Recently, it has been demonstrated on an *in vivo* model system of B16-F10 melanoma in mice that

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T. spiralis infection is a potent reducer of tumour growth and metastasis [15].

Melanoma is the most aggressive form of skin cancer. This tumour is mostly resistant to conventional chemotherapy, which implicates a bad prognosis for patients in the advanced stage of the disease [21, 22]. The resistance of melanoma cells is a consequence of their low potential for spontaneous apoptosis *in vivo* and resistance to its induction by different medicines *in vitro* [23, 24]. Investigations of substances that can affect the apoptotic process in melanoma would be a valuable contribution to finding new therapeutic approaches for this disease. From the modest level of data available in the field it appears that all three stages of *T. spiralis* life cycle contain components that are able to control malignancy [10, 15, 19]. On the other hand, it was shown that this parasite could tame autoimmune disease [6], implying the involvement of completely different mechanisms that create a tolerogenic environment [25, 26]. For the purpose of resolving this enigma, we have started an investigation of the influence of chronic *T. spiralis* infection *in vivo* or muscle larvae excretory-secretory (ES L1) products *in vitro* on melanoma cells expansion and cell death. Infection with *T. spiralis* strongly restrained the growth of the tumour. The mechanisms that this parasite uses to control tumour growth might include apoptosis and/or necrosis. This study, for the first time, demonstrated that in chronic *T. spiralis* infection there is a very limited process of necrosis inside the slow-growing tumour tissue compared to infection independent tumour development in control animals and that ES L1 antigen inhibits proliferation and enhances the apoptosis of melanoma cells *in vitro*.

Material and methods

Animals and parasites

C57BL/6 mice were bred and housed at the Military Medical Academy in Belgrade. All animal experiments were performed according to institutional guidelines, with age and sex-matched animals. C57BL/6 mice, 8-12 weeks old, were submitted to the oral infection with 200 *T. spiralis* muscle larvae. Forty days after infection, the tumour cells (B16 melanoma) were administered subcutaneously in the right hip, at 5×10^5 cells/mouse, in 200 µl of phosphate buffer. During the period of 25 days after the tumour cell application, the mice were monitored daily and checked for tumour development, and on days 10, 13, 15, 18, 21, and 25 the tumour size was measured over the skin (with a micrometer) and the volume was determined. The volume was calculated using the formula $V = 0.52 \times a \times b^2$, where *a* is the long axis, and *b* is the short axis. Non-infected animals with subcutaneously applied B16 melanoma cells were used as controls. Animals were sacrificed and tumours were removed and measured after 15 and 25 days of tumour application.

Trichinella spiralis strain (ISS 161) was maintained by passage in Wistar rats. Parasites were recovered from infected Wistar rats by digestion of the carcasses in pre-warmed gastric juice [27]. Muscle larvae were kept under controlled conditions (37°C, 5% CO_2) in complete Dulbecco modified Eagle medium (DMEM) (Sigma), for 18 hours. Excretory-secretory products of the muscle larvae (ES L1) were obtained by dialysis and concentration of culture supernatants (Amicon ultrafiltration cell, Millipore, USA).

Histological analyses of tumour tissue

Infected and non-infected mice that received B16 melanoma cells were sacrificed on day 15 and 25 after B16 cells application. Tumours, along with the surrounding tissue, were extracted, fixed in buffered formalin, paraffin embedded, and cut into 5-µm-thick tissue sections. Sections were stained by haematoxylin and eosin (H&E), and analysed on an Olympus BX50 light microscope. Pictures were taken with an Olympus DP70 digital camera using the Olympus Cell^B program.

Analysis of pictures taken from H&E slides was done using ImageJ software. Surface of necrotic area(s) and whole tumour were measured and compared. The amount of tumour necrosis was expressed as a percentage of necrotic tissue in the tumour. The degree of apoptosis was determined by TUNEL staining (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling) using an apoptosis detection kit (Tumor TACS in situ apoptosis kit, R&D, USA). Sections of paraffin embedded tumour tissue were stained according to the manufacturer's instructions. Each field was randomly selected without significant necrosis in 10 high-power fields (400× magnification) for counting TUNEL-positive cells. The index of TUNEL was calculated as a percentage of the cells with brown nuclei within the number of the total nuclei in the section.

Inflammation in and around necrosis was scored as follows: 0 – no inflammation, 1 – rare inflammation foci in and/or around necrosis, 2 – inflammation incompletely surrounds necrosis and rare inflammation foci out of it, and 3 – inflammation completely surrounding necrosis and out of necrosis either in the foci or seamless.

Melanoma cell cultivation with ES L1 antigen

Mouse (B16) melanoma cells were cultivated in culture flasks in RPMI 1640 medium (Lonza, Belgium) supplemented with 10% of foetal calf serum (FCS) (Sigma, Germany), at 37°C with 5% CO_2.

The viability of cells was checked by staining with Trypan blue. When the number of viable cells was established, the effect of *T. spiralis* ES L1 antigen on their survival was investigated by seeding 2×10^3 cells/well in 96-well flat-bottom plates. To study the impact of ES L1 on cell apoptosis, 2×10^4 B16 cells/well were seeded in

24-well plates. For both procedures, cells were cultivated for 20 hours and then treated with ES L1 in concentration range 0-200 μg/ml. Non-treated melanoma cells cultivated in medium were used as controls. Treatment for determination of ES L1 effect on cell survival lasted for 72 hours, while for apoptosis it lasted 48-72 hours.

A method for determination of cell survival using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

The test for determination of cell survival was performed by adding 10 μl/well of MTT (5 mg/ml in PBS containing 10% FCS) in 96-well plates with 2×10^3 cells/well. Staining with MTT lasted for 4 hours at 37°C, and the reaction was stopped by adding 1% SDS. After 6 more hours in the incubator, the absorbance at 570 nm was measured using a Victor multi-plate reader (Perkin-Elmer, USA). This absorbance correlated with the number of living cells in the sample, and it was expressed as a percentage as S% (S% = (N/Nk) × 100). In order to calculate the S% value, the absorbance of the treated sample was divided with the absorbance of the un-treated sample and multiplied by 100. The obtained values for cell survival were put in the chart in the function of applied ES L1 concentrations. The mean effective concentration (IC_{50}) was estimated from the chart as the concentration of agent that reduces the survival to 50% in comparison with the control sample. All experiments were performed in triplicate.

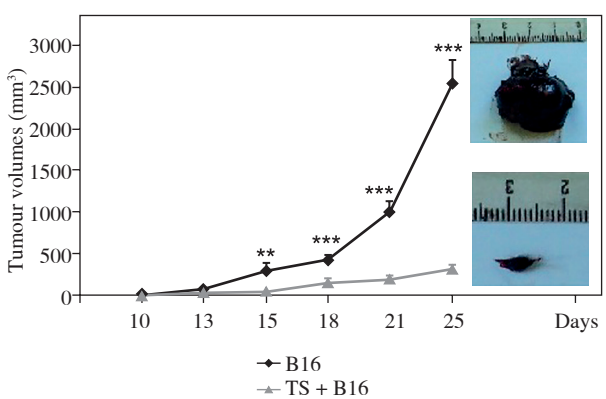

Fig. 1. The effect of chronic *T. spiralis* infection on melanoma development *in vivo*. Mice were infected per os with 200 L1 larvae of *T. spiralis*. During the chronic phase of infection, on day 40, B16 melanoma cells (5×10^5 cells in 200 μl of PBS per mouse) were applied subcutaneously in the right hip of infected and uninfected mice (10 per group). Tumours were measured over the skin with micrometre on days 10, 13, 15, 18, 21, and 25 after the application of tumour cells. Tumour volumes were calculated by the formula $0.52 \times a \times b^2$. Results are presented as mean value of tumour volume ± SEM compared to the control from one out of three experiments with similar results. $^{**}p < 0.01$; $^{***}p < 0.001$

Measurement of cell apoptosis *in vitro*

Melanoma cells were cultivated with different concentrations of ES L1 antigen (0-200 μg/ml) and after 48 and 72 hours of incubation, treated and un-treated cells were harvested and the level of apoptosis was determined. Induction of apoptosis was detected by staining the cells with annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI). For this purpose, the commercial annexin V-FITC/PI apoptosis assay kit (R&D, USA) was used according the manufacturer's instructions. Samples were evaluated on an EPICS – XL – MCL flow cytometer, and data were analysed with FlowJo software (TreeStar, Ashland, OR). Fluorescein isothiocyanate-positive cells were classified as early apoptotic cells and FITC- and PI-double-positive cells were considered as late apoptotic cells. All experiments were performed in triplicate.

The apoptotic pathway was investigated by the application of caspase –3, –8, and –9 inhibitors. B16 cells were pre-treated with inhibitors of caspase –3, –8, and –9 (R&D, USA) according to the manufacturer's instructions. In the next step, cells were treated with ES L1 antigen, and after incubation they were stained with PI (Sigma, Germany) in a final concentration of 100 μg/ml. The percentage of apoptosis was determined using flow cytometry.

Statistical analysis

Statistical comparisons between groups were made using the Student's unpaired or paired t-test and the Mann-Whitney test. The results are presented as mean ± SEM. Differences with probability (p) values less than 0.05 were considered statistically significant.

Results

Chronic infection with *Trichinella spiralis* inhibits tumour development

In the group of mice where the chronic *Trichinella* infection was established, 7 out of 10 mice developed tumours, which indicated that the infection could prevent tumour appearance. In infected animals that developed tumours, the tumour growth was slower and consequently the tumour size was reduced compared to controls (Fig. 1). The difference in tumour size between groups was statistically significant starting from day 15 of the experiment. The tumour volumes evaluated in *T. spiralis* infected animals on 10th, 13th, 15th, 18th, 21st, and 25th day of monitoring were: 0.4 ±0.13, 21.5 ±6.80, 37.1 ±11.74, 143.2 ±51.42, 184.9 ±50.64, and 301.0 ±57.85 mm^3, respectively. In the

control group, 10 out of 10 mice developed tumours, and tumour volumes at the same time points were: 1.8 ±0.69, 68.4 ±25.36, 286.0 ±89.52, 420.8 ±54.55, 1006.8 ±111.54, and 2542.0 ±282.61 mm^3, respectively.

Inhibition of melanoma growth in *T. spiralis* infected mice was confirmed by direct measuring of tumour tissue isolated from sacrificed animals on day 15 and day 25 after the application of tumour cells. Determination of isolated tumour volumes showed significantly lower values ($p < 0.001$) in infected mice compared to the values observed in control animals (Fig. 2). Tumour volumes in *T. spiralis* infected mice on day 15 and 25 were 16.6 ±9.95 and 226.6 ±72.24 mm^3, while in control group they were 203.3 ±61.59 and 1885.5 ±825.55 mm^3, respectively. The obtained data emphasise strong impact of *T. spiralis* infection on tumour development, since the final volumes of extracted tumours were 8-10 times lower than in the control group.

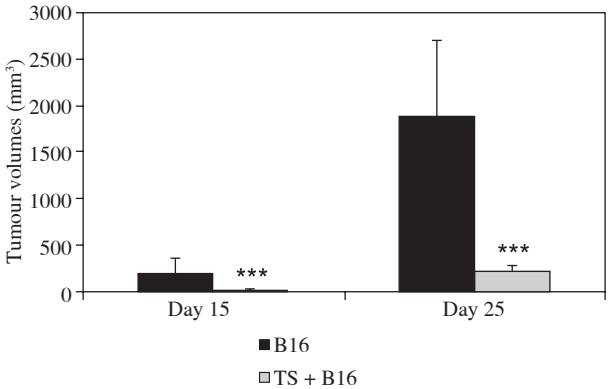

Fig. 2. The volume of extracted melanoma under the influence of chronic *T. spiralis* infection. Mice were infected per os with 200 L1 *T. spiralis* larvae. During the chronic infection, at day 40, B16 melanoma cells (5×10^5 in 200 µl PBS) were applied subcutaneously to the right hip of infected and non-infected mice. After 15 and 25 days mice were sacrificed and tumours were isolated and measured with a micrometre. The results are presented as mean value of tumour volume ± SEM compared to the control from one out of two experiments with similar results. $^{**}p < 0.01$; $^{***}p < 0.001$

Trichinella spiralis controls necrosis and inflammation in the early phase of tumour development

In the *T. spiralis* infected group of animals, the size of necrotic surfaces in tumour tissue was significantly lower compared to the control group at both examination time points ($p < 0.001$) (Fig. 3). On day 15 the degree of tumour necrosis in infected mice was 7.8 ±1.50% compared to 27.8 ±3.70% in the control group, while on day 25 the degree of tumour necrosis in infected mice was 40.1 ±1.80% compared to 54.0 ±2.50% in the control group.

Inflammation, which normally accompanies tumour necrosis, was lower only on day 15 in tumours extracted from infected animals (score 1) compared to the control group (score 2). On day 25 after tumour induction, inflammatory infiltrates had similar sizes in tumours from *T. spiralis*-infected and non-infected animals (score 2 in both cases). At both time points, infiltrates in tumours from infected animals consisted mainly of mononuclear cells, while in tumours from non-infected animals they contained both mononuclears and polymorphonuclears. In infected animals, inflammation was first located inside necrotic fields (day 15), while later it was found around them (day 25). In control animals, inflammation remained located in and out of necrotic fields during the whole course of the experiment (Fig. 4).

Trichinella spiralis infection induces low levels of apoptosis *in vivo*

To analyse whether *T. spiralis* infection induced apoptosis, TUNEL assay was performed on tumour sections. Results obtained by microscopic examination of TUNEL-stained sections (day 15 after tumour induction) revealed significantly higher numbers ($p < 0.001$) of apoptotic cells in tumours extracted from *T. spiralis*-infected animals (5.4 ±0.81%) compared to the control group (3.3 ±1.56%) (Fig. 5). The number of apoptotic cells in tumours extracted from *T. spiralis* infected animals 25 days after tumour induction was higher than in the control group (2.0 ±0.55% vs. 1.4 ±0.24%), but the difference was not statistically significant (data not shown).

Trichinella spiralis ES L1 antigen inhibits the survival and induces mild apoptosis of melanoma cells *in vitro*

In parallel with the examination of the effect of *T. spiralis* infection on melanoma development, we monitored the impact of ES L1 products on the viability and apoptosis of mouse (B16) melanoma cells *in vitro*.

After the incubation of mouse (B16) melanoma cells with *T. spiralis* ES L1 antigen, analysis of cell survival was performed by MTT method. The obtained results demonstrated that ES L1 antigen can inhibit cell survival, starting from the concentration of 100 µg/ml. Concentrations of 100 and 200 µg/ml of ES L1 antigen induced statistically significant inhibition of cell survival ($p < 0.01$ for 100 µg/ml, $p < 0.001$ for 200 µg/ml). IC_{50} could not be calculated, but the dose of 200 µg/ml of ES L1 antigen decreased the survival of malignant cells to 65.3 ±3.0% (Fig. 6). ES L1 antigens did not affect the survival of healthy cells (fibroblasts of green monkey, cell line COS-7) even if ap-

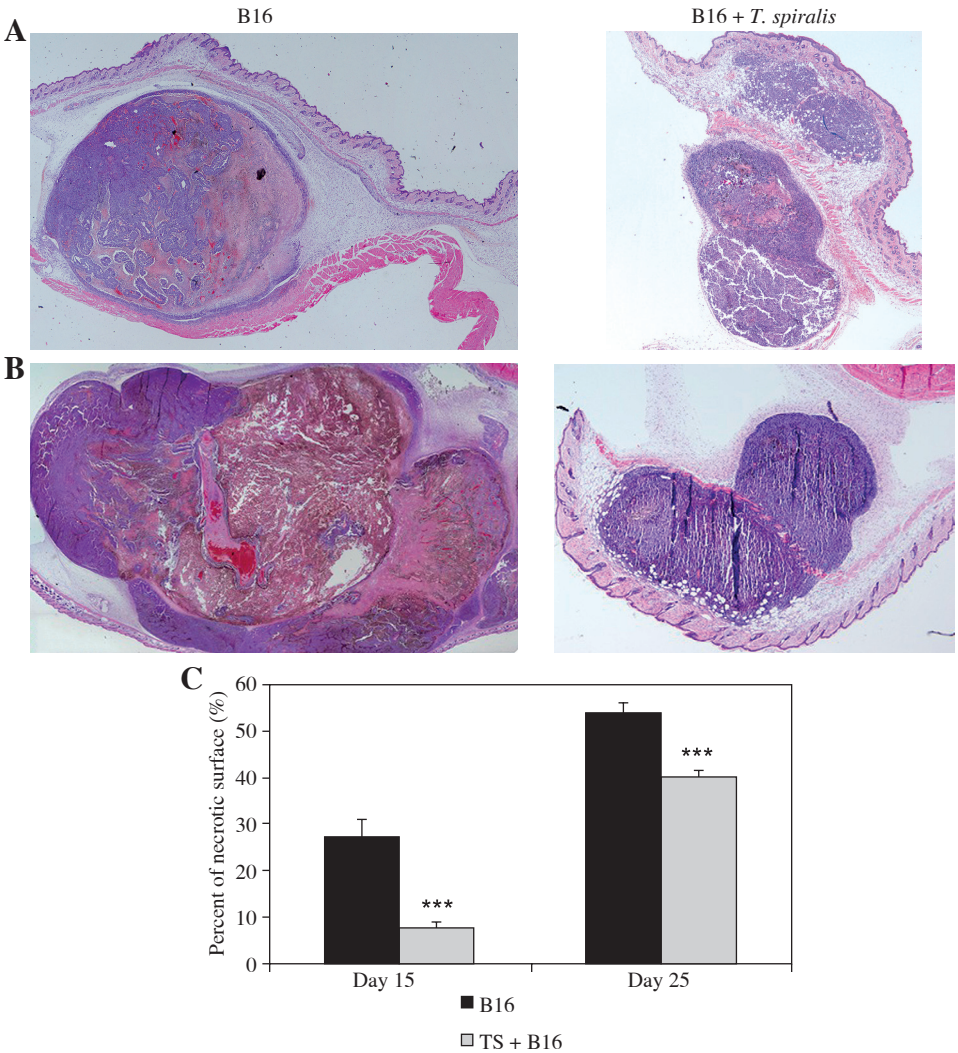

Fig. 3. The impact of *T. spiralis* infection on B16 melanoma necrosis. Necrosis presence in B16 melanoma tumour sections of *T. spiralis* infected and non-infected animals (magnification 100×): **A)** 15 days and **B)** 25 days after the tumour induction. **C)** Size of the necrotic fields in tumour sections of *T. spiralis* infected ($n = 7$) and non-infected mice ($n = 10$) is presented as mean ± SEM from one out of two experiments with similar results. *$p < 0.05$, ***$p < 0.001$

plied in the highest concentration (200 µg/ml) and in the same conditions as for melanoma cells (data not shown).

Microscopic observation of the tumour cell line in the culture plates indicated reduced density and outspread as well as slightly modified morphology of cells that were subjected to the treatment with ES L1 antigen, reflected in the shortened diameter and tendency of cells to de-attach (Fig. 7).

Analysis of apoptosis induced by incubation of B16 melanoma cells with ES L1 antigen revealed that ES L1 antigens applied in concentrations of 100 and 200 µg/ml induced statistically significant apoptosis ($p < 0.01$) of mouse melanoma cells (Fig. 8A). This effect was time dependent ($p < 0.01$, Fig. 8B). The percentage of apoptotic B16 cells after 48 hours and 72 hours was 8.0 ±0.70% and 10.2 ±0.40% in control (un-treated) cells, while for ES L1 treated cells (200 µg/ml) it was 16.4 ±1.10% and 20.4 ±0.60%, respectively. Simultaneous staining of cells with annexin V-FITC/PI enabled detection of the intact cells, cells in early apoptosis and in late apoptosis, as well as cell death. A representative plot is showen in Fig. 8C. The obtained results indicate that treatment with ES L1 antigen induced mild apoptosis of B16 melanoma cells, which are mainly in late apoptotic phase.

Trichinella spiralis ES L1 antigen induces activation of outer caspase-dependent apoptotic pathway

The apoptosis of melanoma cells, pre-treated with inhibitors of caspase-3, -8, and -9, and afterwards incubated

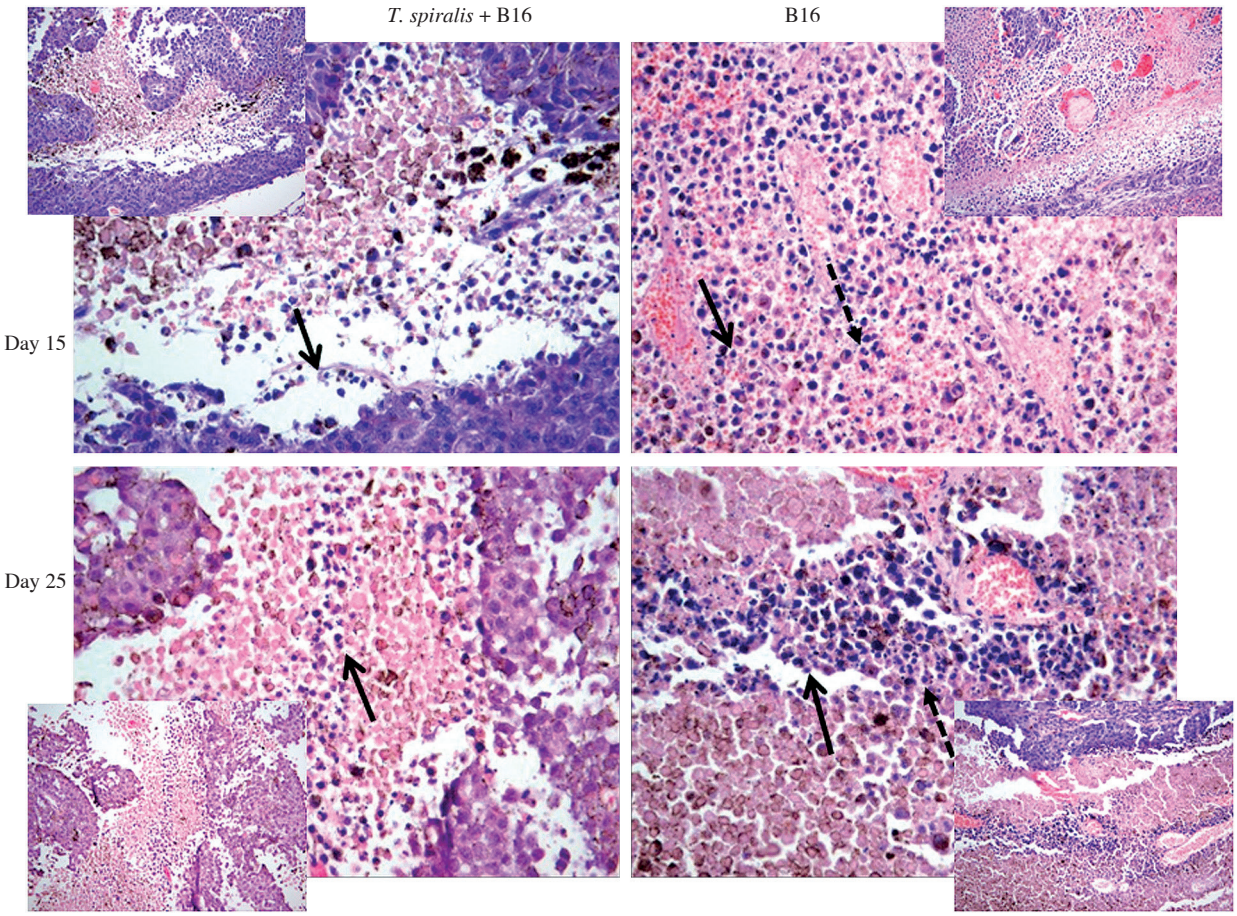

Fig. 4. *Trichinella spiralis* infection affects the inflammation process in the tumour tissue. Presence of inflammatory cells in B16 melanoma tumour sections of *T. spiralis* infected and non-infected animals (magnification 400×, pictures in the corners – magnification 200×). Arrows point to mononuclear infiltrate; dashed arrows point to polymorphonuclear infiltrate

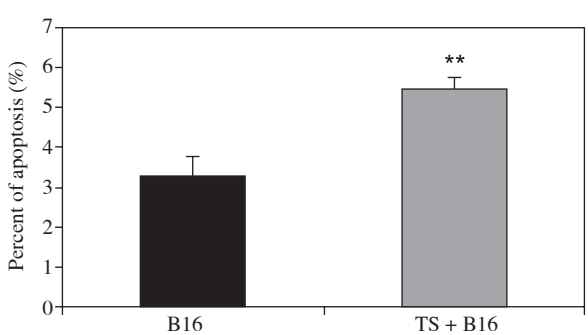

Fig. 5. *Trichinella spiralis* infection promotes apoptosis of B16 melanoma cells. The number of TUNEL-positive cells was expressed as mean ± SEM from one out of two experiments with similar results. $^{**}p < 0.01$ compared to the control group (non-infected animals)

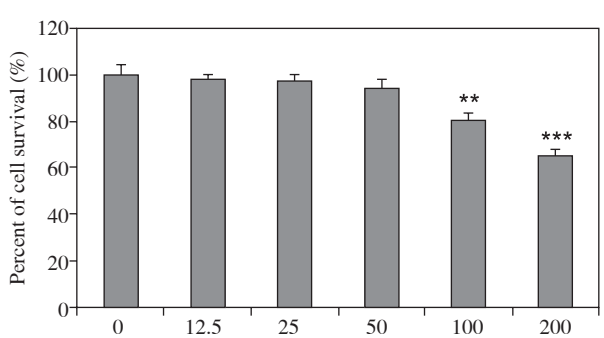

Fig. 6. The effect of ES L1 antigen on survival of mouse melanoma cells. Melanoma cells (2000/well) were seeded in 96-well plates, and after 20 hours they were treated with ES L1 antigen in growing concentrations. The cell survival was determined by MTT method after 72 hours of incubation. Results are presented as percentage of cell survival compared to control (mean value ± SD) from one out of three experiments done in triplicate. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$

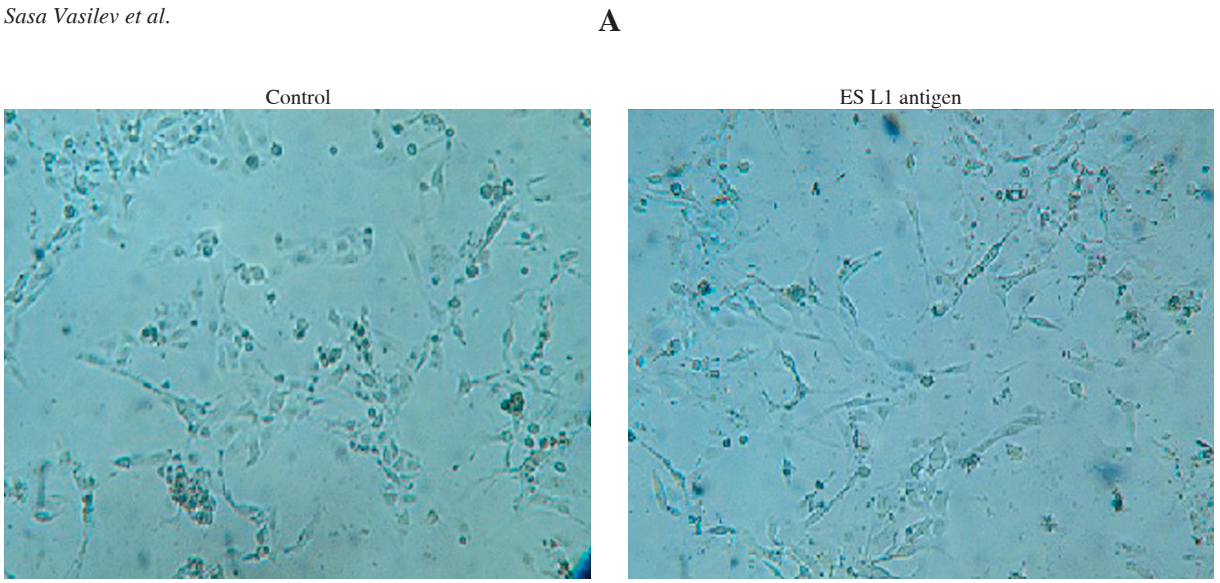

Fig. 7. Morphology of mouse melanoma cells cultivated with ES L1 antigen, light microscopy (100× magnification): Tumour cells after 72 hours of cultivation in medium (left) and with 200 µg/ml ES L1 antigen (right)

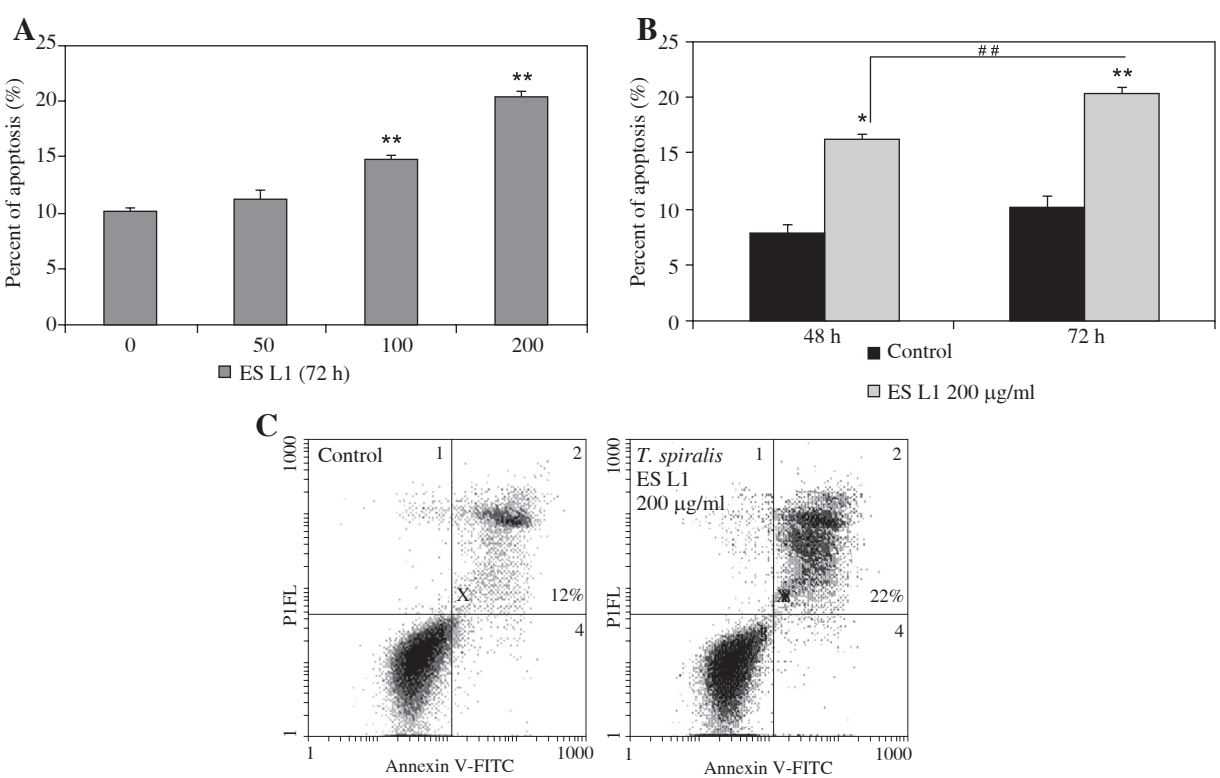

Fig. 8. The effect of ES L1 antigen on apoptosis of mouse melanoma cells. Melanoma cells (2000 cells/well) were seeded in 24-well plates for 20 hours. Afterwards cells were treated with ES L1 antigen, stained with annexin V-FITC kit, and apoptosis was evaluated by flow cytometry. **A)** ES L1 in growing concentrations (50-200 µg/ml) for 72 hours; **B)** Time-dependent effect of ES L1 antigen on apoptosis of mouse melanoma cells determined by the treatment of cells with 200 µg/ml of ES L1 for 48 and 72 hours; **C)** Representative plot – 200 µg/ml of ES L1 for 72 hours. Results are presented as percentage of apoptotic cells compared to the control i.e. untreated cells (mean value ± SEM) from one out of three experiments performed in triplicate **(A, B)**. Results are presented as the histogram of cell distribution, and a percentage of apoptotic cells from quadrant 2 and 4 are presented in quadrant 2. The presented histogram is from one representative experiment out of three with similar results (C). *$p < 0.05$; **$p < 0.01$; $^{\#\#}p < 0.01$

with *T. spiralis* ES L1 antigen, was analysed on a flow cytometer after staining with PI. The obtained results (Fig. 9) showed that pre-treatment with inhibitors of caspase –3 and –8 significantly ($p < 0.01$) inhibited ES L1-induced apoptosis of B16 melanoma cells, suggesting the involvement of outer caspase-dependent pathway in apoptosis induction. The value of apoptosis induced by ES L1 antigen was decreased from 20.4 ±0.40% to 8.4 ±0.90% in cells pre-treated with inhibitor of caspase –3, and to 10.4 ±0.60% in cells pre-treated with inhibitor of caspase –8. The obtained values of apoptosis correspond to those observed in control cells cultivated only in medium (10.2 ±0.40%). Pre-treatment with inhibitor of caspase –9 mildly reduced the percentage of ES L1-induced apoptosis, but the reduction was not statistically significant (Fig. 9).

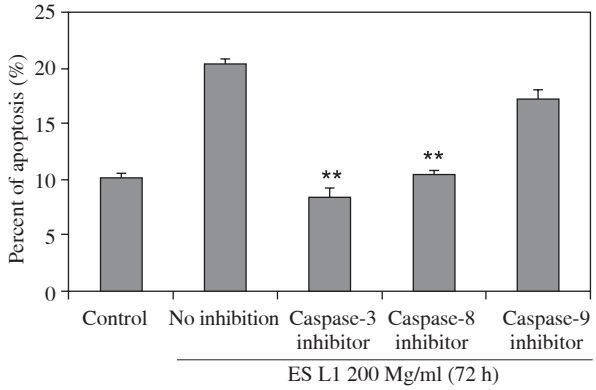

Fig 9. The effect of pre-treatment with caspase inhibitors on ES L1 antigen-induced apoptosis of mouse melanoma cells. Melanoma cells (2000 cells/well) were seeded in 24-well plate for 20 hours and treated with inhibitors of caspase-3, -8, and -9 for 2 more hours. Afterwards, the apoptosis was induced by ES L1 antigen (200 μg/ml) and evaluated by flow cytometry after staining with PI. Results are presented as percentage of apoptotic cells compared to the control, i.e. untreated cells (mean value ± SEM), from one out of three experiments performed in duplicate. $^{**}p < 0.01$

Discussion

Conventional methods aimed to reduce malignancies encompass chemotherapy, radiotherapy, and surgery. These approaches have limited success, and some new therapeutic strategies to supplement the conventional treatment have been explored. It has been observed that chemo- and radiotherapy cannot affect cancer stem cells (CSCs), which present the origination of tumour growth [28], so it appears that the strategy for tumour eradication should include targeting these cells. A variety of experimental models covering the development of dendritic cell (DC)-based vaccines, adoptive transfer of tumour specific T cells, and monoclonal antibodies aimed to eliminate CSC and tumours have been established. Although most of them are still in the phase of preclinical *in vitro* and *in vivo* models, a number of potential cancer immunotherapies have reached a phase III clinical trials in the last 25 years [29]. Dendritic cells-based vaccination with the potential to boost cancer-specific effector T cells and target CSCs, like those for glioblastoma and melanoma, are either in preclinical *in vitro* phase [30, 31] or in clinical phase I or II [32, 33]. The only DC-based vaccine approved by the Food and Drug Administration (FDA) is Provenge for prostate cancer treatment. Adoptive transfer of tumour-specific T cells presents a direct delivery of effector cells, which accurately targets CSCs, and the development of this strategy for treatment of different tumours, like those for cervical, breast, colon, and lung cancer [34-37], is in preclinical *in vivo* phase. Monoclonal antibodies are, up to now, the most successfully used tumour immunotherapies. They have the capacity to inhibit signalling pathways activated by tumour cells in order to interfere with interactions between tumour and stroma [29]. In addition to a lot of antibodies used in preclinical and clinical trials, a few, like rituximab, cetuximab, trastuzumab, ipilimumab, used for treatment of solid and haematological tumours, were approved by the FDA. Besides conventional methods and the above-mentioned immunotherapeutic tools for tumour reduction that are still developing, a new angle in the treatment of malignancies has been opened. It was noticed that living organisms such as helminths and leeches contain molecules with antimicrobial and antineoplastic activity, which is in line with the opinion that the cure for a variety of diseases might be found in nature [38, 39].

Infections caused by different pathogens, including parasites, have initially been recognised as risk factors for the development of malignancies, and 25% of human cancers are considered to be a consequence of chronic inflammation provoked by infection [40, 41]. Among the parasitic agents proven to be carcinogenic for humans are *Schistosoma haematobium* and *Schistosoma japonicum*, associated with the occurrence of bladder cancer and hepatocellular carcinoma [42, 43], and *Opisthorchis viverrini* and *Clonorchis sinensis* associated with cholangiocarcinoma [44, 45]. In contrast to these data, there is a growing body of evidence that parasitic infections or application of parasite-derived proteins can suppress tumour growth. For example, infection with the malaria parasite has anti-tumour and anti-metastatic effects in a model of lung cancer [18]; intratumoural injection of attenuated *Toxoplasma gondii* causes the regression of established melanoma [46]; infection with *Trypanosoma cruzi* lowers the rate of colon cancer in rats [47]; calreticulin from *Trypanosoma cruzi* has anti-tumour and anti-angiogenic properties [48], and the anti-tumour activity of *Echinococcus granulosus*

has been documented through the suppressive effect of human hydatid cyst fluid on the colon carcinoma growth [49]. The impact of *T. spiralis* infection, as well as crude antigens derived from this parasite, on the growth of solid tumours *in vivo* or malignant cell lines *in vitro* has been shown by a limited number of studies [10, 19]. Studies of anti-tumour mechanisms used by *Trichinella* are even more modest and cover only a small segment of immune response (the role of chemokines, increasingly expressed due to infection, in tumour regression) [15].

Our studies were focused on the impact of *T. spiralis* infection or its ES L1 products on tumour development and were conducted both *in vivo* and *in vitro*. ES L1 antigen was chosen since this muscle larva product influences the hosts' organism on a systemic level and for much longer period, compared to the antigens from other life cycle stages of *Trichinella* explored so far.

Chronic *T. spiralis* infection in animals, which preceded injection with melanoma cells, caused significant reduction in tumour size. These observations are consistent with the results of other authors who explored the impact of *T. spiralis* infection on tumour growth, starting with Molinari and Ebersole [19], who demonstrated that mice with chronic *T. spiralis* infection survived for a significantly longer time and had smaller tumours after application of B16 melanoma cells, compared to animals that were not infected prior to the tumour induction. Our analysis of necrosis and apoptosis processes, as potential means of tumour growth suppression, revealed smaller necrotic surfaces in tumour tissue, in mice infected with *T. spiralis* and a low, although significant, increase in the level of apoptosis. Tumour necrosis, as a common solid tumour feature, appears as a consequence of rapid tumour growth and ischaemia of tumour tissue [50]. Increased intra-tumour necrosis correlates with the increased metastatic potential and bad prognosis [51]. It has already been described that tumour necrosis presents an adverse risk factor for the survival of patients with lung cancer, i.e. tumour necrosis correlates with the poor outcomes [52]. Also, high tumour necrosis is an indicator of bad prognosis in breast cancer [53] and in renal cancer [54]. Hence, the reduction of areas under necrosis within the tumours in animals with *T. spiralis* infection could be interpreted as a positive effect of the infection, which should favour the survival of mice with B16 melanoma and be considered as a promising prognostic factor. It seems reasonable to assume that *T. spiralis* chronic infection interferes with the solid tumour development by reducing the degree of necrosis, which implies a reduction in the metastatic potential and spreading of the tumour. In the early phase of tumour development, infection with *T. spiralis* successfully controls the degree of necrosis, keeping it at a level 4 times lower than in controls. Later on, the necrotic surfaces under the influence of *T. spiralis* infection, although significantly reduced compared to the controls, become much larger than in the previous stage of tumour development. The observed results indicate that necrosis cannot be considered as one of the primary mechanisms of *T. spiralis* action in modulation of tumour growth since the phenomenon of necrosis decrease provoked by *Trichinella* seems to be more temporary than permanent in character. Also, during the early tumour development phase, the level of tumour inflammation is reduced in infected animals, while in the later phase the inflammation degree becomes similar to that seen in the control group. It is reasonable to expect that only identification of immune cells at the site of inflammation will provide an answer to the role of the parasite in this process.

Evaluation of the impact of chronic *T. spiralis* infection on apoptotic process in melanoma *in vivo* revealed a low, but still significant, increase in the percentage of apoptotic cells in B16 melanoma in the earlier phase of tumour development (day 15), indicating that apoptosis could be a feature of *T. spiralis*-induced suppression of tumour growth, but cannot be regarded as an important mechanism of controlling the tumour development. It is known that different parasites have different impacts on apoptosis in tumours. In case of the parasite *Schistosoma japonicum*, apoptosis may be considered as one of the main ways in which the parasite's recombinant protein (rSj16) influences the tumour regression [55]. The malaria parasite (*Plasmodium* spp.), although affecting tumour growth suppression mainly by inducing potent innate and adaptive anti-tumour immunity, also induces increased apoptosis in tumour tissue [18]. On the other hand, calreticulin from *Trypanosoma cruzi* did not induce apoptosis, and its anti-tumour activity is attributed to a cytotoxic effect rather than an apoptotic one [48]. Since apoptosis is considered to be one of the main mechanisms employed by anti-tumour therapies and pro-apoptotic effect of parasite products seems promising in the sense of developing new therapeutic approaches, we examined also the cytotoxic and apoptotic potential of isolated *T. spiralis* ES L1 antigen *in vitro* on B16 melanoma cells.

Our results have shown that ES L1 antigen inhibited the survival of B16 melanoma cells in a dose-dependent manner while it did not affect the survival of healthy cells, indicating that ES L1 could influence either cell cycle or cell death. In regard to the impact of ES L1 on cell death, the obtained results demonstrated a mild pro-apoptotic effect of ES L1 on B16 melanoma cells *in vitro*. *Trichinella spiralis* ES L1 antigen triggers apoptosis through initiator caspase-8 and effector caspase –3, suggesting the induction of apoptosis through the outer pathway (death receptor activation). This is in agreement with the literature statements about the ability of *T. spiralis* to induce apoptosis during the establishment of the immuno-privileged place in the hosts' body (the nurse cell formation), but for this purpose *Trichinella* is using both outer and inner pathways [56, 57].

Our findings only partially correlate with the results obtained by Wang *et al*. [10], which demonstrated higher pro-apoptotic influence using antigens from two other life stages of *T. spiralis* (adult worm and new-born larvae crude extract) on two different cell lines (K562 and H7402). These authors also demonstrated that crude extract derived from adults and newborn larvae can slow or inhibit the development of tumours induced with different tumour cell lines (stomach cancer – MFC, hepatoma – H22, and sarcoma – S180). Given that the proportion of apoptotic cells in tumours from mice infected with *T. spiralis* was insufficient to explain the phenomenon of tumour growth suppression, as demonstrated in our *in vivo* experiment, the ability of ES L1 antigen to increase apoptosis in tumour tissue may be considered as less important mechanism, among other, yet unrevealed mechanisms in *T. spiralis*-induced modulation of the course of malignancies. Nevertheless, the fact that we and other authors succeeded in tumour growth suppression by *T. spiralis* infection indicates the possibility that products of *T. spiralis* contain an active component with an anti-tumour effect. Gong *et al.* [58] investigated the presence of myeloma-associated antigens in *T. spiralis* and they found that tropomyosin, a component of *T. spiralis* myofibrils, is the molecule they searched for, and that it has an anti-tumour effect as well as a role in eliciting cross-reactive immunity. Treatment of mice with *T. spiralis* crude antigens, ES L1 antigens, tropomyosin, and infection with 400 L1 larvae had very similar effect on tumour growth, i.e. all these treatments inhibited the development of myeloma SP2/0. Our findings are in agreement with the results of the above-mentioned authors, suggesting that components of ES L1 antigen that is produced during the chronic phase of infection with *T. spiralis* possess anti-tumour activity.

Studies on the potential of *T. spiralis*, as well as other parasites and their products, to reduce tumour growth are not yet numerous, and those that have been conducted are still in the experimental phase. Certainly, for any potential treatment of malignant diseases, in order to avoid the application of the whole parasite, which would be accompanied by various side effects and complications, particular molecules or components from parasite-derived products with the capacity to suppress tumour development and dissemination should be extensively studied and characterised. As it stands, application of parasitic antigens or their components as a therapeutic strategy for tumour targeting is still a long way from clinical trials.

In conclusion, chronic *T. spiralis* infection drastically decreases tumour volume and amount of necrosis in tumour, which might reflect on the reduction of metastatic potential. It also promotes apoptosis but at a level at which it cannot be considered as a major mechanism of controlling tumour development. *Trichinella spiralis* is able to supresses the development of B16 melanoma through the action of ES L1 antigens, a product characteristic for the chronic phase of this infection. In vitro studies revealed that ES L1 antigens possess anti-survival and pro-apoptotic effect on B16 melanoma cells; however, those are not key mechanisms of tumour suppression. Therefore, the mechanisms underlying the ability of this helminth to act in an anti-tumour manner will be revealed by future investigations.

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References

1. McSorley HJ, Maizels RM (2012): Helminth infections and host immune regulation. Clin Microbiol Rev 25: 585-608.
2. Harris NL (2011): Advances in helminth immunology: optimism for future vaccine design? Trends Parasitol 27: 288-293.
3. Johnston CJ, McSorley HJ, Anderton SM, et al. (2014): Helminths and immunological tolerance. Transplantation 97: 127-132.
4. Maizels RM, Balic A, Gomez-Escobar N, et al. (2004): Helminth parasites: Masters of regulation. Immunol Rev 201: 89-116.
5. van Riet E, Hartgers FC, Yazdanbakhsh M (2007): Chronic helmint infections induce immunomodulation: Consequences and mechanisms. Immunobiology 212: 475-490.
6. Gruden-Movsesijan A, Ilic N, Mostarica-Stojkovic M, et al. (2010): Mechanisms of modulation of experimental autoimmune encephalomyelitis by chronic Trichinella spiralis infection in Dark Agouti rats. Parasite Immunol 32: 450-459.
7. Yazdanbakhsh M, Kremsner PG, van Ree R (2002): Allergy, parasites and hygiene hypothesis. Science 296: 490-494.
8. Maizels RM, Pearce EJ, Artis D, et al. (2009): Regulation of pathogenesis and immunity in helminth infections. J Exp Med 206: 2059-2066.
9. Brum C, Barbosa G, Graeff-Teixeira C, et al. (2014): Helminth extracts inhibit eosinophilic inflammation in a murine model of allergic rhinitis. Allergol Imunopathol (Madr) 42: 632-634.
10. Wang XL, Fu BQ, Yang SJ, et al. (2009): Trichinella spiralis – a potential anti-tumor agent. Vet Parasitol 159: 249-252.
11. Wang XL, Liu MY, Sun SM, et al. (2013): An anti-tumor protein produced by Trichinella spiralis induces apoptosis in human hepatoma H7402 cells. Vet Parasitol 194: 186-188.
12. Wu Z, Sofronic-Milosavljevic Lj, Nagano I, Takahashi Y (2008): Trichinella spiralis: nurse cell formation with emphasis on analogy to muscle cell repair. Parasit Vectors 1: 27.
13. Venugopal PG, Nutman TB, Semnani RT (2009): Activation and regulation of toll-like receptors (TLRs) by helminth parasites. Immunol Res 43: 252-263.
14. Bienvenu AL, Gonzalez-Rey E, Picot S (2010): Apoptosis induced by parasitic diseases. Parasit Vectors 3: 106.
15. Kang YJ, Jo JO, Cho MK, et al. (2013): Trichinella spiralis infection reduces tumor growth and metastasis of B16-F10 melanoma cells. Vet Parasitol 196: 106-113.
16. Kim JO, Jung SS, Kim SY, et al. (2007): Inhibition of Lewis lung carcinoma growth by Toxoplasma gondii through induc-

tion of Th1 immune responses and inhibition of angiogenesis. J Korean Med Sci 22 (Suppl.): S38-S46.
17. Ramírez G, Valck C, Aguilar L, et al. (2012): Roles of Trypanosoma cruzi calreticulin in parasite–host interactions and in tumor growth. Mol Immunol 52: 133-140.
18. Chen L, He Z, Qin L, et al. (2011): Antitumor effect of malaria parasite infection in a murine lewis lung cancer model through induction of innate and adaptive immunity. PLoS One 6: e24407.
19. Molinari JA, Ebersole JL (1977): Antineoplastic effects of long term Trichinella spiralis infection on B16 melanoma. Int Arch Allergy Appl Immunol 55: 444-448.
20. Pocock D, Meerovitch E (1982): The anti-neoplastic effect of trichinellosis in a syngeneic murine model. Parasitology 84: 463-473.
21. Grossman D, Altieri DC (2001): Drug resistance in melanoma: mechanisms, apoptosis, and new potential therapeutic targets. Cancer Metast Rev 20: 3-11.
22. Russo AE, Torrisi E, Bevelacqua Y, et al. (2009): Melanoma: molecular pathogenesis and emerging target therapies. Int J Oncol 34: 1481-1489.
23. Soengas MS, Lowe SW (2003): Apoptosis and melanoma chemoresistance. Oncogene 22: 3138-3151.
24. Gray-Schopfer V, Wellbrock C, Marais R (2007): Melanoma biology and new targeted therapy. Nature 445: 851-857.
25. Ilic N, Gruden-Movsesijan A, Sofronic-Milosavljevic L (2012): Trichinella spiralis: shaping the immune response. Immunol Res 52: 111-119.
26. Aranzamendi C, Fransen F, Langelaar M, et al. (2012): Trichinella spiralis-secreted products modulate DC functionality and expand regulatory T cells in vitro. Parasite Immunol 34: 210-223.
27. Gamble HR, Bessonov AS, Cuperlovic K, et al. (2000): International commission on Trichinellosis: recommendations on methods for the control of Trichinella in domestic and wild animals intended for human consumption. Vet Parasitol 93: 393-408.
28. Moncharmont C, Levy A, Gilormini M, et al. (2012): Targeting a cornerstone of radiation resistance: cancer stem cell. Cancer Lett 322: 139-147.
29. Kwiatkowska-Borowczyk EP, Gąbka-Buszek A, Jankowski J, Mackiewicz A (2015): Immunotargeting of cancer stem cells. Contemp Oncol (Pozn) 19: A52-A59.
30. Pellegatta S, Poliani PL, Corno D, et al. (2006): Neurospheres enriched in cancer stem-like cells are highly effective in eliciting a dendritic cell-mediated immune response against malignant gliomas. Cancer Res 66: 10247-10252.
31. Ning N, Pan Q, Zheng F, et al. (2012): Cancer stem cell vaccination confers significant antitumor immunity. Cancer Res 72: 1853-1864.
32. Phase I study of a dendritic cell vaccine for patients with either newly diagnosed or recurrent glioblastoma. ClinicalTrials.gov available at: http://clinicaltrials.gov/show/NCT02010606.
33. Dillman RO, Cornforth AN, Depriest C, et al. (2012): Tumor stem cell antigens as consolidative active specific immunotherapy: a randomized phase II trial of dendritic cells versus tumor cells in patients with metastatic melanoma. J Immunother 35: 641-649.
34. Liao T, Kaufmann AM, Qian X, et al. (2013): Susceptibility to cytotoxic T cell lysis of cancer stem cells derived from cervical and head and neck tumor cell lines. J Cancer Res Clin Oncol 139: 159-170.
35. Mine T, Matsueda S, Li Y, et al. (2009): Breast cancer cells expressing stem cell markers CD44+ CD24 lo are eliminated by Numb-1 peptide-activated T cells. Cancer Immunol Immunother 58: 1185-1194.
36. Inoda S, Hirohashi Y, Torigoe T, et al. (2011): Cytotoxic T lymphocytes efficiently recognize human colon cancer stem-like cells. Am J Pathol 178: 1805-1813.
37. Visus C, Wang Y, Lozano-Leon A, et al. (2011): Targeting ALDH (bright) human carcinoma initiating cells with ALDH1A1- specific CD8+ T cells. Clin Cancer Res 17: 6174-6184.
38. Cooper EL, Ru B, Weng N. (2004). Earthworms: sources of antimicrobial and anticancer molecules. Adv Exp Med Biol 546: 359-389.
39. Cherniack EP (2011). Bugs as drugs, part two: worms, leeches, scorpions, snails, ticks. Centipedes and spiders. Altern Med Rev 16: 50-58.
40. Coussens LM, Werb Z (2002): Inflammation and cancer. Nature 420: 860-867.
41. Ohnishi S, Ma N, Thanan R, et al. (2013): DNA damage in inflammation-related carcinogenesis and cancer stem cells. Ox Med Cell Longev 2013: 387014, doi: 10.1155/2013/387014.
42. Khurana S, Dubey ML, Malla N (2005): Association of parasitic infections and cancers. Indian J Med Microbiol 23: 74-79.
43. El-Tonsy MM, Hussein HM, Helal T, et al. (2013): Schistosoma mansoni infection: Is it a risk factor for development of hepatocellular carcinoma? Acta Trop 128: 542-547.
44. Kamsa-ard S, Laopaiboon M, Luvira V, Bhudhisawasdi V (2013): Association between praziquantel and cholangiocarcinoma in patients infected with Opisthorchis viverrini: a systematic review and meta-analysis. Asian Pacific J Cancer Prev 14: 7011-7016.
45. Suarez-Munoz MA, Fernandez-Aguilar JL, Sanchez-Perez B, et al. (2013): Risk factors and classifications of hilar cholangiocarcinoma. World J Gastrointest Oncol 5: 132-138.
46. Baird JR, Byrne KT, Lizotte PH, et al. (2013): Immune-mediated regression of established B16F10 melanoma by intratumoral injection of attenuated Toxoplasma gondii protects against rechallenge. J Immunol 190: 469-478.
47. Oliveira EC, Leite MS, Miranda JA, et al. (2001): Chronic Trypanosoma cruzi infection associated with low incidence of 1,2-dimethylhydrazine-induced colon cancer in rats. Carcinogenesis 22: 737-740.
48. Lopez NC, Valck C, Ramirez G, et al. (2010): Antiangiogenic and antitumor effects of Trypanosoma cruzi calreticulin. Plos Negl Trop Dis 4: e730.
49. Berriel E, Russo S, Monin L, et al. (2013): Antitumor activity of human hydatid cyst fluid in a murine model of colon cancer. Sci World J 2013:230176, doi: 10.1155/2013/230176.
50. Pollheimer MJ, Kornprat P, Lindtner RA, et al. (2010): Tumor necrosis is a new promising prognostic factor in colorectal cancer. Human Pathol 41: 1749-1757.
51. Swinson DE, Jones JL, Richardson D, et al. (2002): Tumor necrosis is an independent prognostic marker in non-small cell lung cancer: correlation with biological variables. Lung Cancer 37: 235-240.
52. Park SJ, Lee HS, Jang HJ, et al. (2011): Tumor necrosis as a prognostic factor for stage IA non-small cell lung cancer. Ann Thorac Surg 91: 1668-1673.
53. Leek RD, Landers RJ, Harris AL, Lewis CE (1999): Necrosis correlates with high vascular density and focal macrophage infiltration in invasive carcinoma of the breast. Br J Cancer 79: 991-995.

54. Lam JS, Shvarts O, Said JW, et al. (2005): Clinicopathologic and molecular correlations of necrosis in the primary tumor of patients with renal cell carcinoma. Cancer 103: 2517-2525.
55. Yang F, Sun X, Shen J, et al. (2013): A recombined protein (rSj16) derived from Schistosoma japonicum induces cell cycle arrest and apoptosis of murine myeloid leukemia cells. Parasitol Res 112: 1261-1272.
56. Boonmars T, Wu Z, Nagano I, Takahashi Y (2004): Expression of apoptosis-related factors in muscle infected with Trichinella spiralis. Parasitology 128: 323-332.
57. Wu Z, Nagano I, Boonmars T, Takahashi Y (2005): A spectrum of functional genes mobilized after Trichinella spiralis infection in sceletal muscle. Parasitology 130: 561-573.
58. Gong P, Zhang J, Cao L, et al. (2011): Identification and characterization of myeloma-associated antigens in Trichinella spiralis. Exp Parasitol 127: 784-788.